A histidine kinase sensor protein gene is necessary for induction of low pH tolerance in *Sinorhizobium* sp. strain BL3

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Abstract

The aim of this investigation was to identify and isolate genes involved in acid tolerance from *Sinorhizobium* sp. strain BL3. It was hypothesized that acid tolerance of strain BL3 could be enhanced by high level expression of certain genes involved in acid tolerance, following insertion of these genes in a multiple copy plasmid. A cosmid clone library of BL3 was introduced into BL3, and the transconjugant colonies were selected at low pH. A single cosmid containing genes for acid tolerance was isolated from 40 different colonies. By transposon–insertion mutagenesis, subcloning and DNA sequencing, a gene involved in acid tolerance, *actX*, was identified in a 4.4-kb fragment of this cosmid. The *actX* mutant of BL3 showed increased acid sensitivity and was complemented by the 4.4-kb subcloned fragment. *Phaseolus lathyroides* seedlings inoculated with recombinant strains containing multiple copies of *actX* showed increased symbiotic performance at low pH. By constructing an *actX::gus* fusion, it was shown that *actX* was induced at low pH. *actX* encodes a putative histidine kinase sensor protein of a two-component regulatory system. The method of gene identification used in this study for isolation of *actX* may be applied for the isolation of other genes involved in tolerance to adverse environmental factors.

Introduction

Soil acidity is one of the environmental factors that adversely affect *Rhizobium*–legume symbiosis. Acid-sensitive rhizobial inoculants may not grow well in acidic soils, resulting in poor nodulation and nitrogen fixation of the host legume. The development of acid tolerant rhizobia is one key to solve the inefficient inoculant problem in low pH soils. Some literature indicates that during growth on media at moderate acidity, the acid-tolerant rhizobia maintain slightly alkaline intracellular pH, whereas the acid-sensitive strains fail to maintain a neutral or alkaline intracellular pH (O'Hara et al. 1989; Chen et al. 1993). Various mechanisms, including decreasing membrane permeability, amelioration of extracellular pH and proton extrusion may be involved in the tolerance

to low pH conditions. Moreover, acid tolerance may require mechanisms to detoxify heavy metal ions, to restrict their uptake or to actively pump them out, since mutants of root nodule bacteria that show decreased acid tolerance often show greater sensitivity to metal ions such as Cu^{2+}, Cd^{2+}, or Zn^{2+} (Dilworth and Glenn 1999). Gram-negative bacteria, including rhizobia, are also known to show enhanced levels of tolerance to acidity if the cells are grown in moderately acid conditions before exposure to more highly acidic conditions (Goodson and Rowbury 1989; Foster and Hall 1990; Foster 1991). However, the mechanism for this response, known as adaptive acid tolerance (ATR), has not been identified.

At least three new proteins were induced when *Rhizobium* cells were exposed to low pH conditions (Dilworth et al. 2001). Several genes involved in acid tolerance in *Rhizobium*, including *actA*, *actP*, *actR*, *actS*, *phrR* and *lpiA*, have been previously identified and characterized (Tiwari et al. 1996a, 1996b; Reeve et al. 1998, 2002; Vinuesa et al. 2003). Three different approaches have been so far used to identify and isolate genes involved in acid tolerance. These are (i) generation of acid-sensitive mutants from acid-tolerant strains by using transposon mutagenesis, then isolating the genes involved in acid tolerance by complementation (Goss et al. 1990; Tiwari et al. 1992); (ii) using random insertion of a reporter gene into the rhizobial genome for creation of mutants in which the expression of the reporter gene is induced by low pH conditions, so that the interrupted genes can be identified by sequencing (Reeve et al. 1999); and (iii) determining the pH-dependent differential expression of proteins in *Rhizobium* by one- or two-dimensional SDS–PAGE, followed by N-terminal sequencing of the proteins to allow identification of their genes (Vinuesa et al. 2003). In this study, we have used a novel method to isolate a gene involved in acid tolerance from *Sinorhizobium* sp. strain BL3. BL3 was isolated from the nodules of *Phaseolus lathyroides* plants that were found growing under saline conditions in the northeastern region of Thailand. It can grow well in YEM broth containing up to 300 mmol l^{-1} NaCl. It shows moderate tolerance to acidic conditions and is effective in N_2 fixation (W. Payakapong, P. Tittabutr and N. Boonkerd, unpublished). We hypothesized that acid tolerance of BL3 can be enhanced by increasing the expression of certain genes involved in ATR. We constructed derivatives of the acid-sensitive strain BL3 by transferring the cosmid clone library of BL3 genomic DNA into itself. Each of these derivatives contained multiple copies of a number of genes that were cloned into the cosmid. By screening these derivatives for enhanced acid tolerance, we have isolated a cosmid clone containing a gene for acid tolerance.

