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The *Rhizobium etli* RpoH1 and RpoH2 sigma factors are involved in different stress responses

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The physiological role and transcriptional expression of *Rhizobium etli* sigma factors *rpoH1* and *rpoH2* are reported in this work. Both *rpoH1* and *rpoH2* were able to complement the temperature-sensitive phenotype of an *Escherichia coli rpoH* mutant. The *R. etli rpoH1* mutant was sensitive to heat shock, sodium hypochlorite and hydrogen peroxide, whereas the *rpoH2* mutant was sensitive to NaCl and sucrose. The *rpoH2 rpoH1* double mutant had increased sensitivity to heat shock and oxidative stress when compared with the *rpoH1* single mutant. This suggests that in *R. etli*, RpoH1 is the main heat-shock sigma factor, but a more complete protective response could be achieved with the participation of RpoH2. Conversely, RpoH2 is involved in osmotic tolerance. In symbiosis with bean plants, the *R. etli rpoH1* and *rpoH2 rpoH1* mutants still elicited nodule formation, but exhibited reduced nitrogenase activity and bacterial viability in early and late symbiosis compared with nodules produced by *rpoH2* mutants and wild-type strains. In addition, nodules formed by *R. etli rpoH1* and *rpoH2 rpoH1* mutants showed premature senescence. It was also determined that *fixNf* and *fixKf* expression was affected in *rpoH1* mutants. Both *rpoH* genes were induced under microaerobic conditions and in the stationary growth phase, but not in response to heat shock. Analysis of the upstream region of *rpoH1* revealed a σ^{70} and a probable σ^{E} promoter, whereas in *rpoH2*, one probable σ^{E}-dependent promoter was detected. In conclusion, the two RpoH proteins operate under different stress conditions, RpoH1 in heat-shock and oxidative responses, and RpoH2 in osmotic tolerance.

INTRODUCTION

The heat-shock response in bacteria is controlled at the transcriptional level by the alternative sigma factor RpoH (σ^{32}) (Arsène *et al.*, 2000; Yura *et al.*, 1996). Genes encoding RpoH have been found in the majority of the bacterial genomes sequenced to date. The RpoH sigma factors recognize a promoter sequence different from that recognized by the housekeeping RpoD (σ^{70}). A conserved region known as the 'RpoH box' characterizes the RpoH protein family; they also contain conserved sequences in regions 2.4 and 4.2 that are involved in the recognition of the −10 and −35 promoter elements (Nakahigashi *et al.*, 1995; Wösten, 1998).

In *Escherichia coli*, RpoH controls the expression of about 91 genes (Nonaka *et al.*, 2006). Among these are the genes encoding chaperones (GroEL, GroES, DnaK, DnaJ and GrpE) and proteases (FtsH and Lon) (El-Samad *et al.*, 2005; Gross, 1996; Taylor *et al.*, 1984; Yamamori & Yura, 1980). In addition to the response to high temperatures, RpoH is involved in the response to oxidative stress, and has also been implicated in symbiosis and pathogenic lifestyles (Delory *et al.*, 2006; Mitsui, *et al.*, 2004).

Commonly, bacterial genomes contain a single *rpoH* gene, but several α-proteobacteria have two or three *rpoH* homologues. Two *rpoH* genes have been identified in

Abbreviations: AO, acridine orange; RACE, rapid amplification of cDNA ends.

Brucella melitensis and *Rhodobacter sphaeroides*, and in the nitrogen-fixing symbionts *Mesorhizobium loti*, *Sinorhizobium meliloti* and *Rhizobium etli* (Galibert *et al.*, 2001; González *et al.*, 2006; Kaneko *et al.*, 2000; Oke *et al.*, 2001; Ono *et al.*, 2001); *Bradyrhizobium japonicum* possesses three *rpoH*-like genes (Kaneko *et al.*, 2002; Narberhaus *et al.*, 1997). Each of the *rpoH* genes of *Bradyrhizobium japonicum*, *S. meliloti*, *Brucella melitensis* and *Rhodobacter sphaeroides* is able to complement, totally or partially, the temperature-sensitive phenotype of an *E. coli rpoH* mutant, thus suggesting that they are functionally similar to *E. coli* σ^{32} (Delory *et al.*, 2006; Green & Donohue, 2006; Narberhaus *et al.*, 1997; Oke *et al.*, 2001; Ono *et al.*, 2001). The reason for the presence of multiple *rpoH* genes is not understood. In *Bradyrhizobium japonicum*, the *rpoH2* gene seems to be essential, since no viable cells are recovered after knockout of this gene (Narberhaus *et al.*, 1997), while *rpoH1* and *rpoH3* (phylogenetically related to the Rhizobiaceae *rpoH1* and *rpoH2*, respectively) mutants display a phenotype indistinguishable from that of the wild-type under aerobic growth conditions or during root-nodule symbiosis; however, the gene expression and the complementation of an RpoH-deficient *E. coli* suggest that *rpoH1* and probably *rpoH3* are involved in the heat-shock response (Narberhaus *et al.*, 1997). In *S. meliloti*, it has been shown that only the product of *rpoH1* is sufficient for response to heat shock. Furthermore, an *rpoH1* mutant renders the Nod+Fix− phenotype, whereas the *rpoH2 rpoH1* double mutation abolishes the ability to nodulate alfalfa plants (Oke *et al.*, 2001; Ono *et al.*, 2001). These studies suggest that the heat-shock response in *Rhizobium* could overlap the response to other stimuli.

Since *R. etli* also possesses two *rpoH* genes, they could be expected to play a similar role in the heat-shock response to those of other bacteria. Nevertheless, in this work it was found that RpoH1 is mainly involved in heat-shock and oxidative responses, while RpoH2 participates in osmotic tolerance. *rpoH1* inactivation affects the nitrogen fixation activity in symbiosis, and *fixNf* and *fixKf* expression under microaerobic and aerobic conditions. Furthermore, the predicted regulatory elements in the upstream regions of *R. etli rpoH1* and *rpoH2* suggest that they are regulated by different sigma factors. This contribution expands previous observations and allows a more comprehensive view of the interplay between two related regulators.

METHODS

Bacterial strains and microbiological methods. The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown at 37 °C in Luria–Bertani medium. *R. etli* strains were grown at 25 °C in peptone–yeast extract (PY) medium (Noel *et al.*, 1984) or minimal medium (MM; 1.2 mM K_2HPO_4, 0.8 mM $MgSO_4$, 10 mM succinic acid, 10 mM NH_4Cl, 1.5 mM $CaCl_2$ and 0.0005 % $FeCl_3$, pH 6.8; Bravo & Mora, 1988). Microaerobic conditions were as described by Girard *et al.* (2000). Antibiotics were added at the following final concentrations (μg ml^{-1}): gentamicin, 30; chloramphenicol, 25; ampicillin, 100; nalidixic acid, 20; spectinomycin, 100; kanamycin, 30; tetracycline, 10. Sucrose was used at final concentration ranging from 7.5 to 15 % (w/v). Sodium hypochlorite (NaOCl) and hydrogen peroxide (H_2O_2) (Sigma) were added at final concentrations ranging from 0.4 to 0.6 % and from 2 to 80 mM, respectively.

