Rhizobium tropici response to acidity involves activation of glutathione synthesis

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**Rhizobium tropici** CIAT899 displays intrinsic tolerance to acidity, and efficiently nodulates *Phaseolus vulgaris* at low pH. By characterizing a *gshB* mutant strain, glutathione has been previously demonstrated to be essential for *R. tropici* tolerance to acid stress. The wild-type *gshB* gene region has been cloned and its transcription profile has been characterized by using quantitative real-time PCR and transcriptional gene fusions. Activation of the *gshB* gene under acid-stress conditions was demonstrated. *gshB* is also induced by UV irradiation. Upstream from *gshB* a putative σ70 promoter element and an inverted repeat sequence were identified, which are proposed to be involved in expression under neutral and acidic conditions, respectively. Gel retardation assays indicate that transcription in acid conditions may involve protein binding to an upstream regulatory region.

**INTRODUCTION**

Rhizobia are soil bacteria able to induce the formation of nitrogen-fixing nodules in symbiosis with different leguminous plants. As such, they are constantly challenged by a variety of stresses, including nutrient limitation and exposure to physical stresses, e.g. elevated temperature, high osmolarity, acidity, or oxidative shock. The capacity of *Rhizobium* spp. to adapt to these adverse conditions is fundamental for the establishment of an efficient symbiosis. More than one-quarter of the world’s cultivable soils are acidic, which makes the study of the mechanisms implied in the survival to acid stress of great agricultural relevance (Tiwari *et al.*, 1996a).

During the different steps of infection, leading to the formation of nitrogen-fixing nodules, the bacteria have to face different stressors. In the first steps of the recognition between the symbiotic partners, the plant produces a defence response against invasion, generating a low level of reactive oxygen species (ROS), such as O$_2^-$, which the bacteria have to overcome (Santos *et al.*, 2000). Once the bacterium has differentiated into a nitrogen-fixing bacteroid, a low level of ROS becomes a requirement for the functioning of the nitrogenase enzymes, which are highly oxygen sensitive. At the same time, ROS are continuously being generated by the high rate of respiration that is taking place (Escudero *et al.*, 1996). Different mechanisms of protection against these ROS have been described, which include catalases, superoxide dismutases, peroxidases and enzymes such as peroxiredoxin (Sigaud *et al.*, 1999; Santos *et al.*, 2000; Jamet *et al.*, 2003; Dombrecht *et al.*, 2005). Small molecules, such as ascorbate and glutathione (γ-glutamylcysteinylglycine), also participate in the protection of nodules against oxidative stress (Moran *et al.*, 2000; Iturbe-Ormaetxe *et al.*, 2001; Matamoros *et al.*, 2003). In *Sinorhizobium meliloti*, glutathione has recently been shown to play a fundamental role in growth and symbiotic efficiency (Harrison *et al.*, 2005). Acidity also becomes a challenge in nodule development, affecting both the bacteria and the plant, as well as their interaction. The level of ROS increases during nodule senescence (Matamoros *et al.*, 1999).

On the other hand, the tripeptide glutathione plays key roles in the physiology of bacterial cells. Indeed, glutathione ensures correct folding, synthesis, regulation and degradation of proteins, affords protection against oxidative stress, and participates in the detoxification of numerous xenobiotics, such as the electrophile methylglyoxal. It is the major low-molecular-mass thiol found in most organisms, where levels can reach concentrations exceeding 10 mM (Penninckx & Elskens, 1993; Sherrill & Fahey, 1998; Carmel-Harel & Storz, 2000; Noctor *et al.*, 2002; Allocati *et al.*, 2003; Lillig *et al.*, 2003; Neumann *et al.*, 2003).

We had previously reported the isolation and characterization of a *Rhizobium tropici* CIAT899 mutant in the *gshB* gene (strain CIAT899-13T2), encoding the enzyme glutathione synthetase, which catalyses the second step of the metabolic synthesis of glutathione (Ricciolo *et al.*, 2000). Growth of the...
mutant strain CIAT899-13T2 is sensitive to external and intracellular acidification. It is also sensitive to salt stress, oxidative stress and the electrophile methylglyoxal.

This work presents further analysis of the *R. tropici* gshB gene. The molecular characterization of the gshB and its 5’-upstream sequence is presented, and the responsiveness of the gene to acidity and other environmental stresses is shown. The studies described indicate that gshB is activated under acid-stress conditions and that the promoter sequence forms stable complexes with proteins.