Materials and methods

Bacterial strains, plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions

Sinorhizobium strains were grown in yeast extract mannitol (YEM) medium (Vincent 1970) at 28 °C. *Escherichia coli* strains were grown in Luria Bertani (LB) medium (Maniatis et al. 1982) at 37 °C. For internal pH determination, *Sinorhizobium* strains were grown in modified minimal salts medium (Howieson 1985), which contained (mg l^{-1}) Na_2SO_4, 100; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2 \cdot 2H_2O$, 5; $MnSO_4 \cdot 4H_2O$, 1.11; K_2HPO_4, 4.35; KH_2PO_4, 3.4; $FeSO_4 \cdot 7H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 1; $Na_2MoO_4 \cdot 2H_2O$, 1; $CuSO_4 \cdot 5H_2O$, 0.5; thiamine HCl, 1; pantothenic acid, 1; biotin, 2×10^{-3}. For growth medium, carbon and nitrogen sources were added (g l^{-1}) arabinose, 2; galactose, 2; glutamate, 0.4. The medium was buffered with 30 mmol l^{-1} MES for the pHs between 5.0 and 6.0 or 5.46 mmol l^{-1} HEPES for the pHs between 6.5 and 7.0. The pH of media was adjusted to 5.0, 5.5, 6.0, 6.8 and 8.0 after autoclaving. The final pH did not change much from initial pH (± 0.2 point) at the end of growth experiment. Media were supplemented as appropriate with antibiotics at final concentration ($\mu g\,ml^{-1}$); ampicillin (Ap) 100; chloramphenicol (Cn) 30; gentamicin (Gm) 10; kanarmycin (Km) 25; rifampicin (Rf) 40; streptomycin (Sm) 100; and tetracycline (Tc) 10.

Table 1 Bacterial strains and plasmids.

Sinorhizobium strains and plasmids	Relevant characteristics	Source or reference
BL3	*Sinorhizobium* sp., wild-type, highly salt tolerant, isolated from Thailand, Rf^r, Sm^r	W. Payakapong and N. Boonkerd, Thailand (unpublished)
RUH175	*act*X::Tn*3*H*ogus* insertion mutant of BL3, defective in growing at low pH, Rf^r, Sm^r, Km^r	This study
RUH176	Transconjugant of RUH175 containing plasmid pUHR326, Rf^r, Sm^r, Km^r, Tc^r	This study
RUH177	Transconjugant of RUH175 containing plasmid pUHR327, Rf^r, Sm^r, Km^r, Tc^r	This study
BL3:pUHR324	BL3 containing cosmid pUHR324, Rf^r, Sm^r, Tc^r	This study
Plasmids		
pLAFR3	Wide-host-range P1 group cloning vector, used for BL3 genomic DNA library construction, Tc^r	Staskawicz et al. (1987)
pRK404	Wide-host-range P1 group cloning vector, Tc^r	Ditta et al. (1985)
pRK2013	Helper plasmid used for mobilizing plasmids to *Sinorhizobium*, Km^r	Figurski and Helinski (1979)
pPH1JI	P1 group plasmid used to eliminate pLAFR3-based cosmid from *Rhizobium* spp., Gm^r	Beringer et al. (1978)
pUC19	Cloning vector, Ap^r	Maniatis et al. (1982)
pUHR324	pLAFR3-based cosmid clone isolated from the genomic library of BL3 containing genes for ATR, Tc^r	This study
pUHR325	pUHR324::Tn*3*H*ogus*-13	This study
pUHR326 and pUHR327	pRK404 with the 4.4 kb *Hind*III fragment from pUHR324 in both directions, contain *act*X , Tc^r	This study

Triparental mating for transferring cloned DNA into Sinorhizobium

Genomic DNA library of BL3 was introduced into BL3 background by the triparental mating method (Johnston et al. 1978) using pRK2013 as a helper plasmid (Figurski and Helinski 1979) and the transconjugants were selected on YEM medium containing Tc as a selective marker. The triparental mating method was also used to transfer plasmid DNA from *E. coli* into the acid-sensitive mutant for complementation.