To determine the survival rates with NaOCl and H_2O_2, the *R. etli* strains were grown in PY medium at 25 °C and agitated at 200 r.p.m. Aliquots were taken at 12 and 36 h post-inoculation (OD_{600} ~0.4 and ~1.4, respectively) and incubated with different concentrations of NaOCl or H_2O_2 for 45 min at 25 °C. After treatment, samples were diluted in 10 mM $MgSO_4$, 20 mM Tween 40, and plated in PY medium.

Conjugative mobilization of plasmids from *E. coli* to *Rhizobium* was done by triparental mating using pRK2013 plasmid as a helper (Simon *et al.*, 1983).

DNA and RNA isolation and manipulation. Genomic DNA was isolated using the GenomicPrep cells and tissue DNA isolation kit (Amersham Biosciences) following the manufacturer's instructions. Plasmid DNA was isolated as described by Sambrook *et al.* (1989). Restriction enzymes and T4 DNA ligase were used as specified by the manufacturer (Invitrogen). *Pfu* and *Taq* DNA polymerase (Altaenzymes) were used for PCRs. RNAs were isolated using the High Pure RNA Isolation kit (Roche). All the primers used are listed in Table 2 (purchased from Unidad de Biosíntesis, Instituto de Biotecnología, Universidad Nacional Autónoma de México).

Plasmid construction. The 1.65 kb *R. etli rpoH1* and 2.02 kb *R. etli rpoH2* regions were amplified by PCR from the CE3 strain and cloned into the *Xba*I site of the pK18mobsacB plasmid (Schafer *et al.*, 1994) to give pJMS16 and pJMS17, respectively. pJMS18 is a pJMS16 derivative harbouring the *R. etli rpoH1* gene interrupted at the *Bam*HI site (at codon 250) by insertion of 2.3 kb *Bam*HI *loxP*Sp interposon from pJMS2 (Martínez-Salazar & Romero, 2000). pJMS19 is a pJMS17 derivative harbouring the *R. etli rpoH2* gene interrupted at the *Bcl*I site (at codon 10) by insertion of 2.3 kb *Bam*HI *loxP*Sp interposon from pJMS2. For both *R. etli rpoH1–uidA* and *rpoH2–uidA* transcriptional fusions, pBBMCS53 (Girard *et al.*, 2000) derivatives carrying the native promoters of *R. etli rpoH1* and *rpoH2* were constructed. A 477 bp PCR fragment containing 217 bp upstream and 260 bp of the *rpoH1* gene was cloned into the *Xba*I and *Sal*I sites of pBBMCS53 to give pGUSprpoH1. For the *rpoH2* promoter, a 657 bp fragment generated by PCR that carried 548 bp upstream and 109 bp of the *rpoH2* gene was cloned into the *Xba*I and *Sal*I sites of pBBMCS53 to give pGUSprpoH2. A 936 bp PCR fragment containing the *R. etli rpoH1* gene from CE3 was cloned into the *Pst*I and *Eco*RI sites of pRK415 (Keen *et al.*, 1988) to give pRK415ReH1. For *R. etli rpoH2*, an 894 bp fragment generated by PCR that carried the gene, was cloned into the *Bam*HI and *Eco*RI sites of pRK415 to give pRK415ReH2. An 885 bp PCR fragment containing the *E. coli rpoH* gene from MC4100 was cloned into the *Bam*HI and *Eco*RI sites of pRK415 to give pRK415EcH. The three *rpoH* genes were cloned downstream of the *lac* promoter present in pRK415, and no IPTG was used due to high basal expression in this vector.

Construction of *R. etli* mutants. The *R. etli rpoH1* and *rpoH2* mutants were obtained by gene replacement of the wild-type allele by *rpoH1*::*loxP*Sp (pJMS18) or *rpoH2*::*loxP*Sp (pJMS19) alleles, respectively. For this purpose, the corresponding plasmid was mobilized from *E. coli* to *R. etli* by triparental mating, and double recombinants were screened by $Sp^rSac^rKm^s$ phenotype. The *rpoH2 rpoH1* double mutant was generated by a two-step procedure. In the first step, the Sp^r marker was excised from the *loxP*Sp interposon in strain CFNXH2 (*rpoH2*::*loxP*Sp) by using the *loxP*-specific Cre recombinase located in pJMS8 (J. M. Martínez-Salazar and others, unpublished data). Losses of the Sp^r marker and pJMS8 were screened

Table 1. Strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
***R. etli* strains**		
CE3	Streptomycin-resistant, nodulates *P. vulgaris*	Noel *et al.* (1984)
CFNXH1	CE3 derivative, *rpoH1*::*loxP*Sp	This work
CFNXH2	CE3 derivative, *rpoH2*::*loxP*Sp	This work
CFNXH2lox	CEXH2 derivative, Sp deletion, *rpoH2*::*loxP*	This work
CFNXH2H1	CEXH2lox derivative, *rpoH1*::*loxP*Sp	This work
***E. coli* strains**		
DH5α	Host strain for plasmids	Hanahan (1983)
BW21038	*uidA* derivative	Metcalf & Wanner (1993)
S17.1	Host strain used for conjugation	Simon *et al.* (1983)
JMH1025	MC4100 Δ*rpoH*::kan derivative	Díaz-Acosta *et al.* (2006)
Plasmids		
pBBMCS53	Gm^r, replicable in *R. etli*, carrying a promoterless β-glucuronidase gene	Girard *et al.* (2000)
pRK2013	Conjugation helper plasmid, Km^r	Ditta *et al.* (1980)
pRK415	pRK290 derivative, Tc^r	Keen *et al.* (1988)
pK18mobsacB	Km^r, used for gene replacement	Schafer *et al.* (1994)
pJMS2	Plasmid harbouring the *loxP*Sp interposon	Martínez-Salazar & Romero (2000)
pJMS8	pRK7813 derivative, harbouring *cre* gene	J. M. Martínez-Salazar and others, unpublished data
pLG1	pBBMCS53 derivative carrying the *R. etli fixKf–uidA* transcriptional fusion	Girard *et al.* (2000)
pLG2	pBBMCS53 derivative carrying the *R. etli fixNf–uidA* transcriptional fusion	Girard *et al.* (2000)
pLG4	pBBMCS53 derivative carrying the *R. etli fixNd–uidA* transcriptional fusion	Girard *et al.* (2000)
pGUSprpoH1	pBBMCS53 derivative carrying the *R. etli rpoH1–uidA* transcriptional fusion	This work
pGUSprpoH2	pBBMCS53 derivative carrying the *R. etli rpoH2–uidA* transcriptional fusion	This work
pRK415ReH1	pRK415 derivative carrying a 936 bp fragment with the *R. etli rpoH1* gene	This work
pRK415ReH2	pRK415 derivative carrying an 894 bp fragment with the *R. etli rpoH2* gene	This work
pRK415EcH	pRK415 derivative carrying an 885 bp fragment with the *E. coli rpoH* gene	This work
pJMS16	pK18mobsacB derivative carrying a 1.65 kb fragment containing the *R. etli rpoH1* gene	This work
pJMS17	pK18mobsacB derivative carrying a 1.65 kb fragment containing the *R. etli rpoH2* gene	This work
pJMS18	pJMS16 derivative carrying the *R. etli rpoH1* gene interrupted at the *Bam*HI site (at codon 250) by insertion of 2.3 kb *Bam*HI *loxP*Sp	This work
pJMS19	pJMS17 derivative carrying the *R. etli rpoH2* gene interrupted at the *Bcl*I site (at codon 10) by insertion of 2.3 kb *Bam*HI *loxP*Sp	This work