**METHODS**

**Bacterial strains, plasmids and growth media.** Strains and plasmids used are described in Table 1. *R. tropici* strains were grown at 28°C in TY medium (Beringer, 1974) or minimal GTS medium (Kiss *et al.*, 1979). For acid-stress experiments, GMS minimal medium was used (pH 5.0 or 4.8 as stated) (Riccillo *et al.*, 2000). Antibiotics were added when needed: 400 μg streptomycin ml⁻¹, 100 μg neomycin ml⁻¹, 5 μg tetracycline ml⁻¹ and 50 μg gentamicin ml⁻¹. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth at 37°C supplemented with the required antibiotics: 100 μg ampicillin ml⁻¹, 25 μg kanamycin ml⁻¹, 10 μg tetracycline ml⁻¹, 5 μg gentamicin ml⁻¹. For complementation studies, cells were grown overnight in GTS and then diluted in fresh GTS or GMS medium to an OD600 of 0.3; below 5.1, a pH that does not allow growth of the mutant strain CIAT899-13T2. For UV induction experiments, cells were grown like those described for complementation studies to an OD600 of 0.1. Growth was followed by reading OD600. The pH of the GMS broth after 24 h incubation remained like those described for complementation studies to an OD600 of 0.3; 20 ml aliquots of culture were withdrawn and laid on the bottom of a sterile Petri dish and gently agitated. This volume was enough to form a thin film that covered the bottom of the Petri dish. Cultures were then exposed to UV radiation for 30 s with a laminar-flow UV lamp (G30T8 Germicidal) and after that the culture was transferred to a flask for further incubation under the conditions applied before irradiation. Appropriate aliquots were sampled over time. Except for the irradiation time, throughout the experiment cells were kept in the dark.

**DNA isolation and manipulations.** Total genomic *R. tropici* DNA isolation, restriction enzyme digestion, ligation, plasmid isolation and Southern blotting experiments were carried out according to procedures described previously (Sambrook *et al.*, 1989; Aguilar & Grasso, 1991).

**Cloning of the wild-type gshB gene region.** A *R. tropici* CIAT899 partial genomic library, of fragments larger than 5 kb, was constructed. Colony blotting was performed using Hybond-N+ membrane (Amersham Biosciences) according to the manufacturer’s instructions. A *R. tropici* gshB 500 bp DNA fragment was amplified and labelled by PCR using specific primers PR13T2R3 (Riccillo *et al.*, 2000) and Met (5’-CAACATGGGTTGATCC-3’), in the presence of 3.7 x 10⁶ Bq [α-³²P]dATP (1.11 x 10¹² Bq mmol⁻¹) (Amersham Biosciences) in the reaction mix, and used to probe the blots. A bacterial clone carrying a plasmid with a 7.5 kb *R. tropici* DNA insert was confirmed to have the gshB gene region. The recombinant plasmid was named pCM1. The 7.5 kb *R. tropici* DNA fragment from pCM1 was cloned in vector pSUP204, generating plasmid pCM2. A 3.2 kb Xbal–SalI fragment encompassing the gshB gene and ORF1 was subcloned from pCM2 into pSUP204 to yield plasmid pCM6.

**Construction of a mutant strain in ORF1.** An internal fragment of ORF1 was amplified with specific primers 418F (5’-CGGCAAAATAGGCGACATC-3’) and 418R (5’-ATTGCAT-GTCTTGAAACTGTA-3’), using pCM1 as template DNA, and cloned in vector pGem-T-Easy. The PCR fragment was then subcloned in plasmid pSUP102 and transferred by conjugation into strains *R. tropici* CIAT899 and CM11b. Single-event recombinants were selected in LB medium supplemented with tetracycline and alteration of the ORF1 sequence of these recombinants was confirmed by Southern blot analysis. Strain CM11b was constructed as follows. A BanHI fragment, which includes ORF1, was deleted from plasmid pCM11. This plasmid was used for conjugation into strain CIAT899 and single-event recombinants were selected.

**RNA isolation and primer extension.** Total RNA was isolated, using a phenol/chloroform/macaloid clay extraction as described by Raya *et al.* (1998); it was treated with RNase-free DNase (Promega) and repurified using TRIZOL reagent (Invitrogen), following the manufacturer’s instructions. The quality of the RNA obtained was assessed by examining the integrity of rRNA bands in 0.8% agarose gels. For primer extension analysis, approximately 20 μg RNA was mixed with the 33 base primer PrExt (5’-ATACAGCGCATAAGT-CGGCCTCCTGCTGATT-3’). This mixture was incubated at 85°C for 5 min and then cooled to 63°C. After this annealing procedure, extension products were generated with the avian myeloblastosis virus reverse transcriptase (Promega). Products of the primer extension reactions were labelled by including 3.7 x 10⁷ Bq [α-³²P]dATP (1.11 x 10¹² Bq mmol⁻¹) in the extension mixture (Amersham Biosciences) and then they were separated on sequencing gels.

**Construction of gene fusions between gshB and lacZ.** The Xbal–SalI DNA fragment from pCM6 was cloned in pK18Mob. The resulting plasmid was digested with Xhol and ligated to the lacZ-Gm cassette from plasmid pAB2001. The orientation of the inserted fragment was verified by restriction enzyme analysis. This plasmid was named pCM11. Plasmids were transferred to strain CIAT899 by conjugation, and transconjugants were selected on gentamicin-supplemented TY medium. Recombinants derived from the wild-type strain that resulted from a single crossover of plasmid pCM11 were named strain CM11. Plasmids containing the lacZ-Gm cassette inserted in a 3’–5’ orientation with respect to gshB were also transferred to strain CIAT899 to use as controls.

