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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 σ^{70} 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 (Riccillo *et al.*, 2000). Growth of the

Abbreviations: qRT-PCR, quantitative real-time reverse transcriptase PCR; ROS, reactive oxygen species.

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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 pH 4.8 medium to an OD_{600} of 0.1. Growth was followed by reading OD_{600}. The pH of the GMS broth after 24 h incubation remained 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 OD_{600} 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′-CAACATTGCGGGTGATTCCAC-3′), in the presence of 3.7×10^5 Bq [α-^{32}P]dATP (1.11×10^{11} 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 *Xba*I–*Sal*I 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′-CCGGCAAAATAGGCGACATC-3′) and 418R (5′-ATTGCATG-GTCTTGAAACTGA-3′), using pCM1 as template DNA, and cloned in vector pGemT-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 *Bam*HI 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′-ATACCAGCCATAAGTC-AGCCCTCGCTCGGATTT-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 3.7×10^5 Bq [α-^{32}P]dATP (1.11×10^{11} Bq $mmol^{-1}$) in the extension mixture (Amersham Biosciences) and then they were separated on sequencing gels. The sequence reaction of the 5′-*gshB* region by using the primer PrExt was performed and the mixture was run side by side with the primer extension products.

Construction of gene fusions between *gshB* and *lacZ*. The *Xba*I–*Sal*I DNA fragment from pCM6 was cloned in pK18Mob. The resulting plasmid was digested with *Xho*I 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 F53 (5′-CTCGAGCAACTTCGT-TCGGC-3′) and F51 (5′-GCCTCTCGAGCAAACCTCC-3′), respectively, and the reverse primer R3 (5′-GGATTTGCCTCGAGATGTA-3′) (the underlined bases denote nucleotide positions that were changed in order to generate *Xho*I restriction sites). The identity of the cloned fragments was confirmed by sequencing analysis. The fragments were then excised with *Eco*RI and cloned in the *Eco*RI 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 *Hin*dIII recognition site. This was achieved by megaprimer PCR mutagenesis as described by Tyagi *et al.* (2004) using the forward primer Mut (5′-GCACACAGGGTAAGCTTGCTGAGTCG-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

Table 1. Strains and plasmids used

Strain	Relevant characteristics*	Reference
E. coli DH5α	*supE44* Δ*lacU169* (*ϕ*80 *lacZ*ΔM15) *hsdR17 recA endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
E. coli S17-1	*thi pro hsdR*$^-$ *hsdM*$^+$ *recA* carrying RP4 2-Tc::Mu integrated in the chromosome	Simon *et al.* (1983)
R. tropici strains		
CIAT899	Sm, wild-type strain	Martínez-Romero *et al.* (1991)
CIAT899 derivative strains		
CIAT899-13T2	Sm Nm, *gshB*::Tn*5-luxAB* mutant	Riccillo *et al.* (2000)
CM11	Sm Gm, Nm, *gshB–lacZ* transcriptional fusion	This work
CM11b	Sm Gm Nm, same as CM11 with a unique copy of ORF1	This work
CM20	Sm Tc, ORF1 : pCM20	This work
CM21	Sm Tc Gm Nm, *gshB–lacZ* transcriptional fusion, ORF1 : pCM20	This work
CM84D	Sm Tc, harbouring pCM84D	This work
CM84L	Sm Tc, harbouring pCM84L	This work
CM152D	Sm Tc, harbouring pCM152D	This work
CM152L	Sm Tc, harbouring pCM152L	This work
CIAT899MC18	Sm Nm, *guaB–lacZ* transcriptional fusion	This laboratory
CIAT899Mut	Sm Tc, harbouring pMut	This work
CM15	CIAT899-13T2 derivative strain with a *gshB–lacZ* transcriptional fusion	This work
Plasmids		
pAB2001	Gm Ap, contains *lacZ*-Gm cassette used for the construction of transcriptional fusions	Becker *et al.* (1995)
pCM1	Ap, pIC20H wild-type *gshB* 7.5 kb *Eco*RI fragment	This work
pCM2	Tc, pSUP104 with 7.5 kb *Eco*RI fragment from pCM1	This work
pCM6	Tc, pSUP104 with *Xba*I–*Sal*I DNA fragment from pCM1 which contains *gshB* gene region	This work
pCM19	Ap, pGEM-T Easy with an ORF1 350 bp internal fragment	This work
pCM20	Tc, pSUP102 with an ORF1 350 bp internal fragment	This work
pCM84D	Tc, pMP220 derivative, containing an 84 bp fragment from upstream *gshB* gene fused to *lacZ*, forming a transcriptional fusion	This work
pCM84L	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	This work
pCM152D	Tc, pMP220 derivative, containing a 152 bp fragment from upstream *gshB* gene fused to *lacZ*, forming a transcriptional fusion	This work
pCM152L	Tc, pMP220 derivative, containing a 152 bp fragment from upstream *gshB* gene fused to *lacZ* in a 3′–5′ orientation, forming a transcriptional fusion	This work
pCMMut	Tc, pMP220 derivative, containing 152 bp Mut fragment fused to *lacZ*, forming a transcriptional fusion	This work
pCR 2.1-TOPO	Ap, cloning vector with 3′-T overhangs for cloning	Invitrogen Life Technologies
pGEM-T Easy	Ap, vector with 3′-T overhangs for cloning	Promega
pIC20H	Ap, *lacZ* vector with multiple cloning sites	Marsh *et al.* (1984)
pK18Mob	Km, pK18 derivative; mobilizable vector	Schafer *et al.* (1994)
pMP220	Tc, appropriate replicative vector for constructing transcriptional fusions in *Rhizobium*	Spaink *et al.* (1987)
pSUP102	Tc Cm, Mob$^+$ suicide vector in *Rhizobium*	Simon *et al.* (1986)
pSUP104	Cm Tc, Mob$^+$ broad-host-range pACYC184 derivative	Priefer *et al.* (1985)