by Sp^sTc^s phenotype. In a second step, the *rpoH1*::*loxP*Sp (pJMS18) allele was introduced into the CFNXH2lox strain (*rpoH2*::*loxP*) by a marker-exchange procedure.

Nodulation, nitrogen fixation determination and bacterial viability on nodules. *Phaseolus vulgaris* 'Negro Jamapa' seeds were surface-sterilized and germinated on sterile trays containing sterile vermiculite. Three-day-old seedlings were transferred to 1 l plastic pots containing sterile vermiculite and inoculated with the desired *R. etli* strain. Plants were kept in a culture room at 25 °C under a 12 h light/dark period, and watered with a nitrogen-free nutrient solution (Fahraeus, 1957). Acetylene reduction assays for nitrogenase activity were carried out as described by Romero *et al.* (1988). Nodules were surface-sterilized with NaOCl 2.0 % (w/v) and disrupted with 10 mM $MgSO_4$, 20 mM Tween 40, and dilution series were plated on PY medium.

Fluorescence microscopy by acridine orange (AO)/ethidium bromide staining. Nodule cell death was studied morphologically by using fluorescent DNA-binding dyes. AO stains DNA bright green, allowing visualization of the nuclear chromatin (Vento *et al.*, 1998). Nodules were disrupted in stabilization solution (50 mM PIPES, pH 6.9, 5 mM EGTA, 2 mM $MgSO_4$, 100 mM *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester and 0.1 % Triton X-100), stained with AO/ethidium bromide solution (10 μg AO ml^{-1}, 10 μg ethidium bromide ml^{-1}, in PBS), mixed gently, and then examined through a Zeiss LSM 510 Meta confocal microscope attached to an Axiovert 200 M inverted microscope, using an argon laser and an HFT UV 488/542/633 nm dual dichroic excitation mirror with an LP 505 nm emission filter for detection.

β-Glucuronidase activity measurements. The cultures were grown overnight in the presence of the appropriate antibiotic selection, and then diluted in fresh PY medium or MM to OD_{540} 0.01 and grown to exponential and stationary phases (about 12 and 24 h, respectively). Quantitative β-glucuronidase assays were performed with 4-nitrophenyl β-D-glucuronide substrate as described by Girard *et al.* (2000). Nodules were isolated and stained for β-glucuronidase activity using X-gluc (5-bromo-4-chloro-indolyl glucuronide) as substrate, as described by Pichon *et al.* (1992).

Transcription start site determination. Transcription start sites were mapped by means of 5′ rapid amplification of cDNA ends (RACE) kit version 2.0 (Invitrogen), as previously described

Table 2. Primers used in this work

Primer	Sequence (5′–3′)*	Relevant characteristics
RPOH1-UP	GCTCTAGACCGCTGCCGGAC GATATGGTG	Sense primer complementary to positions 217–239 upstream from *R. etli rpoH1*
RPOH1-LW	GCGTCGACCATCAGACCGACATTGCCTTC	Antisense primer complementary to positions 240–260 from *R. etli rpoH1*
RPOH1-LW2	GCATCTAGATGAAGCGCACCGAGC	Antisense primer complementary to positions 483–502 downstream from *R. etli rpoH1*
RPOH2-UP	GCTCTAGAGAGAATCAGCCCGAGCAGTCC	Sense primer complementary to positions 548–569 upstream from *R. etli rpoH2*
RPOH2-LW	GCGTCGACCGTCGTGATCTTTCCAGCGGG	Antisense primer complementary to positions 80–109 from *R. etli rpoH2*
RPOH2-LW2	ACTTCTAGATCGACAACAAGGTCGA	Antisense primer complementary to positions 556–575 downstream from *R. etli rpoH2*
ReRPOH1-UP	AACTGCAGAAGAAGGAGATCTTGCCG	Sense primer complementary to first 21 nt of *R. etli rpoH1*
ReRPOH1-LW	CGGAATTCTCAGGCCGTGGCTTCGACGACG	Antisense primer complementary to last 22 nt of *R. etli rpoH1*
ReRPOH2-UP	CGGGATCCAGAAGGAGATATCATATGTTCCGGAAGACG	Sense primer complementary to first 15 nt of *R. etli rpoH2*
ReRPOH2-LW	CGGAATTCTCACGCGTAGGC TGCCATATGC	Antisense primer complementary to last 22 nt of *R. etli rpoH2*
EcRPOH-UP	CGGGATCCAGAAGGAGATATCATATGACTGACAAAATGC-AAAGTTTAG	Sense primer complementary to first 22 nt of *E. coli rpoH*
EcRPOH-LW	CGGAATTCTTACGCTTCAATGGCAGCAGCGAAT	Antisense primer complementary to last 25 nt of *E. coli rpoH*
GUS-LW5	CGATCCAGACTGAATGCCCAC	Antisense primer complementary to positions 96–117 of *uidA*
GUS-LW4	GTAACATAAGGGACTGACCTGC	Antisense primer complementary to positions 28–49 of *uidA*
GUS-LW2	GCTTGGCGTAATCATGGTCAT	Antisense primer complementary to positions 1–21 of *uidA*
APP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	Complementary homopolymer tail primer used in 5′ RACE method

*Underlining denotes restriction sites.

(Ramírez-Romero *et al.*, 2006). The *R. etli* RNA was isolated from strains containing plasmid-borne transcriptional pGUSprpoH1 or pGUSprpoH2. Single-chain DNA (scDNA) was synthesized using the primer GUS-LW5 (Table 2). PCR amplification was accomplished by using a second antisense primer GUS-LW4 and a complementary homopolymer tail primer AAP (Table 2). When secondary bands were obtained, an additional PCR was done using AAP primer and antisense primer GUS-LW2 (Table 2). The PCR products were sequenced in an automatic 3730xl DNA sequencer (Applied Biosystems). The DNA sequencing reactions were performed using appropriate primers and BigDye Terminator kit version 3.1.