**Construction of fusions between the gshB promoter and lacZ.** Two PCR fragments of 84 bp and 152 bp were cloned in vector plasmid pCR 2.1-TOPO. The 84 bp fragment corresponds to the 5’-upstream region of gshB whereas the 152 bp fragment extends further upstream from the 5’-end of the 84 bp sequence to include the putative gshB promoter sequence described in this work. The primers used were forward primers F3 (5’-CTGAGAGACTTCTGT-TCGC-3’) and F31 (5’-GCCTCTGAGCAAACTTCC-3’), respectively, and the reverse primer R3 (5’-GGATTTGCGGAAATAGGCGACATC-3’) (the underlined bases denote nucleotide positions that were changed in order to generate Xhol restriction sites). The identity of the cloned fragments was confirmed by sequencing analysis. The fragments were then excised with EcoRI and cloned in the EcoRI site of vector pMP220. The orientation of the inserts was checked by sequencing analysis. Finally, the plasmids containing the desired fusions were introduced by conjugative mating into strain CIAT899.

**Site-directed mutagenesis.** The sequence corresponding to the 5’-arm of the hairpin structure was replaced by a *HindIII* recognition site. This was achieved by megaprimer PCR mutagenesis as described by Tyagi *et al.* (2004) using the forward primer Mut (5’-GACACAAGGTAAAGCTGCTGATCG-3’, where the underlined bases correspond to the introduced mutation) and primer Prom3 to generate the mutation, and primers Prom53 and Prom3 to amplify a
152 bp mutated fragment (named Mut), which was cloned in TOPO 2.1 vector. The correct mutation was confirmed by DNA sequencing of the cloned fragment. The Mut fragment was then cloned in plasmid pMP220 to create a transcriptional fusion between the Mut fragment and the lacZ sequence. This resulting plasmid (pCMMut) was introduced into strain CIAT899 as described above.

### Table 1. Strains and plasmids used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli DH5α</strong></td>
<td>supE44 ΔlacU169 (808 lacZAM15) hsdR17 recA endA1 gyrA96 thi-1 relA1</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td><strong>E. coli S17-1</strong></td>
<td>thi pro hsdR− hsdM+ recA carrying RP4 2-Tc::Mu integrated in the chromosome</td>
<td>Simon et al. (1983)</td>
</tr>
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<td><strong>R. tropici strains</strong></td>
<td></td>
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</tr>
<tr>
<td>CIAT899</td>
<td>Sm, wild-type strain</td>
<td>Martinez-Romero et al. (1991)</td>
</tr>
<tr>
<td>CIAT899 derivative strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIAT899-13T2</td>
<td>Sm Nm, gshB::Tn5-lacZAB mutant</td>
<td>Riccillo et al. (2000)</td>
</tr>
<tr>
<td>CM11</td>
<td>Sm Gm, Nm, gshB-lacZ transcriptional fusion</td>
<td>This work</td>
</tr>
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<td>CM11b</td>
<td>Sm Gm Nm, same as CM11 with a unique copy of ORF1</td>
<td>This work</td>
</tr>
<tr>
<td>CM20</td>
<td>Sm Tc, ORF1:pCM20</td>
<td>This work</td>
</tr>
<tr>
<td>CM21</td>
<td>Sm Tc Gm Nm, gshB-lacZ transcriptional fusion, ORF1:pCM20</td>
<td>This work</td>
</tr>
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<td>CM84D</td>
<td>Sm Tc, harbouring pCM84D</td>
<td>This work</td>
</tr>
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<td>CM84L</td>
<td>Sm Tc, harbouring pCM84L</td>
<td>This work</td>
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<td>CM152D</td>
<td>Sm Tc, harbouring pCM152D</td>
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</tr>
<tr>
<td>CM152L</td>
<td>Sm Tc, harbouring pCM152L</td>
<td>This work</td>
</tr>
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<td>CIAT899MC18</td>
<td>Sm Nm, guaB-lacZ transcriptional fusion</td>
<td>This laboratory</td>
</tr>
<tr>
<td>CIAT899Mut</td>
<td>Sm Tc, harbouring pMut</td>
<td>This work</td>
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<tr>
<td>CM15</td>
<td>CIAT899-13T2 derivative strain with a gshB-lacZ transcriptional fusion</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
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<tr>
<td>pAB2001</td>
<td>Gm Ap, contains lacZ-Gm cassette used for the construction of transcriptional fusions</td>
<td>Becker et al. (1995)</td>
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<td>pCM1</td>
<td>Ap, pIC20H wild-type gshB 7.5 kb EcoRI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pCM2</td>
<td>Tc, pSUP104 with 7.5 kb EcoRI fragment from pCM1</td>
<td>This work</td>
</tr>
<tr>
<td>pCM6</td>
<td>Tc, pSUP104 with XbaI–SalI DNA fragment from pCM1 which contains gshB gene region</td>
<td>This work</td>
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<td>pCM19</td>
<td>Ap, pGEM-T Easy with an ORF1 350 bp internal fragment</td>
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<td>pCM20</td>
<td>Tc, pSUP102 with an ORF1 350 bp internal fragment</td>
<td>This work</td>
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<td>pCM84D</td>
<td>Tc, pMP220 derivative, containing an 84 bp fragment from upstream gshB gene fused to lacZ, forming a transcriptional fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pCM84L</td>
<td>Tc, pMP220 derivative, containing an 84 bp fragment from upstream gshB gene fused to lacZ in a 3′- to 5′-orientation, forming a transcriptional fusion</td>
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</tr>
<tr>
<td>pCM152D</td>
<td>Tc, pMP220 derivative, containing a 152 bp fragment from upstream gshB gene fused to lacZ, forming a transcriptional fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pCM152L</td>
<td>Tc, pMP220 derivative, containing a 152 bp fragment from upstream gshB gene fused to lacZ in a 3′-5′ orientation, forming a transcriptional fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pCMMut</td>
<td>Tc, pMP220 derivative, containing 152 bp Mut fragment fused to lacZ, forming a transcriptional fusion</td>
<td>This work</td>
</tr>
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<td>pCR 2.1-TOPO</td>
<td>Ap, cloning vector with 3′-T overhangs for cloning</td>
<td>Invitrogen Life Technologies</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Ap, vector with 3′-T overhangs for cloning</td>
<td>Promega</td>
</tr>
<tr>
<td>pIC20H</td>
<td>Ap, lacZ vector with multiple cloning sites</td>
<td>Marsh et al. (1984)</td>
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<tr>
<td>pK18Mob</td>
<td>Km, pK18 derivative; mobilizable vector</td>
<td>Schafer et al. (1994)</td>
</tr>
<tr>
<td>pMP220</td>
<td>Tc, appropriate replicative vector for constructing transcriptional fusions in <em>Rhizobium</em></td>
<td>Spaenk et al. (1987)</td>
</tr>
<tr>
<td>pSUP102</td>
<td>Tc Cm, Mob+ suicide vector in <em>Rhizobium</em></td>
<td>Simon et al. (1986)</td>
</tr>
<tr>
<td>pSUP104</td>
<td>Tc Cm, Mob+ broad-host-range pACYC184 derivative</td>
<td>Priefer et al. (1985)</td>
</tr>
</tbody>
</table>

