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^{32} 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; Wösthen, 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 z-proteobacteria have two or three rpoH homologues. Two rpoH genes have been identified in

Abbreviations: AO, acridine orange; RACE, rapid amplification of cDNA ends.
**Role of rpoH sigma factors in Rhizobium etli**

**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 K2HPO4, 0.8 mM MgSO4, 10 mM succinic acid, 10 mM NH4Cl, 1.5 mM CaCl2 and 0.0005 % FeCl3, 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 (H2O2) (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 H2O2, 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 (OD600−0.4 and −1.4, respectively) and incubated with different concentrations of NaOCl or H2O2 for 45 min at 25 °C. After treatment, samples were diluted in 10 mM MgSO4, 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). Pet and Tag 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 Biosintesis, 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 XbaI site of the pK18mobsacB plasmid (Schafer et al., 1994) to give pLM15 and pLM16, respectively. pLM18 is a pLM16 derivative harbouring the *R. etli rpoH1* gene interrupted at the BamHI site (at codon 403) by insertion of 1.2 kb *BamHI*loxPSpI interposon from pLSM2 (Martinez-Salazar & Romero, 2000). pLM19 is a pLM17 derivative harbouring the *R. etli rpoH2* gene interrupted at the EcoRI site (at codon 10) by insertion of 2.3 kb *BamHI*loxPSpI interposon from pLSM2. 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 XbaI and SalI sites of pBBMCS53 to give pGUSprpoH1. For the *rpoH2* promoter, a 657 bp fragment generated by PCR that carried 267 bp upstream and 109 bp of the *rpoH2* gene was cloned into the XbaI and SalI sites of pBBMCS53 to give pGUSprpoH2. A 936 bp PCR fragment containing the *R. etli rpoH1* gene from CE3 was cloned into the PstI and EcoRI 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 BamHI and EcoRI sites of pRK415 to give pRK415ReH2. An 885 bp PCR fragment containing the *E. coli rpoH* gene from MC4100 was cloned into the BamHI and EcoRI 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::loxPSpI* (pLM18) or *rpoH2::loxPSpI* (pLM19) 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′Sac′ Km′ phenotype. The *rpoH2 rpoH1* double mutant was generated by a two-step procedure. In the first step, the Sp′ marker was excised from the *loxPSpI* interposon in strain CFNXH2 (rpoH2::loxPSpI) by using the *loxP*-specific Cre recombinase located in pLM88 (J. M. Martín-Salazar and others, unpublished data). Losses of the Sp′ marker and pLM88 were screened.
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 MgSO₄, 20 mM Tween 40, and dilution series were plated on PY medium. Exponential and stationary phases (about 12 and 24 h, respectively). Transcription start sites

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 by Pichon et al. (1992). 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 MgSO₄, 20 mM Tween 40, and dilution series were plated on PY medium.

Nodules were disrupted in stabilization solution (50 mM PIPES, pH 6.9, 5 mM EGTA, 2 mM MgSO₄, 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 OD₅₄₀ 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).