RESULTS

R. etli rpoH genes complement the heat-sensitive phenotype of an *E. coli rpoH* mutant

To determine whether the *R. etli rpoH* genes can sustain a heat-shock response, they were expressed under *lac* promoter control in *E. coli* JMH1025. This strain is unable to respond to an increase in temperature due to the deletion of the *rpoH* gene and cannot grow at 42 °C. When plasmids containing *R. etli rpoH1* or *rpoH2* were expressed in *E. coli* JMH1025, the strain was able to grow at 42 °C (data not shown). After heat shock (42 °C for 30 min), the survival fraction (ratio of viable cells at 42 °C with respect to 30 °C) was about 0.08 ± 0.08 when RpoH1 was present and 0.18 ± 0.14 for RpoH2. Even though these values were lower than those exhibited by *E. coli* JM1025 with its own *rpoH* gene on the plasmid (survival fraction of 0.36 ± 0.31), they were three orders of magnitude higher than in absence of any *rpoH* gene. These results show that both *rpoH1* and *rpoH2* are able to complement the *E. coli rpoH* mutant under heat-shock stress.

rpoH1 encodes the principal heat-shock sigma factor of *R. etli*

Considering that both *R. etli rpoH* genes partially complement the *E. coli rpoH* mutant, it is likely that their protein products mediate the heat-shock response in the cellular context of *R. etli*. To test this hypothesis, single (*rpoH1* and *rpoH2*) and double (*rpoH2 rpoH1*) mutants

were constructed by gene replacement at 25 °C to avoid secondary mutations (see Methods). Both single and double mutants were viable at 30 °C, the normal growth temperature of *R. etli*, and although we observed that *rpoH1* and *rpoH2 rpoH1* mutants grew slightly slower than the wild-type, no significant differences were determined. Therefore, the *R. etli* strains were grown at 25 °C in the subsequent experiments. Under heat-shock conditions (temperature shift to 42 °C for 30 min), the survival fraction of the *rpoH1* mutant was reduced to $3.7 \pm 2.2 \times 10^{-3}$ (a 270-fold reduction with respect to the wild type), whereas the *rpoH2* mutant remained unaffected (survival fraction 0.93 ± 0.07). Significantly, the *rpoH2 rpoH1* mutation decreased the survival fraction to $1.9 \pm 3 \times 10^{-4}$ (a 5200-fold reduction with respect to the wild-type; a 19-fold reduction with respect to the *rpoH1* mutant) after heat shock. These results indicate that RpoH1 has an important role in the heat-shock response, whereas RpoH2 has a minor role in this type of stress.

R. etli rpoH1 and *rpoH2* genes are involved in the oxidative stress response

In addition to its known role in protection against heat stress, RpoH has been implicated in the oxidative stress response (Bang *et al.*, 2005; Díaz-Acosta *et al.*, 2006). *Rhizobium*, like some pathogens, induces an oxidative burst when it invades plant roots (Santos *et al.*, 2001). In its free-living form, endogenous oxidative molecules are generated as by-products of aerobic metabolism. Therefore, the *R. etli rpoH* mutants were tested for sensitivity to H_2O_2 and NaOCl in exponential and stationary growth phases. The *R. etli* wild-type strain was more resistant to H_2O_2 in stationary phase (80 mM) than in the exponential growth phase (8 mM) (Fig. 1a, c). Under the same conditions, the *rpoH2 rpoH1* double mutant was shown to be sensitive to 8 mM H_2O_2 in the exponential growth phase (Fig. 1a), and also displayed a clear hypersensitivity to 80 mM H_2O_2 during the stationary phase (Fig. 1c). The *rpoH* single mutants did not generate significant differences in response to H_2O_2 in either the exponential or the stationary growth phase.

In the presence of 0.6 % NaOCl, the double mutant displayed a 100-fold viability reduction with respect to the wild-type in exponential growth phase (Fig. 1b), and a 10 000-fold reduction in stationary phase (Fig. 1d). The *rpoH1* single mutant was sensitive (1000-fold viability reduction with respect to wild-type) only in the stationary phase, whereas the *rpoH2* mutant did not show any appreciable phenotype under the conditions tested. These results indicate that both *R. etli* RpoH1 and RpoH2 are involved in resistance to oxidative stress generated by H_2O_2 and NaOCl.

RpoH2 is involved in the osmotic stress response

In the rhizosphere, bacteria are exposed to the detrimental effects of changes in salinity and osmolarity. Salinity affects the survival of *Rhizobium* in the soil, and may also inhibit

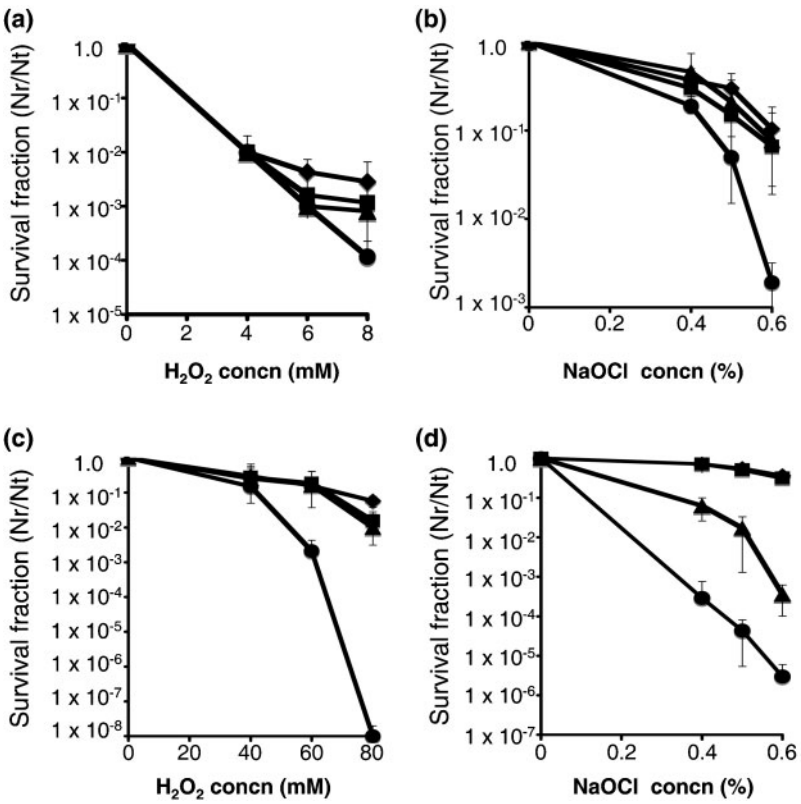


Fig. 1. Sensitivity of RpoH-deficient *R. etli* strains to H_2O_2 and NaOCl. Exponential (a, b) and stationary (c, d) cultures were incubated for 45 min with H_2O_2 (a, c) or NaOCl (b, d). The survival fraction is the number of viable cells after treatment (Nr) divided by the number of viable cells without treatment (Nt). The data are the mean of at least three independent experiments. CE3 strain, ◆; CFNXH2 (*rpoH2*::*loxP*Sp), ■; CFNXH1 (*rpoH1*::*loxP*Sp), ▲; CFNXH2H1 (*rpoH2*::*loxP*-*rpoH1*::*loxP*Sp), ●.