Isolation of Tn3Hogus insertion mutants of BL3

Cosmid pUHR324 was mutagenized with random insertion of transposon Tn*3*H*ogus*, as described previously (Borthakur et al. 2003). The pUHR324::Tn*3*H*ogus* derivatives were transferred to BL3 and the transconjugants were spotted on YEM agar at pH 5.0, 5.5, 6.0 and 6.8. The YEM agar also contained $50\,\mu g\,ml^{-1}$ 5-bromo-4-chloro-3-indolyl-D-glucuronic acid cyclohexylammonium salt (X-glc). Colonies were incubated at 28 °C for 3–7 days. Colonies that showed high expression of glucuronidase (GUS) activity on YEM agar medium at pH 5.0, but showed low expression at neutral pH were selected. The disrupted gene in pUHR324::Tn*3*H*ogus* of the selected derivative was transferred into the BL3 chromosome by marker exchange using the incompatible plasmid, pPH1JI (Beringer et al. 1978), selecting for Km and Gm resistance, and Tc sensitivity, as described by Ruvkun and Ausubel (1981).

Restriction mapping and sequencing

A restriction map of insert DNA in cosmid pUHR324 was developed by subcloning the various *Hin*dIII and *Bam*HI fragments in pUC19, analyzing the cloned fragments by restriction digests with both enzymes and sequencing the

cloned fragments from both ends using M13 forward and reverse primers. The 4.4-kb *Hin*dIII fragment was completely sequenced in both directions with an automated DNA sequencer (Model 373A; Applied Biosystems) at the Biotechnology and Molecular Biology Instrumentation facilities, University of Hawaii. The GenBank accession number for this sequence is AY724677. DNA sequences were analyzed by using the online BLAST program from the National Center for Biotechnology Information (NCBI). The position of the Tn*3*Ho*gus* insertion in DNA was determined by sequencing the PCR fragment generated by using *gus* reverse primer (5′-AATTCCACAGTTTTCGCGATC-3′) and a forward primer from the subcloned fragment.

Determination of internal pH

The internal pH of BL3, RUH175 and BL3: pUHR324 was determined by a fluorescent probe method (O'Hara et al. 1989). Cells grown in 2 l-Erlenmeyer flask containing 200 ml of minimal salts medium pH 6.8 at 28 °C for 5 days were harvested and washed twice with sterile minimal salts medium without carbon and nitrogen sources, which have the same pH as the growth medium. Washed cells were resuspended in the same minimal salts medium without carbon and nitrogen sources, buffered at pH 5.5 and 6.0 with 30 mmol l^{-1} MES, and at pH 6.5 and 7.0 with 5.46 mmol l^{-1} HEPES. The suspension was then incubated for 30 min at 28 °C with100 μmoll^{-1} fluorescein diacetate (Sigma). *S. meliloti* has been shown previously to retain fluorescein during a comparable incubation (O'Hara et al. 1989). Cells were then thoroughly washed and resuspended in the same buffer of each pH. The fluorescence intensity of the samples was recorded with a fluorescence spectrophotometer (model F-2500; Hitachi) at 520 nm after excitation at 490 and 435 nm. The values of internal pH were read from a calibration curve prepared as described by Slavik (1982).

β-Glucuronidase activity assay

Sinorhizobium cells from 5 ml overnight culture in YEM broth at pH 6.8 were spun down and resuspended in 5 ml YEM broth at pH 5.0, 6.8 or 8.0, and grown for 9 h. The cells were precipitated by centrifugation and washed with 1 mol l^{-1} NaCl, and resuspended in GUS extraction buffer containing 50 m mol l^{-1} $NaPO_4$ (pH 7.0), 10 m mol l^{-1} β-mercaptoethanol, 10 m mol l^{-1} EDTA, 0.1% sodium lauryl sarcosine, and Triton X-100. The GUS activities of the samples were determined using 4-methylumbelliferl-β-D-glucuronide (MUG) as the substrate and measuring the fluorescence emitted by the product 7-hydroxy-4-methylcoumarin (MU) with a fluorescence spectrophotometer (PerkinElmer, 1420 Multilabel counter) as described previously (Fox and Borthakur 2001).