*Ap, Km, Gm, Nm, Sm and Tc denote ampicillin, kanamycin, gentamicin, neomycin, streptomycin and tetracycline resistance, respectively.*
Mobility-shift assays. R. tropici cells grown as described above were harvested at mid-exponential phase by centrifugation, washed and resuspended, as described by Schúman et al. (2003). Extracts were obtained with cells grown in GTS medium or with cells that had been harvested by centrifugation and resuspended in GMS medium for 10, 15, 20 and 30 min. The cells were lysed by sonication and the extracts were obtained by centrifugation at 8500 g for 10 min to remove large cell debris and unbroken cells. The supernatants were immediately frozen in liquid N₂ and stored at −80 °C. Protein concentration was determined by using the bicinchoninic acid assay kit from Sigma-Aldrich. Probes were prepared by PCR amplification of the 152 bp, the 84 bp and the Mut DNA fragments, respectively, in the presence of 3.7 × 10⁶ Bq [α-³²P]dCTP (1.11 × 10¹¹ Bq mmol⁻¹) (Amersham Biosciences) in the reaction mix. Crude cell extracts (containing 150 μg total protein) were incubated with the labelled DNA fragment for 15 min at room temperature in buffer A containing 16 μg poly-dIdC ml⁻¹ with the addition of 0.05 % (v/v) Nonidet P40 and 10 mM MgCl₂ as described by Shujman et al. (2003). The mixtures were separated by electrophoresis on 5 % native polyacrylamide gels. The gels were dried and subjected to autoradiography. Alternatively, gels were stained with SYBR Green (Roche). For the competitive binding experiment, the incubation mix contained a 100-fold molar excess of either competitor DNA. The shifted species was undetectable when experiments were run with 1000-fold dilution of whole cells extracts.

Quantitative determination of glutathione. Cellular glutathione content was determined by the method of Anderson (1985) using baker’s yeast glutathione reductase (Sigma-Aldrich).

Biochemical assays. β-Galactosidase specific activity (Miller units: ΔA₄₂₀ min⁻¹ per ml culture × 1000/OD₆₀₀) was assayed as described by Miller (1972). Cells were grown overnight in GTS medium, diluted in fresh GTS medium, and allowed to grow to an OD₆₀₀ of 0.3. Cells were harvested by centrifugation, resuspended in GMS medium for 10, 20, 30 and 60 min. TRIZOL reagent (Invitrogen LifeTechnology) was used for RNA purification according to instructions provided by the manufacturer. Samples were treated with RNase-free DNase (Promega) for 30 min at 37 °C. DNase inactivation was done 10 min at 75 °C. Reverse transcription was carried out using Superscript II reverse transcriptase and specific primers, for 1 h at 42 °C. Quantitative PCR was carried out with 5 μl of a 1/20 dilution of the cDNA, by using a Platinum SYBR Green qPCR Supermix kit (Invitrogen). The primers used for PCR reactions were gshFr₄5 (5'-TGTTCCAGGCTGTTGTCCAGTCA-3') and gshRr₄5 (5'-ATTGCGATGAGGCGGTTCAGAA-3'). Denaturation of cDNA (8 min at 95 °C) and 45 cycles of denaturation, annealing and elongation were run on an iCycler real-time PCR system (Bio-Rad). Relative transcript abundance was calculated on the basis of a standard curve that was included. 16S RNA was chosen as a reference for ratio normalization. Primers used for amplification of 16S cDNA were 16SF (5'-AACGCATTAAACACATTCGCTGCGG-3') and 16SR (5'-TAACGAGATGTCAAGGCGGTG-3'). Every reaction was done at least twice.