the initial steps of symbiosis (root colonization, infection and nodule development) and nitrogen fixation (Nogales *et al.*, 2002; Zahran, 1999). Moreover, *Rhizobium* must adapt to osmotic changes during the infection process and in nodules (Botsford & Lewis, 1990). For that reason, the *rpoH* mutants were tested for survival in NaCl and sucrose in early stationary growth phase. The *R. etli* wild-type and *rpoH1* mutant were resistant to both 80 mM NaCl and 15 % sucrose, whereas the *rpoH2* mutant showed a significantly lower viability with respect to the wild-type strain in both 80 mM NaCl and 15 % sucrose (1000-fold; Fig. 2). The *rpoH2 rpoH1* double mutant was as sensitive to NaCl and sucrose as the *rpoH2* mutant (Fig. 2). These results suggest that *R. etli* RpoH2 has a role in tolerance to the osmotic stress generated by NaCl and sucrose, unlike RpoH1, which does not display any detectable participation.

Role of *rpoH* genes in symbiosis

Earlier studies of the heat-shock response in *S. meliloti* have indicated that RpoH plays a role in symbiosis (Mitsui *et al.*, 2004; Oke *et al.*, 2001; Ono *et al.*, 2001). To investigate whether the *R. etli rpoH* genes participate in symbiosis, we tested the capability of the *R. etli rpoH* mutants to establish nitrogen-fixing nodules in bean plants. In these experiments, white nodules (associated with the absence of leghaemoglobin) in the *rpoH1* and *rpoH2 rpoH1* mutants were obtained, and the number of nodules was similar to that of wild-type strains. These results were obtained in several experiments with low variability, suggesting that the nodules induced by the mutants could not contain suppressor mutations (data not shown).

Twenty-one days after inoculation, the *rpoH1* and *rpoH2 rpoH1* mutants were able to nodulate but were defective in nitrogen fixation (Fig. 3). The *rpoH2* strain was able to form nodules and had a specific nitrogen fixation activity (21.91 ± 1.40) comparable to that of the wild-type strain (22.22 ± 4.65). The *rpoH2 rpoH1* mutant had nitrogen fixation activity 20-fold lower than that of the *rpoH1* mutant (0.05 ± 0.08 and 1.02 ± 0.05, respectively; Fig. 3), suggesting that the two genes act synergistically during symbiosis. Lack of nitrogen fixation capacity in the *rpoH1* mutant could be due to: (1) alteration of *nif* or *fix* gene expression; (2) modification of nodule development; and/or (3) low bacterial viability inside the nodule.

A low number of viable bacteria was recovered from nodules formed by *rpoH1* and *rpoH2 rpoH1* mutants in early and late symbiosis, and after 26 days bacterial viability in nodules generated by the *rpoH2 rpoH1* mutant was drastically affected. Conversely, nodules formed by the *rpoH2* mutant showed a viable cell number similar to that of the wild-type strain. Microscopy analysis showed that nodules produced by *rpoH1* (Fig. 4c) and *rpoH2 rpoH1* (Fig. 4d) mutants were prematurely senescent compared with the nodules generated by the *rpoH2* mutant (Fig. 4b) and wild-type (Fig. 4a) strains. These results suggest that RpoH1 plays a role in bacterial survival inside the nodule.

If RpoH1 takes part in nitrogen fixation, it might be possible to find sequences similar to RpoH promoters upstream of some *nif* or *fix* genes. As a matter of fact, we found sequences that resemble the *E. coli* RpoH consensus promoter upstream of the translation start of *fixNd*, *fixNf* (cytochrome oxidase genes located in p42d and p42f plasmids, respectively) and *fixKf* (an FNR transcriptional regulator located in p42f plasmid) (G. Lopez-Leal and others, unpublished data). To test whether RpoH family members are able to control the expression of these genes, plasmids containing *fixNd–uidA*, *fixKf–uidA* or *fixNf–uidA* transcriptional fusions were transferred into the wild-type, *rpoH1* and *rpoH2 rpoH1* strains. Since *fix* gene expression is controlled by oxygen tension (Fischer, 1994; Girard *et al.*, 2000), the activity of the fusions under aerobic and microaerobic (1 % oxygen) conditions was determined. As expected, in the wild-type strain, the three fusions had increased transcription in microaerobiosis with respect to aerobiosis (Fig. 5). In microaerobiosis, the expression of the *fixNf* and *fixKf* fusions was reduced to 50 % in both the *rpoH1* and the *rpoH2 rpoH1* mutants compared with wild-type, whereas the *fixNd* fusion maintained expression levels similar to those of the wild-type (Fig. 5b). Despite the reduced expression of the fusions under aerobic conditions, the absence of RpoH still produced an observable phenotype. *fixNd*, *fixNf* and *fixKf* gene expression was reduced to about 60 % in the *rpoH1* mutant, whereas no activity was found in the *rpoH2 rpoH1* mutant (Fig. 5a). These results suggest that RpoH1 has either a direct or an indirect influence on the regulation of *fixNf* and *fixKf* genes under aerobic and microaerobic conditions.

Gene expression of the *R. etli rpoH* genes

Given the above results, determination of the growth conditions under which *rpoH1* and *rpoH2* are expressed was desirable. This was achieved by measuring the β-glucuronidase activity using plasmids containing transcriptional fusions of *rpoH1–uidA* and *rpoH2–uidA*. Maximum expression of *rpoH1* and *rpoH2* was obtained in the

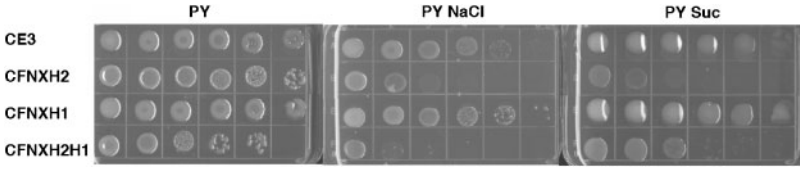
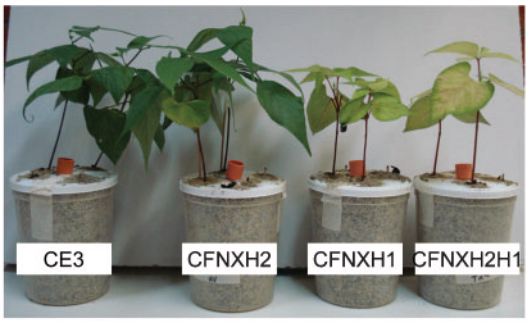
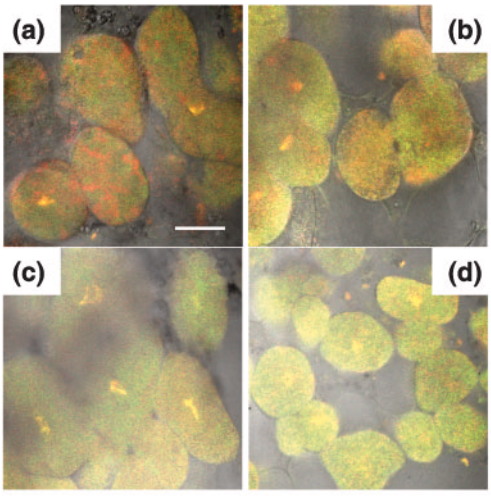