Plant experiments

Sinorhizobium sp. strain BL3 was originally isolated from the nodules of salt-resistant wild *Phaseolus lathyroides* Thailand. Therefore, the plant inoculation experiment for nodulation was also conducted on seedlings of wild *P. lathyroides*, collected from northeastern Thailand. The seeds were surface-sterilized, germinated and grown in modified Leonard jar assemblies containing vermiculite and nitrogen-free nutrient solution (Somasegaran and Hoben 1994). The plant nutrient solution was adjusted to pH 5.0, 6.0 and 7.0. Three days old seedlings were inoculated with 10^8 sinorhizobia, diluted in sterile plant nutrient solution, from a two days old culture. Three replicates were used for each treatment, with each Leonard jar containing three seedlings. Plants were placed in a control environmental growth chamber in a completely randomized block design and grown for 8 weeks. Nodulation was assessed by determining the number of nodules and plant dry weight.

Results

Isolation of acid tolerant derivatives of strain BL3

To isolate genes potentially involved in acid tolerance, genomic DNA library of strain BL3 was introduced into strain BL3, which cannot form single colonies on YEM agar at pH 5.0. Transconjugants containing the cosmid clones were screened for acid tolerance by plating on YEM

agar at pH 5.0. Forty transconjugant colonies that grew at pH 5.0 were selected. Cosmids isolated from all these transconjugants were identical. When one of these cosmids was reintroduced into BL3, the transconjugants could grow on YEM agar at pH 5.0. Thus this cosmid, pUHR324, enhanced the acid tolerance of BL3, indicating that it may contain gene(s) involved in acid tolerance.

Isolation of a gene involved in ATR from plasmid pUHR324

To identify gene(s) involved in acid tolerance, plasmid pUHR324 was mutagenized with random insertions of the transposon Tn*3*Ho*gus*. Various pUHR324::Tn*3*Ho*gus* derivatives were transferred to BL3, and screened for colonies that showed very slow or no growth at pH 5.0. One pUHR324::Tn*3*Ho*gus* derivative (pUHR325) in BL3 that showed very slow growth on YEM agar at pH 5.0, but not at pH 7.0, was selected. A mutant, RUH175, was constructed by transferring the Tn*3*Ho*gus* insertion from pUHR325 into the corresponding homologous position in the genome of BL3 by marker exchange. RUH175 grew slowly on YEM agar at pH 5.0 or 5.5, without forming single colonies. However, it grew well forming single colonies on YEM agar at pH 6.0 or 7.0. The growth of the mutant RUH175 and BL3 was very similar in YEM broth at pH 7.0 (Figure 1a). In YEM broth at pH 5.0, RUH175 showed much slower growth rate than BL3 indicating that the mutant was sensitive to low pH conditions (Figure 1b and 1c). At this pH, the number of colony forming units (CFU) of the cultures of RUH175 decreased rapidly after 72 h. Under acidic conditions, RUH175:pUHR327, a transconjugant derivative of RUH175 containing a 4.4-kb cloned *Hin*dIII fragment from pUHR324 (see below) showed growth characteristics that were intermediate between BL3 and RUH175. When the inoculants were pre-grown at pH5.5, the cultures of BL3 and RUH175:pUHR327 at pH 5.0 showed a relatively shorter lag phase and reached stationary phase sooner (Figure 1b and c). Hence the acid tolerance associated with the genetic locus identified herein is considered to be part of the ATR.