DNA sequence analysis. Sequencing of double-stranded plasmid DNA was performed using the dyeideoxy method of Sanger, using the Sequenase kit (US Biochemicals). Nucleotide sequences were analysed by using programs CLUSTALW version 1.8.1 (Higgins, 1994) and BCM Search Launcher (http://searchlauncher.bcm.tmc.edu) (Smith et al., 1996; Worley et al., 1995, 1998). DNA two-dimensional structure was analysed using mfold (Zuker, 2003; http://www.bioinfo.rpi.edu/applications/mfold/dnaform1.cgi). Algorithms available from server http://www.icgeb.trieste.it/dna were used to evaluate double helix stability parameters (Vlahovic et al., 2003) as described by Ramirez-Romero et al. (2006).

RESULTS

Cloning of the R. tropici CIAT899 gshB gene

In order to clone the wild-type gshB gene, an EcoRI CIAT899 genomic library was probed with a gshB DNA fragment. This analysis resulted in the isolation of a 7.5 kb EcoRI DNA fragment, and an internal 3.2 kb XbaI–SalI fragment was sequenced (GenBank accession no. DQ912168). Analysis of the sequence data revealed two ORFs, one of them corresponding to the gshB gene and the other, named ORF1, showing homology to a Rhizobium etli hypothetical protein (E-value of 10⁻⁶⁶) (accession no. NC_007761.1, locus YP_467870, 11). The 3.2 kb fragment was then subcloned into plasmid pSUP204 to yield plasmid pCM6, which was transferred into the mutant strain CIAT899-13T2. Transconjugants were assessed for their glutathione content and growth under acidic conditions. The glutathione level of transconjugants [85 ± 5 nmol (mg protein)⁻¹] was more than sixfold higher than that of the wild-type [14 ± 2 nmol (mg protein)⁻¹]. The transconjugants recovered the wild-type ability to grow in GMS medium at pH 4.8 (data not shown), demonstrating that the cloned fragment contained a functional gshB.

In order to test whether ORF1 exerts an effect on the transcription of gshB, we altered the ORF1 sequence in the wild-type strain R. tropici CIAT899, by constructing insertion mutants. Strain CM20, which represents one of these ORF1 mutants, was found to be tolerant to acid conditions as well as being phenotypically indistinguishable from the wild-type strain (results not shown). We also assayed β-galactosidase activity of strain CM11, which carries a gshB–lacZ chromosomal transcriptional fusion, and strain CM21, which is isogenic to the former but carries an insertion mutant. Strain CM20, which represents one of these ORF1 mutants, was found to be tolerant to acid conditions as well as being phenotypically indistinguishable from the wild-type strain (results not shown). We also assayed β-galactosidase activity of strain CM11, which carries a gshB–lacZ chromosomal transcriptional fusion, and strain CM21, which is isogenic to the former but carries a deletion within the ORF1 (AORF1, gshB–lacZ). The level of β-galactosidase was comparable between these isogenic strains, having either the wild-type ORF1 or its mutant allele (250 ± 15 and 245 ± 15 units for strain CM11 and CM21, respectively). These results indicate that transcription of gshB is independent of the upstream ORF.

Glutathione increases during acid shock

We have previously shown that glutathione is important for acid tolerance in R. tropici (Riccillo et al., 2000); therefore the cellular glutathione content of CIAT899 subjected to acid stress was determined over time. This analysis showed
that glutathione increased, about twofold, at 60 min (Table 2); it continued high for about 2 h, after which cells recovered their basal levels (not shown).

This finding led us to investigate the effect of overexpressing gshB on the cellular level of glutathione in acid-stress conditions. Strains CIAT899(pCM6) and CIAT899-13T2(pCM6), which containing gshB on the plasmid pCM6, which behaves as a multiple copy replicon, were assayed. The level of glutathione of the mutant strain was found to be seven times higher than that in the wild-type strain (Table 2). The glutathione content of the mutant was not affected by acid stress, indicating that the mechanism that leads glutathione to rise in acid conditions is tightly regulated.

**gshB transcription is induced by UV irradiation**

In order to test whether stresses other than acidity could be effectors of gshB expression, we examined the effect of UV radiation on gshB transcription. We measured β-galactosidase activity of strain CM11 immediately after UV exposure and found higher levels of β-galactosidase (Fig. 1). In order to assess the specificity of this response, we performed the same experiments with *R. tropici* CIAT899MC18, which has a guaB–lacZ chromosomal fusion. The guaB gene encodes the inosine monophosphate dehydrogenase enzyme required in the biosynthetic pathway of guanine. The β-galactosidase activity of strain CIAT899MC18 was unaffected by UV irradiation (Fig. 1), which demonstrates that the effect of UV irradiation is not a general cellular response. These results suggest that gshB is involved in the response to UV irradiation, and that glutathione may form part of the network that in *R. tropici* protects DNA from damage. Finally, we investigated whether gshB transcription is affected by osmotic and oxidative stress, resistance to which is known to be mediated by glutathione (Riccillo et al., 2000). Our results showed no response of gshB to these environmental constraints (data not shown).