**Table 1.** Strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R. etli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CE3</td>
<td>Streptomycin-resistant, nodulates <em>P. vulgaris</em></td>
<td>Noel et al. (1984)</td>
</tr>
<tr>
<td>CFNXH1</td>
<td>CE3 derivative, <em>rpoH1</em>::loxPSp</td>
<td>This work</td>
</tr>
<tr>
<td>CFNXH2</td>
<td>CE3 derivative, <em>rpoH2</em>::loxPSp</td>
<td>This work</td>
</tr>
<tr>
<td>CFNXH2lox</td>
<td>CEXH2 derivative, Sp deletion, <em>rpoH2</em>::loxP</td>
<td>This work</td>
</tr>
<tr>
<td>CFNXH2H1</td>
<td>CEXH2lox derivative, <em>rpoH1</em>::loxPSp</td>
<td>This work</td>
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<td><strong>E. coli strains</strong></td>
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<tr>
<td>DH5α</td>
<td>Host strain for plasmids</td>
<td>Hanahan (1983)</td>
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<td>S17.1</td>
<td>Host strain used for conjugation</td>
<td>Simon et al. (1983)</td>
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<tr>
<td>JMH1025</td>
<td>MC4100 ΔrpoH::kan derivative</td>
<td>Díaz-Acosta et al. (2006)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pBBMCS53</td>
<td>Gm⁺, replicable in <em>R. etli</em>, carrying a promoterless β-glucuronidase gene</td>
<td>Girard et al. (2000)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Conjugation helper plasmid, Km⁺</td>
<td>Ditta et al. (1980)</td>
</tr>
<tr>
<td>pRK415</td>
<td>pRK290 derivative, Tc⁺</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>pK18mobsacB</td>
<td>Km⁺, used for gene replacement</td>
<td>Schafer et al. (1994)</td>
</tr>
<tr>
<td>pJMS8</td>
<td>pRK7813 derivative, harbouring cre gene</td>
<td>J. M. Martínez-Salazar and others, unpublished data</td>
</tr>
<tr>
<td>pLG1</td>
<td>pBBMCS53 derivative carrying the <em>R. etli</em> fixKf–uidA transcriptional fusion</td>
<td>Girard et al. (2000)</td>
</tr>
<tr>
<td>pLG2</td>
<td>pBBMCS53 derivative carrying the <em>R. etli</em> fixNF–uidA transcriptional fusion</td>
<td>Girard et al. (2000)</td>
</tr>
<tr>
<td>pLG4</td>
<td>pBBMCS53 derivative carrying the <em>R. etli</em> fixND–uidA transcriptional fusion</td>
<td>Girard et al. (2000)</td>
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<td>pGUSSporH1</td>
<td>pBBMCS53 derivative carrying the <em>R. etli</em> <em>rpoH1</em>–uidA transcriptional fusion</td>
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<td>pGUSSporH2</td>
<td>pBBMCS53 derivative carrying the <em>R. etli</em> <em>rpoH2</em>–uidA transcriptional fusion</td>
<td>This work</td>
</tr>
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<td>pRK415ReH1</td>
<td>pRK415 derivative carrying a 936 bp fragment with the <em>R. etli</em> <em>rpoH1</em> gene</td>
<td>This work</td>
</tr>
<tr>
<td>pRK415ReH2</td>
<td>pRK415 derivative carrying an 894 bp fragment with the <em>R. etli</em> <em>rpoH2</em> gene</td>
<td>This work</td>
</tr>
<tr>
<td>pRK415SeH</td>
<td>pRK415 derivative carrying an 885 bp fragment with the <em>E. coli</em> <em>rpoH</em> gene</td>
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<td>pJMS16</td>
<td>pK18mobsacB derivative carrying a 1.65 kb fragment containing the <em>R. etli</em> <em>rpoH1</em> gene</td>
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<tr>
<td>pJMS17</td>
<td>pK18mobsacB derivative carrying a 1.65 kb fragment containing the <em>R. etli</em> <em>rpoH2</em> gene</td>
<td>This work</td>
</tr>
<tr>
<td>pJMS18</td>
<td>pJMS16 derivative carrying the <em>R. etli</em> <em>rpoH1</em> gene interrupted at the BamHI site (at codon 250) by insertion of 2.3 kb BamHIloxPSp</td>
<td>This work</td>
</tr>
<tr>
<td>pJMS19</td>
<td>pJMS17 derivative carrying the <em>R. etli</em> <em>rpoH2</em> gene interrupted at the BclI site (at codon 10) by insertion of 2.3 kb BamHiloxyPSp</td>
<td>This work</td>
</tr>
</tbody>
</table>

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

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,
(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.

### Table 2. Primers used in this work

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'–3')*</th>
<th>Relevant characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPOH1-UP</td>
<td>GCTCTAGACCGCTGCCGGAC GATATGGTG</td>
<td>Sense primer complementary to positions 217–239 upstream from R. etli rpoH1</td>
</tr>
<tr>
<td>RPOH1-LW</td>
<td>GCCGTGACCATCAGACGAGCATTTGCTTC</td>
<td>Antisense primer complementary to positions 240–260 from R. etli rpoH1</td>
</tr>
<tr>
<td>RPOH1-LW2</td>
<td>GCATCTAGATGAAGCCGACCGGC</td>
<td>Antisense primer complementary to positions 483–502 downstream from R. etli rpoH1</td>
</tr>
<tr>
<td>RPOH2-UP</td>
<td>GCTCTAGAGAATCCAGCGAAGCTTTGCCG</td>
<td>Sense primer complementary to positions 548–569 upstream from R. etli rpoH2</td>
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<tr>
<td>RPOH2-LW</td>
<td>GCCGTGACGCTCGTTATTCCTGCCAGGG</td>
<td>Antisense primer complementary to positions 80–109 from R. etli rpoH2</td>
</tr>
<tr>
<td>RPOH2-LW2</td>
<td>ACTCTAGATGCCAACCAGGTGCA</td>
<td>Antisense primer complementary to positions 556–575 downstream from R. etli rpoH2</td>
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<tr>
<td>ReRPOH1-UP</td>
<td>AACTCGAGAACAGGAGATCTTGGCG</td>
<td>Sense primer complementary to first 21 nt of R. etli rpoH1</td>
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<tr>
<td>ReRPOH1-LW</td>
<td>CGGAATTCACGGCGTGCTGACGACG</td>
<td>Antisense primer complementary to last 22 nt of R. etli rpoH1</td>
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<tr>
<td>ReRPOH2-UP</td>
<td>CGGGATCCAGAAGGAGATATCATATGTTCGGAAGACG</td>
<td>Sense primer complementary to first 15 nt of R. etli rpoH2</td>
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<td>ReRPOH2-LW</td>
<td>CGGAATTCCTACGGCGTGCTGACGACG</td>
<td>Antisense primer complementary to last 22 nt of R. etli rpoH2</td>
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<tr>
<td>EcRPOH-UP</td>
<td>CGGGATCCAGAAGGAGATATCATATGTACGACAAATGC-AAAGTTTAG</td>
<td>Sense primer complementary to first 22 nt of E. coli rpoH</td>
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<tr>
<td>EcRPOH-LW</td>
<td>CGGAATTCCTACGGCGTGCTGACGACG</td>
<td>Antisense primer complementary to last 25 nt of E. coli rpoH</td>
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<td>GUS-LW5</td>
<td>CGATCCAGACTGAATGCCCAC</td>
<td>Antisense primer complementary to positions 96–117 of uidA</td>
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<td>GUS-LW4</td>
<td>GTAACATAAGGGGACTGACCTGC</td>
<td>Antisense primer complementary to positions 28–49 of uidA</td>
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<tr>
<td>GUS-LW2</td>
<td>GCTTGGCGTAATATGGCTCAT</td>
<td>Antisense primer complementary to positions 1–21 of uidA</td>
</tr>
<tr>
<td>APP</td>
<td>GGCCACGGCTGACTAGTACGGGIGGGIGGGIIGGGIIGG</td>
<td>Complementary homopolymer tail primer used in 5' RACE method</td>
</tr>
</tbody>
</table>