Fig. 2. Saline and osmotic tolerance of *R. etli rpoH* mutants. Serial dilutions of cells in early stationary growth phase were plated on PY (control), or PY containing 80 mM NaCl or 15 % sucrose.



S.A.	22.22	21.91	1.02	0.05
(SD)	(4.65)	(1.40)	(0.05)	(0.08)

Fig. 3. Role of *R. etli rpoH* genes in nodulation and nitrogen fixation. Plants growing for 21 days after inoculation with the strains are shown. Ethylene reduction was used to determine nitrogen fixation specific activity (S.A.; micromoles of ethylene per milligram of nodules per hour). The data are the mean of at least three independent experiments.



(e)

Strain	Median of bacterial viable cells inside the nodule at	
	18 days	26 days
CE3	*3.0 x 10^8	*3.4 x 10^8
CFNXH2	2.8 x 10^8	3.0 x 10^8
CFNXH1	*3.6 x 10^7	*5.2 x 10^7
CFNXH2H1	*1.2 x 10^7	*6.0 x 10^6

Fig. 4. Viability of nodule cells. Nodules of 18-day-old *P. vulgaris* colonized with (a) CE3, (b) CFNXH2 (*rpoH2*::*loxP*Sp), (c) CFNXH1 (*rpoH1*::*loxP*Sp) and (d) CFNXH2H1 (*rpoH2*::*lox-rpoH1*::*loxP*Sp) were stained with AO/ethidium bromide and observed by fluorescence microscopy (×63 magnification). Bar, 50 μm. (e) Numbers of viable bacteria inside nodules for wild-type and *R. etli rpoH* mutants. The data are the median of 10 nodules from at least three independent experiments. The asterisks indicate statistically significant differences according to the Kruskal–Wallis test at the 95 % confidence level.

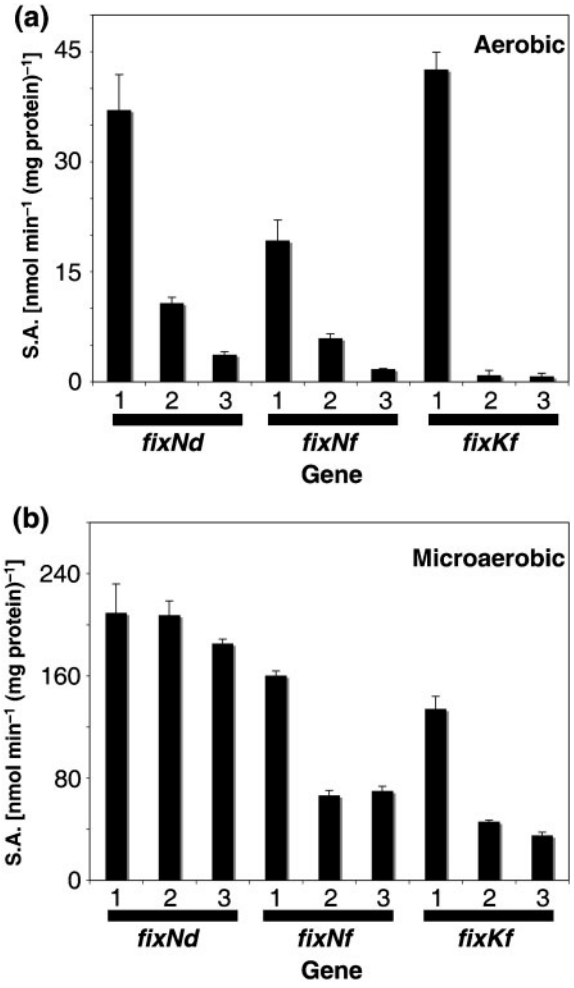


Fig. 5. *R. etli rpoH* genes regulate the expression of *fixKf*, *fixNd* and *fixNf* genes. CE3 (1), CFNXH1 (2; *rpoH1*::*loxP*Sp) and CFNXH2H1 (3; *rpoH2*::*lox-rpoH1*::*loxP*Sp) strains harbouring the plasmids pLG4 (*fixNd*), pLG2 (*fixNf*) and pLG1 (*fixKf*) in exponential-growth cultures under aerobic (a) and microaerobic (b) conditions were used to determinate β-glucuronidase specific activity [S.A.; nmol min^{-1} (mg protein)$^{-1}$]. The data are the mean of at least three independent experiments.

stationary growth phase in both rich and minimal media (Fig. 6). Moreover, the *rpoH1* fusion had a higher level of expression in PY than in MM, whereas the *rpoH2* fusion had similar expression levels in both media. In addition, a twofold increase in expression of *rpoH1* and *rpoH2* in minimal medium at low oxygen concentrations was observed (Fig. 6b, c). In contrast, a temperature shift from 25 to 42 °C for a period ranging from 45 min to 24 h did not have any effect on either *rpoH1* or *rpoH2* expression (Fig. 6, data not shown). As a whole, these results suggest that *rpoH1* and *rpoH2* are transcriptionally upregulated in the microaerobic stationary phase, even though *rpoH1* always had higher expression levels than *rpoH2*. In agreement with the effect of the *rpoH1* mutation on the