**Activation of the *R. tropici* gshB gene by acidity**

In order to compare expression profiles of gshB under neutral and acidic conditions, we assayed the β-galactosidase activity of strain CM11. When strain CM11 was shifted to acidity, β-galactosidase activity increased – the peak of activity occurring 20–30 min after shifting – followed by a decrease to basal level (Fig. 2a). Sixty minutes after the shock, the level of β-galactosidase activity was similar to that detected in neutral growth medium. In addition, the same type of experiment was performed in a gshB genetic background by using strain CM15, which carries a chromosomal gshB–lacZ fusion. Under neutral growth conditions, the level of gshB transcription of the mutant strain was significantly higher than that of the wild-type strain, although the pattern of increase in response to acidity was similar in the two strains (data not shown). As in the case of UV irradiation, we performed a control experiment in which the same experimental treatment was applied to strain *R. tropici* CIAT899MC18 (guaB–lacZ fusion), and found that acidity had no effect on guaB transcription (Fig. 2a).

In order to confirm activation of gshB in response to acidic conditions, we used qRT-PCR to determine level of gshB

![Fig. 1. Effect of UV irradiation on the transcription of the *R. tropici* gshB gene, determined as β-galactosidase activity of the gshB–lacZ fusion. β-Galactosidase activity (Miller units) by strain CM11 in UV irradiated (○) and non-irradiated (●) cultures was assayed. As a control the same experiment was performed with a strain carrying a guaB–lacZ fusion; ▽ and ▼ indicate β-galactosidase levels of irradiated and non-irradiated cells, respectively. Standard deviations are indicated as vertical bars. The β-galactosidase activity of cells of CM11 from irradiated and non-irradiated cultures of strain was significantly different (P<0.05).](image_url)
Acid stress on the transcription of *Rhizobium tropici* gshB reporter gene

The effect of acid stress on the transcriptional activity of *gshB* gene in *R. tropici* CIAT899 was examined. Twenty minutes after acidic exposure, the level of *gshB* transcript had increased about fivefold as compared to time zero. Levels decreased afterwards (Fig. 2b). This profile is consistent with the results obtained with the *gshB*–*lacZ* fusion.

We also measured β-galactosidase activity in cells incubated in the presence of sodium acetate, which produces intracellular acidification (Perez-Galdona & Kahn, 1994; Roe et al., 1998) and found that *R. tropici* displays a positive response similar to that found in conditions of external acidification, with a threefold increase (Fig. 2c). These results add further evidence that acid stress is an inducer of the *R. tropici* *gshB* gene.

The effect of exogenous glutathione on the acid responsiveness of *gshB* was also examined. β-Galactosidase activity of rhizobia that were grown in media supplemented with different concentrations of glutathione followed by acid shock was determined. Under these conditions, the degree of activation of *gshB* decreased as glutathione in the medium increased, in a dose-dependent manner, and was undetectable in cells incubated in medium containing above 0.8 mM glutathione (data not shown). These findings indicate that exogenous glutathione overrides the *gshB* activation under acidic conditions. These results indicate that, in order to cope with acidity, *R. tropici* responds by raising its intracellular glutathione content, most likely by increasing the transcription of its biosynthetic genes.

**Analysis of the 5′-*gshB* upstream region**

It became evident from the preceding analysis that *gshB* is responsive to acid stress. Therefore, in order to characterize the *gshB* upstream region, we first determined the transcriptional initiation site by primer extension analysis using RNA template obtained from *R. tropici* CIAT899. As shown in Fig. 3(a), a signal was detected that corresponds to an adenine located 71 nucleotides upstream from the predicted transcriptional initiation site. The sequence of the upstream region is depicted in Fig. 3(b).

The sequence of the 5′-*gshB* upstream region, which contains four out of the six conserved bases of the canonical −10 box to represent a −35 sequence proposed in both reports. Located 14 bp upstream of the transcription start codon AUG. This upstream region was subjected to further analysis. Its comparison with the σ70 promoter sequences of *S. meliloti* and *R. etli* described by MacLellan et al. (2006) and Ramirez-Romero et al. (2006), respectively, revealed a putative −35 box (CCTGAG) which contains four out of the six conserved bases of the canonical −35 sequence proposed in both reports. Located 14 bp upstream of the transcription start site, a putative −10 element (CAAAAC) was identified, which has only two and one conserved bases of the canonical −10 box described for *S. meliloti* and *R. etli*, respectively. Although this sequence has a very low level of conservation, this seems to be common also for *R. etli* and *S. meliloti* −10 boxes (MacLellan et al., 2006; Ramirez-Romero et al., 2006). We calculated the double helix-stability for this region, and found the value for the proposed −10 box to represent a minimum of local stability, which is in agreement with the overall characteristics shown for these −10 sequences by...
Ramírez-Romero et al. (2006). The features found in the 5′-region of gshB indicate that the basal expression we detected in our assays may take place from a σ70 promoter element. Further experiments are needed to test this possibility.

Further analysis of the gshB upstream region was performed by using the mfold DNA web server (Zuker, 2003), which facilitates identification of potential secondary structures. Thus, a hairpin structure with an estimated value of ΔG approx. −9 kcal mol\(^{-1}\) (−38 kJ mol\(^{-1}\)) was detected between positions −28 and −50 (Fig. 3b). Inspection of this region revealed an 8 bp inverted repeat, which contains the sequence CTTGCCGCN6GCGGCAAG. The observation that this dyad structure and the predicted −35 box share part of the 5′-gshB upstream region suggested its involvement in gshB regulation.