Comparison of the restriction fragments of plasmids pUHR324 and pUHR325 showed that a 4.4-kb *Hin*dIII fragment of pUHR324 was replaced by two larger fragments (4.9 and 6.2 kb) in plasmid pUHR325, indicating that the Tn*3*Ho*gus* insertion was located in the 4.4-kb fragment. Similar comparison of the *Bam*HI digested fragments of these two plasmids showed that the Tn*3*Ho*gus* transposon was inserted in a 1.3-kb *Bam*HI fragment. The 4.4-kb *Hin*dIII fragment of pUHR324 was subcloned into the broad host-range plasmid vector pRK404 in both directions to obtain plasmids pUHR326 and pUHR327. Restriction mapping of these plasmids shows that they contain a 1.3-kb *Bam*HI fragment within the 4.4-kb *Hin*dIII fragment (Figure 2). Both of these plasmids could complement RUH175 for the acid sensitivity defect, because the transconjugants of RUH175 containing either pUHR326 or pUHR327 formed single colonies on YEM agar at pH 5.0. As seen in Figure 1, RUH175:pUHR327 grew to higher cell densities and CFU than RUH175. The RUH175:pUHR327 cultures did not show a decrease in CFU after 72 h. The 4.4-kb *Hin*dIII fragment of pUHR327 was subcloned in pUC19 and sequenced. Sequence analyses of the 4.4-kb fragment revealed four ORFs between nucleotide positions 58 and 4101 (Figure 2). ORF1 shows similarity to the cytochrome *b* of *Burkholderia cepacia* and *Rhodobacter sphaeroides*. The ORF2- and ORF3-encoded proteins resemble a response regulator and a histidine kinase sensor protein, respectively, of a two-component regulatory system from many bacteria including *S. meliloti*. The 1185-bp ORF4 encodes a protein of size 40.98 kDa that has up to 68% identity with CBS-domain containing membrane proteins of *Agrobacterium tumefaciens* and some other bacteria.

ORF3 is located between nucleotide positions 1382 and 2734, and is preceded by a putative Shine–Dalgarno sequence AAGAA, 7 bp upstream of the ATG start codon. The Tn*3*Ho*gus* insertion in plasmid pUHR325 or mutant RUH175 is located within this ORF, 1250-bp downstream from the start codon. Therefore, ORF3 encodes a gene involved in acid tolerance. Since four *Rhizobium* genes involved in acid tolerance have been previously named as *actA*, *actR*, *actS* and *actP*, we name this gene involved in acid tolerance as *actX*, following the similar nomenclature. The ATG start codon of *actX* overlaps with the TGA stop codon of ORF2, and thus these two genes appear to be translationally coupled.