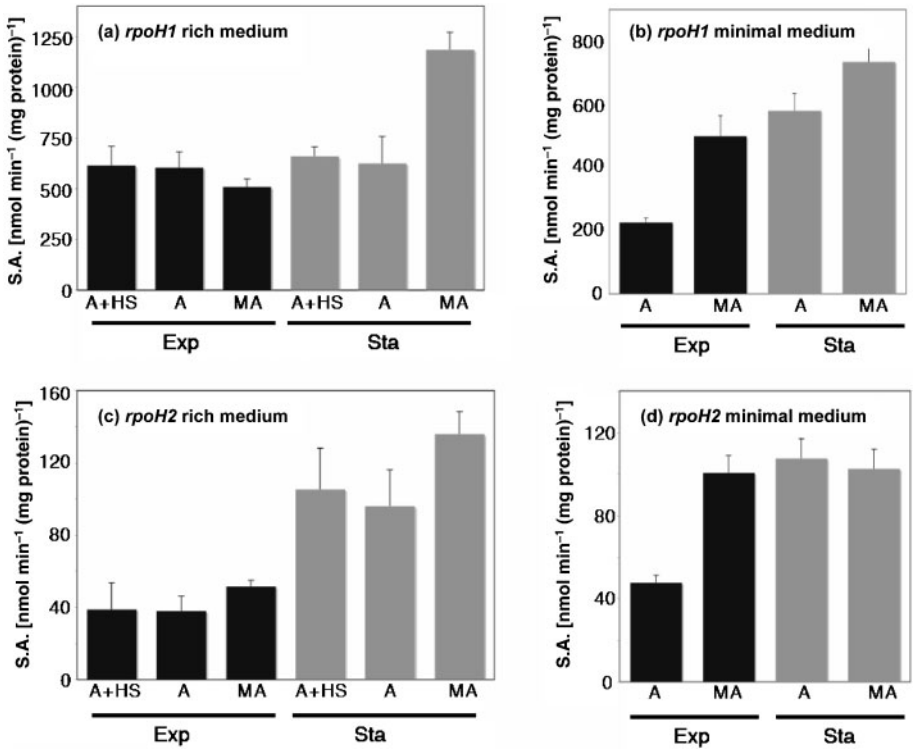


Fig. 6. Transcriptional expression of *R. etli rpoH1* and *rpoH2* genes under different growth conditions. The expression on rich medium (a, c) or minimal medium (b, d) for *rpoH1* (CE3/pGUSprpoH1) (a, b) and *rpoH2* (CE3/pGUSprpoH2) (c, d) is shown. The gene expression in exponential (Exp) and stationary phases (Sta) is indicated by black and grey bars, respectively. A+HS, heat shock at 42 °C for 45 min of aerobic growth cultures; A, aerobic growth cultures; MA, microaerobic growth cultures. The data are the mean of at least three independent experiments. β-Glucuronidase specific activity [S.A.; nmol min^{-1} (mg protein) $^{-1}$] was determined.

capacity of *R. etli* to form nitrogen-fixing nodules in beans, the *rpoH1–uidA* fusion was expressed within the nodule. Twenty-one days after inoculation, the nodule exhibited a clear β-glucuronidase activity, indicating that the *rpoH1* gene was expressed during symbiosis. Similarly, at the same post-inoculation time, the *rpoH2–uidA* fusion was also expressed in nodules, but at lower levels than the *rpoH1–uidA* fusion (data not shown).

Analysis of *rpoH1* and *rpoH2* promoter regions

To identify the probable promoters that control the expression of both *rpoH* genes, the transcription start sites of these genes were determined. This was achieved by isolating total RNA from the wild-type *R. etli* under the following conditions: aerobic–exponential, aerobic–stationary, microaerobic–exponential, microaerobic–stationary, and after heat-shock stress (45 min at 42 °C). Then, 5′ ends of mRNAs corresponding to *rpoH1* and *rpoH2* were identified by 5′ RACE. For *rpoH1*, two RT-PCR products were obtained, one abundant under all tested conditions (Fig. 7a, S1) and a second less abundant product observed only at 42 °C (Fig. 7a, S2). The main transcription start site (S1), previously reported by Ramirez-Romero *et al.* (2006),

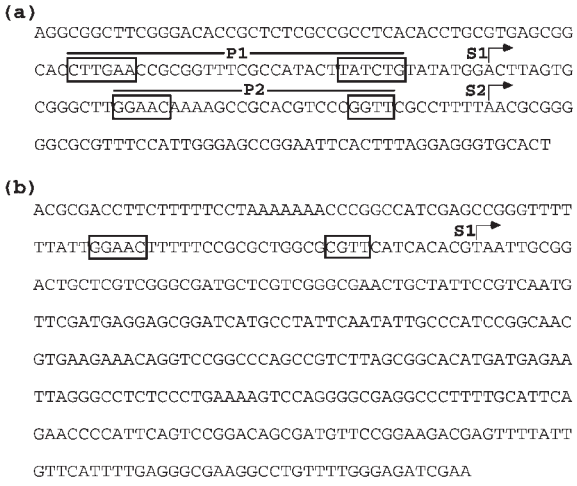


Fig. 7. Transcription start sites and putative promoters of *R. etli rpoH1* (a) and *rpoH2* (b) genes. Transcription start sites are marked with arrows and the letter S above the nucleotide. The −10 and −35 elements are indicated by boxes. The probable promoters recognized by σ^{70} (P1) and σ^{E} (P2) are delimited with lines.

was located at an A nucleotide 101 bp upstream of the translation start codon. Seven basepairs upstream of the transcription start site, an *R. etli* σ^{70} promoter (CTTGAA-N_{18}-TATCTG; P1) was identified. The second putative transcription start site (S2) corresponded to an A nucleotide located 53 nt upstream of the *rpoH1* start codon. We did not find any evidence of an *R. etli* σ^{70} promoter, but a sequence (GGAAC-N_{16}-GGTT, P2) that is identical in eight out of nine positions to the *S. meliloti* RpoE2 consensus promoter (Sauviac *et al.*, 2007) was situated at 8 bp upstream of S2 (Fig. 7a). These results suggest that *rpoH1* transcription is mainly controlled by σ^{70}, but transcripts could also be produced from the putative σ^{E} promoter at 42 °C. The absence of induction of the *rpoH1–uidA* fusion after heat shock could be the result of a differential expression from these promoters.

For the *rpoH2* gene, three conspicuous RT-PCR products were obtained under all conditions tested. The putative transcription start sites corresponded to a T nucleotide (S1), a C nucleotide (S2) and a T nucleotide (S3), located 287, 100 and 36 nt upstream of the gene, respectively. In this region the *R. etli* σ^{70} promoter sequence was not found; however, 9 bp upstream of S1, a perfect match with the *S. meliloti* RpoE2 promoter consensus (GGAAC-N_{16}-CGTT, P1) was identified, whereas for S2 and S3, no significant similarity to any promoter consensus was detected (Fig. 7b). The highest activity of *rpoH2* being achieved in stationary phase and the presence of a putative *S. meliloti* RpoE2 promoter suggest control by the σ^{E} equivalent or another related sigma factor in *R. etli*.

DISCUSSION

In α-proteobacteria genomes, it is common to find more than one *rpoH* gene. The phylogenetic relationship among the entire set of RpoH proteins from 52 α-proteobacterium species showed two well separated clades. Proteins more similar to *E. coli* RpoH and close to the Rickettsiales clade constitute the first group. The second group includes all the alternative RpoH2 proteins (data not shown). It is not clearly understood how these additional RpoH proteins contribute to the heat-shock response and whether they have a role in other cellular processes. In this work, the RpoH-dependent stress responses of *R. etli* CE3 mediated by *rpoH1* and *rpoH2* genes, their expression, and the role of these genes in symbiosis with the common bean, *P. vulgaris,* were characterized. Both *R. etli rpoH1* and *rpoH2* were able to complement the sensitivity to heat of an *E. coli rpoH* mutant, indicating that in *E. coli* both *R. etli rpoH* genes encode elements necessary for the heat-shock response. Similar results have been obtained with other *rpoH* genes from several α-proteobacteria (Delory *et al.*, 2006; Green & Donohue, 2006; Narberhaus *et al.*, 1997; Oke *et al.*, 2001; Ono *et al.*, 2001).