*Underlining denotes restriction sites.

**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 (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 \(0.93 \pm 0.07\)). Significantly, the *rpoH2 rpoH1* mutation decreased the survival fraction to \(1.9 \pm 3 \times 10^{-3}\) (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 \(\text{H}_2\text{O}_2\) and NaOCl in exponential and stationary growth phases. The *R. etli* wild-type strain was more resistant to \(\text{H}_2\text{O}_2\) 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 \(\text{H}_2\text{O}_2\) in the exponential growth phase (Fig. 1a), and also displayed a clear hypersensitivity to 80 mM \(\text{H}_2\text{O}_2\) during the stationary phase (Fig. 1c). The *rpoH* single mutants did not generate significant differences in response to \(\text{H}_2\text{O}_2\) 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 \(\text{H}_2\text{O}_2\) 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...
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, whereas the rpoH1 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 rpoH1 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

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**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.
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. 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.

### Fig. 4
Viability of nodule cells. Nodules of 18-day-old *P. vulgaris* colonized with (a) CE3, (b) CFNXH2 (rpoH2::loxPSp), (c) CFNXH1 (rpoH1::loxPSp) and (d) CFNXH2H1 (rpoH2::lox-rpoH1::loxPSp) 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.

<table>
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<th>Strain</th>
<th>Median of bacterial viable cells inside the nodule at</th>
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<tr>
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<td>18 days</td>
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<td>CE3</td>
<td>3.0 x 10⁹</td>
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<tr>
<td>CFNXH2</td>
<td>2.8 x 10⁹</td>
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<tr>
<td>CFNXH1</td>
<td>3.6 x 10⁹</td>
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<td>CFNXH2H1</td>
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### Fig. 5
*R. etli* rpoH genes regulate the expression of fixKf, fixNd and fixNf genes. CE3 (1), CFNXH1 (2; rpoH1::loxPSp) and CFNXH2H1 (3; rpoH2::lox-rpoH1::loxPSp) 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 determine β-glucuronidase specific activity [S.A.; nmol min⁻¹ (mg protein)⁻¹]. The data are the mean of at least three independent experiments.
capacity of \textit{R. etli} to form nitrogen-fixing nodules in beans, the \textit{rpoH1–uidA} fusion was expressed within the nodule. Twenty-one days after inoculation, the nodule exhibited a clear \(\beta\)-glucuronidase activity, indicating that the \textit{rpoH1} gene was expressed during symbiosis. Similarly, at the same post-inoculation time, the \textit{rpoH2–uidA} fusion was also expressed in nodules, but at lower levels than the \textit{rpoH1–uidA} fusion (data not shown).

**Analysis of \textit{rpoH1} and \textit{rpoH2} promoter regions**

To identify the probable promoters that control the expression of both \textit{rpoH} genes, the transcription start sites of these genes were determined. This was achieved by isolating total RNA from the wild-type \textit{R. etli} under the following conditions: aerobic–exponential, aerobic–stationary, microaerobic–exponential, microaerobic–stationary, and after heat-shock stress (45 min at 42 \(\degree\)C). Then, 5’ ends of mRNAs corresponding to \textit{rpoH1} and \textit{rpoH2} were identified by 5’ RACE. For \textit{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 \(\degree\)C (Fig. 7a, S2). The main transcription start site (S1), previously reported by Ramirez-Romero \textit{et al.} (2006), was determined.
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_{16}-TATCTG; P1) was identified. The second putative transcription start site (S2) corresponded to an A nucleotide located 53 nt upstream of the 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^54^ 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^54^ 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 Rickettsiases 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$_2$O$_2$. 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 (Dominguez-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 (Diaz-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^54^ and σ^70^ (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 aerobic; 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 ς70 (P1, P4 and P5), ς5 (P3) and ς35 (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 ς70 promoter (Ramirez-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 ς5. Further experiments are needed to clarify which promoters are being used to transcribe rpoH1, but the available evidence points to ς70 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 ς5. 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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