In order to assess the significance of the putative promoter elements for the gshB expression, we next applied a functional approach. Thus, two DNA fragments of 84 bp and 152 bp each spanning part of the gshB upstream region were individually cloned to construct transcriptional fusions to the lacZ gene. These two fragments share their 3′-end at nucleotide +58, which fuses to the lacZ gene, whereas they differ in the 5′-end at positions −29 and −85 (Fig. 3b). In this way, plasmid pCM152D but not pCM84D carries the putative regulatory elements that were described above. These plasmids were transferred to R. tropici CIAT899 and the transconjugants obtained were used to determine β-galactosidase activity in GTS-grown cells (Fig. 4a). Significant activity was detected in cells carrying pCM152D whereas basal level of activity was detected with pCM84D. In addition, β-galactosidase activity produced by pCM152D was quantified in GTS-grown cells after exposure to GMS acid medium. After 20 min of acid exposure, β-galactosidase increased about threefold above the background level (Fig. 4b). These results indicate that the upstream sequence includes signals within the 152 bp fragment, needed for gshB to show an acid response.

**Investigation of the protein-binding ability of the gshB promoter**

The evidence described above, which indicated activation of the gshB promoter under acidic conditions, prompted us to investigate whether cell-free extracts from acid-treated R. tropici CIAT899 may contain proteins that bind to the promoter sequence. In order to examine this possibility, we performed gel mobility-shift assays.

The radioactive labelled 152 bp DNA fragment containing the promoter sequence was incubated with cell extracts of R. tropici CIAT899 grown in neutral and in acid media, and samples of each of these mixtures were then run on a polyacrylamide gel. The probe incubated with extracts obtained from neutral medium migrated similarly to the free probe. By contrast, a retardation band was observed with the extracts obtained from acid-treated cells. The retardation band was detectable by using extracts from cells that had been acid shifted for 10, 15, 20 and 30 min (Fig. 5a). This species was resistant to competition by excess of non-specific DNA (100-fold by weight), but not when the 152 bp fragment was used as competitor (data not shown).
We also assayed protein-binding ability of the 84 bp fragment described above, which was incubated with cell extracts from *R. tropici* CIAT899 grown in neutral media, and from cells that had been shifted and incubated for 20 min in acid media. The 152 bp fragment was included as a control. The results of these experiments are shown in Fig. 5(b). The 84 bp fragment migrated equally with extracts from either neutral or acid-treated cells, and it was indistinguishable from that of the free probe. In order to assess the importance of the imperfect inverted repeat for the formation of the 152 bp DNA fragment–protein complex we have detected, the inverted repeat was altered. The 5'-arm of the repeat was replaced by a *Hin* III restriction site, which prevents formation of the secondary structure, although it should be noted that the mutation does not alter the 235 box (Fig. 3b). The mutant fragment obtained was named Mut. Retardation assays were performed by using the Mut fragment as a probe. No DNA–protein complexes were detectable in this case (Fig. 5b). In addition, the effect of the mutation was assessed by in vivo experiments. The Mut fragment was also cloned in plasmid pMP220 to construct a Mut–*lacZ* transcriptional fusion, generating plasmid pCMMut, which was transferred by conjugation to strain CIAT899. We compared the β-galactosidase activity produced by cells containing the mutant plasmid pCMMut or the wild-type plasmid pCM152. Under conditions of neutral growth, we found no differences between plasmids pCMMut and pCM152.
However, β-galactosidase activity of cells carrying the wild-type plasmid pCM152, unlike the mutant pCMMut, was found to increase when cells were transferred to acid medium (data not shown), which demonstrated that integrity of the inverted repeat is needed in order to promote transcription under acidic conditions.

These results indicated that extracts of acid-treated cells contain protein(s) that bind the upstream region of gshB and that in order to show protein-binding properties, the integrity of the 8 bp inverted repeat is required. From our results with the transcriptional fusions, we propose this element to play a role in regulation of gshB expression in response to acidity.

**DISCUSSION**

In a previous report, we demonstrated that gshB is important for acid tolerance in *R. tropici* (Riccillo et al., 2000). It was proposed that glutathione may play a role in controlling the K⁺ flux and therefore enable cells to achieve the intracellular potassium levels needed to survive in low-pH environments (Ferguson & Booth, 1998; Riccillo et al., 2000; Masip et al., 2006). Without glutathione either produced by its own biosynthetic metabolism or supplied externally, *R. tropici* is unable to grow under acidic conditions. In this work, we present data on the regulation of the expression of the *R. tropici* gshB gene. Our results provide evidence that the intracellular level of glutathione is increased in response to low pH by a mechanism that involves the transcriptional activation of the gshB gene. Furthermore, if glutathione is supplied in the culture medium or alternatively over-produced from a gshB gene carried on a multicopy plasmid, there is no detectable effect of acidity on gshB transcription, suggesting that from this point of view the level of available glutathione is enough to overcome acid shock. These results reinforce the idea that glutathione participates in acid resistance in *R. tropici*.