*Ap, Km, Gm, Nm, Sm and Tc denote ampicillin, kanamycin, gentamicin, neomycin, streptomycin and tetracycline resistance, respectively.

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.

Mobility-shift assays. *R. tropici* cells grown as described above were harvested at mid-exponential phase by centrifugation, washed and resuspended, as described by Schujman *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 N_2 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 3.7×10^5 Bq [α-^{32}P]dCTP (1.11×10^{11} 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 $MgCl_2$ 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 specific (the 152 bp fragment) or non-specific (poly-dIdC) unlabelled 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: ΔA_{420} min^{-1} per ml culture × 1000/OD_{600}) 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 OD_{600} of 0.3. Cells were harvested by centrifugation, resuspended in GMS medium pH 5.0, incubated at 28 °C with agitation and sampled over time for 24 h. In intracellular acidification studies, cells were grown as before, except that at OD_{600} 0.3 the medium was supplemented with sodium acetate to a final concentration of 7 mM and sampled over time. Effects of methylglyoxal, osmotic and oxidative challenges were assayed as described by Riccillo *et al.* (2000). All data shown represent the means of at least three replicate experiments. Assays were performed at least three times in independent experiments. Error bars represent standard deviations of replicate values obtained in an experiment. Data were analysed using Student's *t*-test with $P<0.05$.

Quantitative real-time reverse transcriptase PCR (qRT-PCR). RNA for reverse transcription was isolated from cells grown in GTS medium or cells that have been shifted to GMS medium and incubated 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 gshbFrt (5′-TCGTCACCGAATTTGCCGATCTCA-3′) and gshbRrt (5′-ATTGCCGTAGAGCGGCTTCAGAAT-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 rRNA was chosen as a reference for ratio normalization. Primers used for amplification of 16S cDNA were 16SF (5′-AACGCATTAAACATTCCGCCTGGG-3′) and 16SR (5′-TAACACAGGATGTCAAGGGCTGGT-3′). Every reaction was done at least twice.

DNA sequence analysis. Sequencing of double-stranded plasmid DNA was performed using the dideoxy 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/dna/form1.cgi). Algorithms available from server http://www.icgeb.trieste.it/dna were used to evaluate double helix stability parameters (Vlahovicek *et al.*, 2003) as described by Ramírez-Romero *et al.* (2006).