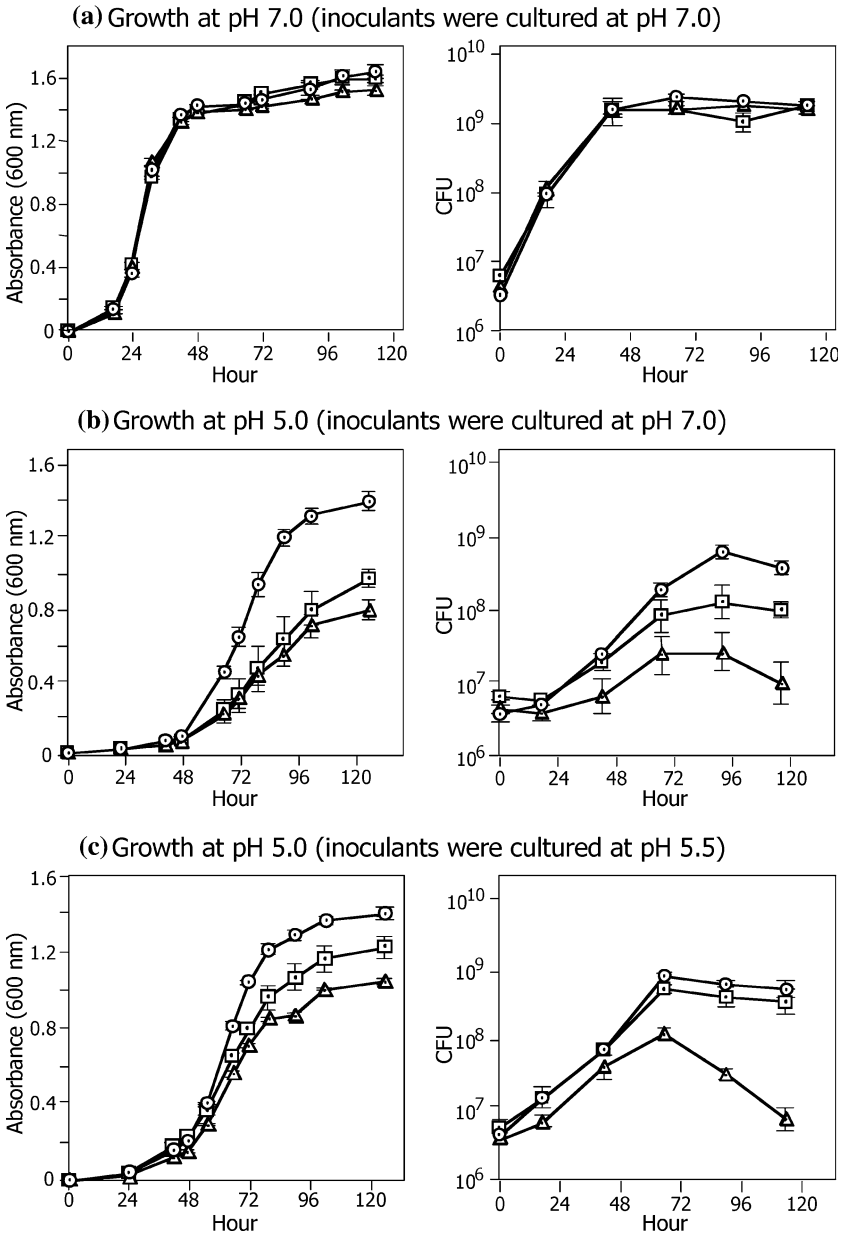

Figure 1. Growth of sinorhizobia in YEM liquid medium at (a) pH 7.0 (inoculants were cultured at pH 7.0); (b) pH 5.0 (inoculants were cultured at pH 7.0); and (c) pH 5.0 (inoculants were cultured at pH 5.5). Symbols: BL3 (circle); RUH175 (triangle); and RUH175:pUHR327 (square).

The *actX*-encoded protein has a molecular mass of 49.4 kDa with pI 6.2. The deduced amino acid sequence of *actX* shows 75 and 30% identities with putative histidine kinase sensor proteins of a two-component regulatory system in *Sinorhizobium meliloti* and *Mesorhizobium loti*, respectively (Kaneko et al. 2000; Finan et al. 2001). It shows 25–31% identities with two-component sensor proteins from various Gram-negative bacteria including *B. cepacia* (31%), *E. coli* (27%), and *A. tumefaciens* (25%). ActX has all the characteristic sequence features of a histidine kinase sensor protein of a two-component regulatory system (Tiwari et al. 1996b). It has a single hydrophobic

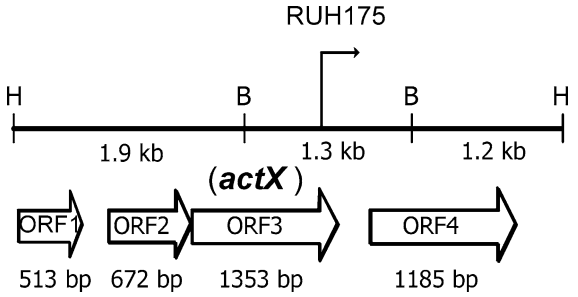

Figure 2. Restriction map of the 4.4-kb *Hin*dIII fragment of pUHR324 showing the position and the direction of four ORFs (open arrows). The vertical arrow indicates the position of the Tn*3*H*ogus* insertion in ORF3 with the attached side arrow showing the direction of the *gus* gene in Tn*3*H*ogus*. H: *Hin*dIII; B: *Bam*HI.

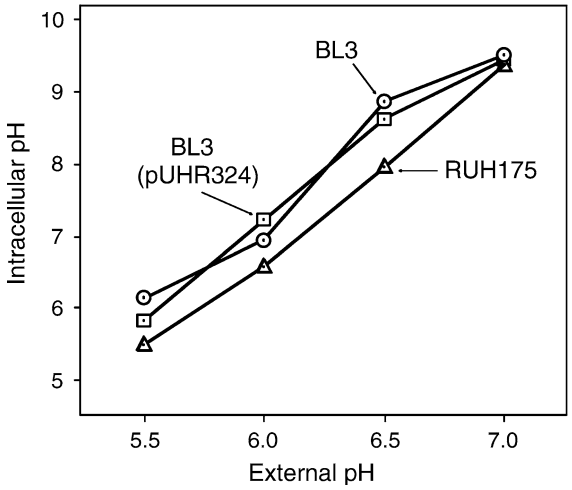

Figure 3. The intracellular pHs of BL3 (circle), RUH175 (triangle) and BL3:pUHR324 (square) under different external pH conditions. The standard deviations for the intracellular pHs were less than 1%.

transmembrane domain at positions 172–192 and a conserved histidine residue (site of autophosphorylation) at position 257. A conserved Asn at position 363 is located 106 residues apart from the conserved His-257. There is a conserved Asp–X–Gly–X–Gly domain at positions 388–392, 24 residues downstream of Asn-363, followed by another Gly–X–Gly domain at positions 413–415.