Generally, the mechanisms microbes have to overcome acidity are known as acid resistance (AR), which has mainly been investigated in *E. coli*. In *Rhizobium*, several genes have been characterized as essential for AR (Tiwari et al., 1996a,b; Riccillo et al., 2000; Vinuesa et al., 2003; Rojas-Jiménez et al., 2005; Reeve et al., 2006). Genes involved in CO₂ fixation, hydantoin utilization and microaerobic respiration have been found in *Sinorhizobium medicae* to be regulated by the acid-sensitive ActS/ActR regulatory system (Fenner et al., 2004). By using a transcriptome approach, Tiwari et al. (2004) have reported genes of *S. medicae* to be induced about 2–25-fold by shifting the pH from 7.0 to 5.7. By applying alternative experimental procedures to determine transcriptional activity, namely gene fusion to lacZ and quantitative PCR, we have shown gshB transcription to increase under acidic conditions. It should be noted that β-galactosidase activation under acidic conditions was detectable at a lower level than that determined by qRT-PCR and that it declined after 1 h of exposure to acidity. We have no explanation for this phase of decline that follows activation, as the β-galactosidase protein should be expected to be stable. Taking these results altogether, it is possible to conclude that a small increase in the pattern of expression is still important to compensate environmental constraints. This observation suggests that following the acid shock in which early responses such as that of glutathione are detectable, other mechanisms may participate. Given the role of glutathione in acid tolerance and acid responsiveness, it is clear that gshB is an additional gene participating in the AR of *R. tropici*. Although several authors have shown the complex nature of the response caused by acidity, we believe this is the first report demonstrating the involvement of glutathione biosynthetic genes in such a response.

We found that gshB is induced by UV irradiation. Glutathione has previously been shown to be related to protection against UV irradiation, apparently by means of scavenging the ROS generated (Masip et al., 2006). Glutathione has also been involved in the modulation of the hydrogen peroxide-induced transcriptional activator OxyR, which may in turn protect against UV-induced oxidative stress (Masip et al., 2006). In *E. coli*, glutathione reductase levels have been demonstrated to be enhanced after a low UV dose (Hoerter et al., 2005). Although the *R. tropici* gshB mutant showed survival rates after UV exposure similar to those of the wild-type strain (data not shown), this may reflect the fact that mutants in the glutathione synthetase gene usually show high levels of γ-glutamylcysteine, the biosynthetic precursor of glutathione, which has also been proposed to act as an antioxidant (Harrison et al., 2005). Nevertheless, our data showing that gshB transcription increases after exposure to UV suggest that this gene could be part of the *R. tropici* SOS response (O’Reilly & Kreuzer, 2004).

The responsiveness of gshB to acidity led us to characterize its 5′-upstream sequence. Generally, prokaryotic transcriptional activation requires sites for binding the RNA polymerase and activators, which aligned at the promoter, interact with each other. We have identified a 152 bp fragment with promoter activity which is implied in gshB acid responsiveness. By sequence comparison, a putative σ70 has been identified within the 152 bp of the 5′-upstream region, which also overlaps a dyad symmetrical element. This fragment forms a stable DNA–protein complex with proteins present in extracts of acid-treated cells. The importance of this inverted repeat sequence is evidenced by the drastic negative effect that its alteration has on the protein-binding properties as well on the gshB activation after acid stress. These results indicate that the hairpin sequence we have identified is a key element in the gshB acid response. Although the understanding of the mechanisms by which gshB expression is controlled is still incomplete, we believe our data provide strong evidence of its regulation by acidity, and we speculate that under normal neutral media conditions, transcription takes place from the σ70 promoter whereas under acidic conditions the inverted repeat
element is important for gshB expression. Certainly, further experiments are needed to demonstrate this mechanism. Transcriptional activators that participate in the acid response were not identified in *S. meliloti* and *S. medicae* after extensive studies by Reeve et al. (2002) and Tiwari et al. (2004). More recently, Reeve et al. (2006) have described the transcriptional activation of gene *IpsA* of *S. medicae* by FsrR, which is involved in acidic conditions. In addition, positive regulation in other micro-organisms has been reported such as that of the two-component system ArsRS in up-regulating regulation in other micro-organisms has been reported such as that of the two-component system ArsRS in up-regulating some genes of *Helicobacter pylori* (Pfloek et al., 2006).

Summarizing, we have found that glutathione is essential for *R. tropici* to grow at low pH and that expression of acid tolerance involves transcriptional activation of the gshB gene in which protein(s) binds the 5′-upstream region, a feature in which inverted repeat sequences appear to be important. We are currently pursuing the identification of proteins present in acid-treated cells that have a regulatory role in gshB expression.

**ACKNOWLEDGEMENTS**

This work was supported by grants from Agencia de Promoción Científica y Tecnológica, Argentina (PICT no. 7072/99) and Consejo Nacional de Investigaciones Científicas y Tecnológicas-CONICET, Argentina. O.M.A. and C.I.M. were supported by CONICET, Argentina. The authors would like to thank Mónica Collavino for providing strain CIAT899MC18, Herman Spaink and Angeles Zurreguieta for providing plasmid pMP220, and Pablo Riccillo for the *R. tropici* CIAT899-13T2 strain and for the 3.2 kb EcoRI-fragment sequence. C.I.M. would like to thank Gustavo Schujman for his help with the mobility-shift assays.

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Edited by: C. W. Ronson