RESULTS

Cloning of the *R. tropici* CIAT899 *gshB* gene

In order to clone the wild-type *gshB* gene, an *Eco*RI CIAT899 genomic library was probed with a *gshB* DNA fragment. This analysis resulted in the isolation of a 7.5 kb *Eco*RI DNA fragment, and an internal 3.2 kb *Xba*I–*Sal*I 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^{-60}) (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 a deletion within the ORF1 (ΔORF1, *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

Table 2. Effect of acid shock on the glutathione content of cells with multiple copies of *gshB*

Cells were grown to exponential phase in GTS medium and resuspended in GMS (pH 5) or GTS medium at time zero. Samples were periodically withdrawn, and their glutathione content was measured.

Minutes after acid shock	Cellular glutathione content [nmol glutathione (mg protein)$^{-1}$]					
	CIAT899		CIAT899(pCM6)		CIAT899-13T2(pCM6)	
	GTS	GMS	GTS	GMS	GTS	GMS
0	13±3	13±3	96±5	94±4	83±5	80±3
30	10±2	21±3	95±4	95±3	85±5	84±4
60	11±3	26±2	96±5	91±5	80±3	82±5

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*

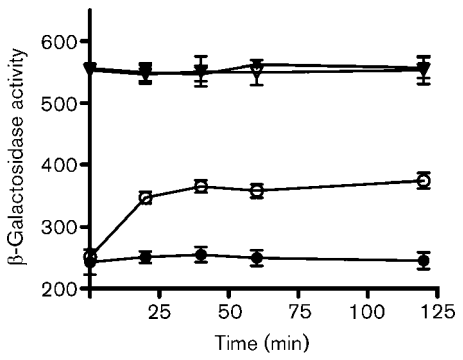

Fig. 1. Effect of UV irradiation on the transcription of the *R. tropici gshB* gene, determined as β-galactosidase activity of the *gshB–lacZ* fusion. β-Galactosidase activity (Miller units) by strain CM11 in UV irradiated (○) and non-irradiated (●) cultures was assayed. As a control the same experiment 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 ($P<0.05$).

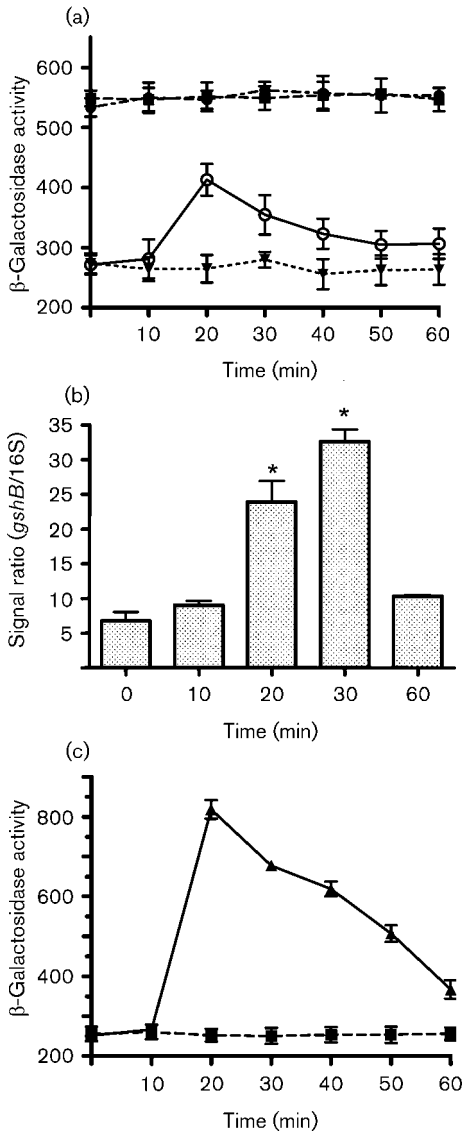

Fig. 2. Effect of acid stress on *gshB* expression. (a) β-Galactosidase activity (Miller units) in strains CM11 and CIAT899CM18, which were separately grown to exponential phase in GTS medium and resuspended in GMS medium (pH 5) at time zero (● and ○, respectively); control samples were resuspended in GTS (▼ and ■, respectively). (b) Expression level of *gshB* was measured by qRT-PCR in cells of strain CIAT899 that had been grown in GTS medium or that had been exposed to GMS medium (pH 5) for 10, 20, 30 or 60 min. Values were normalized to the constitutively expressed gene 16S rRNA. Asterisks on columns show values that were similar to each other, but significantly different from the rest ($P<0.05$). (c) β-Galactosidase activity of strain CM11 grown as in (a) except that sodium acetate to a final concentration of 7 mM was added at time zero (▲). A control of untreated cells was included (■). Standard deviations are indicated as vertical bars. The β-galactosidase activity of strain CM11 from GTS and GMS media (a) and from cells treated and not treated with sodium acetate (c) was significantly different ($P<0.05$).