R. etli rpoH null mutants were obtained by gene replacement at 25 °C in order to avoid suppressor mutations. Both *rpoH1* and *rpoH2 rpoH1* mutations compromised seriously the cell survival after heat shock. A similar behaviour was observed when the mutants were exposed to oxidative compounds such as NaOCl and H_2O_2. Also, in *Brucella melitensis*, an *rpoH2* mutant was sensitive to oxidative stress (Delory *et al.*, 2006). Considering that the *R. etli rpoH2 rpoH1* double mutant was even more sensitive to heat shock and oxidative agents than the *rpoH1* single mutant, a synergistic effect might be necessary for a complete stress response. In contrast, the *R. etli* RpoH2 has a role in the tolerance to NaCl and sucrose without a clear participation of RpoH1. These results indicate a function for RpoH2 in the osmotic stress response. In *S. meliloti* strain 1021, an induction of *rpoH2* expression after osmotic stress has been reported (Domínguez-Ferreras *et al.*, 2006), and in *Sinorhizobium* sp. strain BL3, an *rpoH2* mutant is sensitive to salt stress (Tittabutr *et al.*, 2006). Additionally, in *Rhizobium* sp. strain Tal1145, exopolysaccharide biosynthesis and expression of *exo* genes are RpoH2-dependent (Kaufusi *et al.*, 2004). As a whole, these data suggest that in rhizobia, the *rpoH2* gene is implicated in different stress responses, depending on the species.

In the present study we found that *R. etli* was more resistant to oxidative stress in the stationary phase than in the exponential phase. These results are in agreement with those described for *E. coli* and *Rhizobium leguminosarum* bv. *phaseoli.* These bacteria are more resistant to stress (oxidants, pH, heat and osmotic shock) in the stationary phase than in the exponential growth phase (Díaz-Acosta *et al.*, 2006; Ishihama, 1997; Nystrom, 2004; Thorne & Williams, 1997). In *E. coli,* different mechanisms for stress resistance have been described; some of them are dependent on the stationary growth phase and on different sigma factors, σ^{32}, σ^{E} and σ^{S} (Bang *et al.*, 2005).

In the soil, *R. etli* deals with many environmental variations that could induce physiological survival responses. In the nodule, *Rhizobium* also encounters an oxidizing environment. It has been shown that inactivation of genes related to oxidative stress in *Rhizobium*, such as *katG* (catalase), *prxS* (peroxiredoxin) and *groELS* (chaperones), affects the symbiotic process (Bittner & Oke, 2006; Davies & Walker, 2007; Dombrecht *et al.*, 2005). It has been shown here that the *R. etli rpoH2* mutant had a Nod+Fix+ phenotype, while *rpoH1* and *rpoH2 rpoH1* mutants were able to nodulate *P. vulgaris* plants, causing a Nod+Fix− phenotype. In addition, both *rpoH1* and *rpoH2 rpoH1* mutants showed a reduction in viable cell number in early and late nodulation states. Lack of nitrogen fixation could be a consequence of the poor bacterial growth inside the nodule. In addition, nodules formed by *R. etli rpoH1* and *rpoH2 rpoH1* mutants showed premature senescence. This phenotype resembles the one obtained with *fix* mutants, which also render nodules prematurely senescent (Brewin, 1991). The premature nodule senescence could be a consequence of the low capacity of the bacteria to respond to oxidative stress,

although no satisfactory hypothesis has yet emerged. Looking for a connection between RpoH and nitrogen fixation, it was found that *fixNf* and *fixKf* expression was affected by *rpoH1* inactivation. Accordingly, both *rpoH1–uidA* and *rpoH2–uidA* fusions were expressed throughout the nodule, suggesting that RpoH proteins are involved in symbiosis. Consequently, it is proposed that both RpoH proteins are regulators that might connect the stress response with nitrogen fixation directly or indirectly; however, future experiments are needed to elucidate this function.

In *R. etli*, the *rpoH1* and *rpoH2* genes were expressed under all the conditions tested, and induced in the microaerobic stationary growth phase. Thus, the *R. etli rpoH* genes are induced by starvation and oxygen tension, which may be similar to the *Rhizobium* environment during nodule development. Moreover, *rpoH1* was expressed at high levels in rich-medium aerobiosis; this could be due to the fact that aerobic metabolism generates molecules that cause oxidative stress as by-products. In contrast, *rpoH1–uidA* and *rpoH2–uidA* expression was not affected after heat shock. In *S. meliloti*, similar results have been obtained in experiments with an *rpoH1–uidA* fusion (Oke *et al.*, 2001). These results could be due to the existence of one or more transcriptional control elements.

In *E. coli*, *rpoH* transcription is very sophisticated. There are five promoters upstream of the *rpoH* gene, recognized by σ^{70} (P1, P4 and P5), σ^{E} (P3) and σ^{54} (P6) (Erickson *et al.*, 1987; Janaszak *et al.*, 2007). In *R. etli*, upstream of the *rpoH1* gene, two promoter sequences have been identified. P1 is a strong promoter that resembles the −10 and −35 consensus boxes of the *R. etli* σ^{70} promoter (Ramírez-Romero *et al.*, 2006). Transcription from the P2 promoter was observed only in heat-shock stress. It has −10 and −35 boxes similar to the *S. meliloti* RpoE2 consensus promoter (Sauviac *et al.*, 2007). In *S. meliloti*, RpoE2 is activated in stationary phase and after heat shock. Considering that *R. etli rpoH1* transcription seems to be upregulated in microaerobic stationary phase, this expression is probably controlled by a σ^{E}. Further experiments are needed to clarify which promoters are being used to transcribe *rpoH1*, but the available evidence points to σ^{70} as the main element of *rpoH1* expression.

For the *R. etli rpoH2* gene, three 5′ RACE products were detected. They allow for the identification of one (P1) probable promoter identical to the *S. meliloti* RpoE2 promoter consensus sequence (Sauviac *et al.*, 2007). For the other putative transcription start sites, we were unable to detect sequences similar to known promoters. Recently, it has been reported that *S. meliloti rpoH2* is expressed under the control of RpoE2 (Sauviac *et al.*, 2007). Taking into consideration all data, we suggest that *R. etli rpoH2* is regulated by σ^{E}. Finally, based on our results, we propose that RpoH1 and RpoH2 are involved in different stress responses, and that this provides a major capacity to adapt to different environments.

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