transcript in wild-type cells incubated in neutral or in acidic medium. As shown in Fig. 2(b), acidity induced significant changes in *gshB* transcription. 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 (Pérez-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 translational start codon AUG. This upstream region was subjected to further analysis. Its comparison with the σ^{70} promoter sequences of *S. meliloti* and *R. etli* described by MacLellan *et al.* (2006) and Ramírez-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 (CAAACC) 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; Ramírez-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

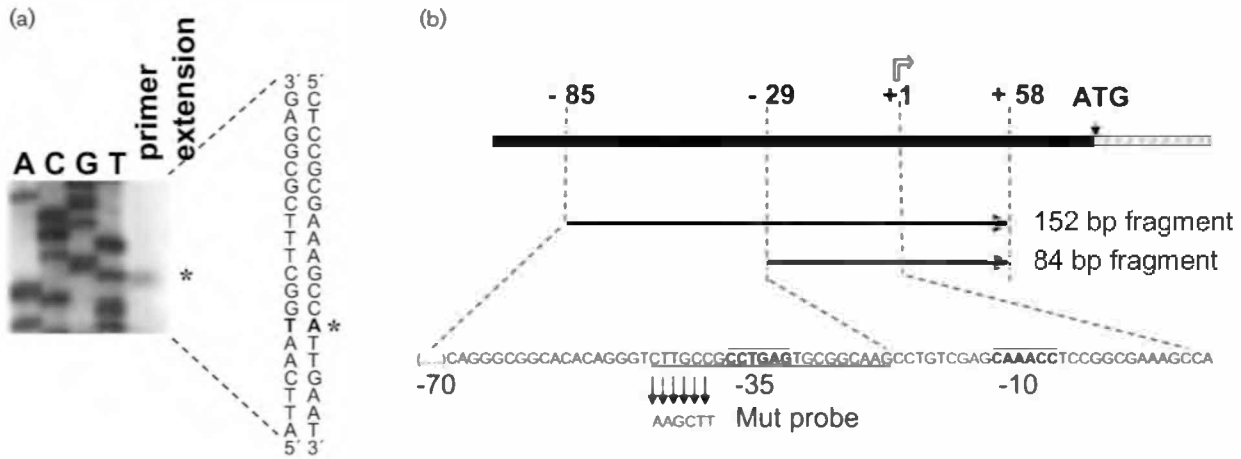

Fig. 3. Analysis of the 5′-*gshB* upstream region. (a) Primer extension analysis was performed to determine the 5′-end of the *gshB* transcript. Lanes A, C, G and T represent the DNA sequencing ladder. The transcriptional start point is indicated with an asterisk. (b) Schematic drawing of the upstream region of *gshB*. Fragments amplified for cloning (152 and 84 bp, respectively) or used as probes for the electrophoretic mobility-shift assays (152 and 84 bp, respectively) are shown with black arrows. Nucleotide positions are numbered relative to the transcription start site. The transcription start site is indicated with a grey arrow (+1). The hairpin structure mentioned in the text is underlined. The −35 and −10 elements are indicated in bold type and overlined. The sequence that was mutated in order to generate Mut probe is shown below the corresponding modified bases.

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 σ^{70} 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 CTTGCCGCN$_6$GCGGCAAG. 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).

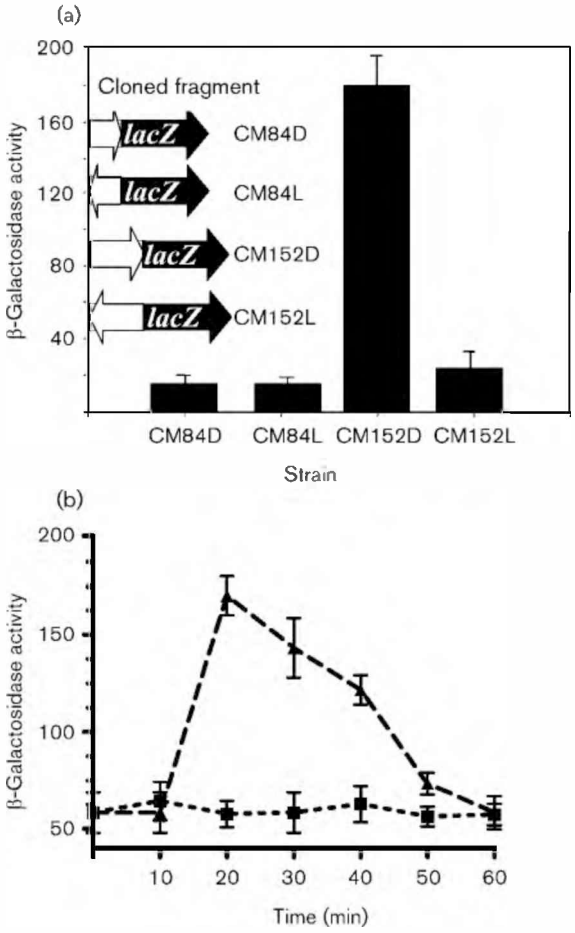

Fig. 4. Functional analysis of the *gshB* upstream region. (a) Levels of β-galactosidase activity (Miller units) were assayed for strains carrying *lacZ* fused to a 152 bp (CM152D) or 84 bp (CM84D) DNA fragment of the *gshB* upstream region. Strains CM152L and CM84L carrying respectively the same regions fused to *lacZ* in a 3′–5′ orientation were also assayed. The black arrow represents the *lacZ* gene, the white arrow the fused fragment. (b) Cells of strain CM152D were grown to exponential phase in GTS medium and resuspended in GMS (pH 5) at time zero; β-galactosidase activity was determined over time (▲). Control samples were resuspended in GTS (■). Standard deviations are indicated as vertical lines. The β-galactosidase activity of strain CM152D in GTS and GMS media was significantly different ($P<0.05$).

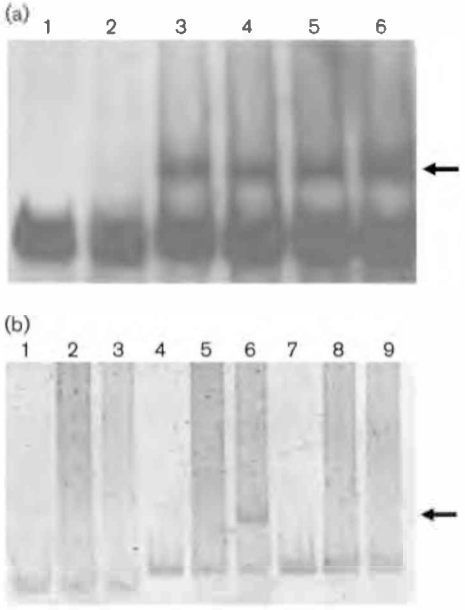

Fig. 5. Gel mobility-shift assays with the *R. tropici gshB* promoter. (a) The 152 bp DNA probe, which was labelled with [^{32}P]dCTP, was incubated with cell extracts prepared from GTS medium (lane 2) and from cells that had been resuspended in GMS medium (pH 5) and incubated for 10, 15, 20 and 30 min, respectively (lanes 3–6). The migration of the free probe is shown in lane 1. (b) The DNA fragments were incubated with lysates prepared from cells grown in GTS medium (lanes 2, 5 and 8, for the 84, 152 bp and Mut fragments, respectively) and from cells that had been acid-shifted for 20 min (lanes 3, 6 and 9, for the 84, 152 bp and Mut fragments, respectively). The migration of the free probe incubated in the absence of cell extract is shown in lanes 1 (84 bp fragment), 4 (152 bp fragment) and 7 (Mut fragment). The gel was SYBR Green stained. The arrows indicate the retardation species described in the text.

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*dIII restriction site, which prevents formation of the secondary structure, although it should be noted that the mutation does not alter the −35 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 CO_2 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 σ^{70} 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 places from the σ^{70} 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 trancriptional activation of gene *lpiA* 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 some genes of *Helicobacter pylori* (Pflock *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.

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