Intracellular pH of BL3, actX mutant, and BL3:pUHR324

Since some acid-tolerant strains are known to maintain higher intracellular pH, we examined whether strains BL3, RUH175 and BL3:pUHR324 can maintain higher intracellular pH under low external pH conditions. At external pH 7.0, all three strains maintained a similar internal pH of approximately 9.4. However, at pH 5.5, 6.0, or 6.5, the mutant RUH175 maintained a significantly lower pH than BL3 and BL3:pUHR324 (Figure 3). These results indicate that the disruption of *actX* in mutant RUH175 makes it unable to maintain intracellular pH at the same level as BL3 at low external pH conditions. The presence of multiple copies of *actX* in BL3:pUHR324 did not increase its intracellular pH to a level higher than that of BL3.

The actX gene is inducible at low pH

To determine if *actX* is induced at low pH, the *actX* mutant RUH175 with a single copy *actX::gus* fusion in the chromosome, and the transconjugant strain BL3:pUHR325 containing multiple copies of *actX::gus* fusion in a plasmid, were tested for GUS activity at pH 5.0, 6.8 and 8.0 (Table 2). Both strains showed the highest level of GUS activities at pH 5.0 and slightly higher levels of GUS activities at pH 8.0 compared to pH 6.8. RUH175 showed 13-fold higher GUS activity at pH 5.0 compared to pH 6.8. Similarly, BL3:pUHR325 showed about 300-fold higher levels of GUS activity at pH 5.0 than at pH 6.8.

Table 2 Induction of GUS activity due to the *actX::gus* fusion in RUH175 and BL3:pUHR325, grown in YEM broth at different pHs.

Strains	Number of *actX::gus* per cell	GUS activity (nmol l^{-1} of MU ml^{-1} h^{-1})		
		pH 6.8	pH 5.0	pH 8.0
BL3	0	0.0	0.0	0.0
RUH175	1	23.6 ± 6.9	514.2 ± 33.8	143.2 ± 107.8
BL3:pUHR325	4–7	14.2 ± 12.0	4162.6 ± 89.1	337.5 ± 92.5

These results demonstrate that *actX* is induced in acidic conditions.

Nodulation at low pH condition

To determine the role of the *actX* gene in symbiosis, strains BL3, mutant RUH175, and the transconjugants RUH175:pUHR326 (RUH176) and RUH175:pUHR327 (RUH177) were inoculated on seedlings of *P. lathyroides*, grown in Leonard jars in a controlled growth chamber. The *P. lathyroides* seedlings were grown at pH 5.0, 6.0 and 7.0. At pH 7.0, all four strains formed 12–19 nodules per plant and fixed nitrogen, as apparent from the vigor and green color of the plants. The plant dry weight, number of nodules and nodule dry weight for RUH175 were slightly lower than for BL3 at pH 7.0 (Figure 4). At pH 6.0 and 5.0, the plants inoculated with RUH175 remained stunted and produced much less nodules and plant dry weight than the plants inoculated with BL3, suggesting that sensitivity of RUH175 to low pH might adversely affect its ability to develop an effective symbiosis at low pH. The plants inoculated with RUH175:pUHR326 and RUH175:-pUHR327 and grown at pH 5.0 and 6.0 produced dry weight, nodule number and nodule weight that were comparable to those plants inoculated with BL3. This suggests that the symbiotic defects in RUH175 were complemented by the 4.4-kb *Hin*dIII fragment cloned in plasmids pUHR326 and pUHR327. Thus, multiple copies of *actX* in RUH175:pUHR326 and RUH175:pUHR327 appear to provide some buffering capacity to the strains for symbiotic performance at lower pH.

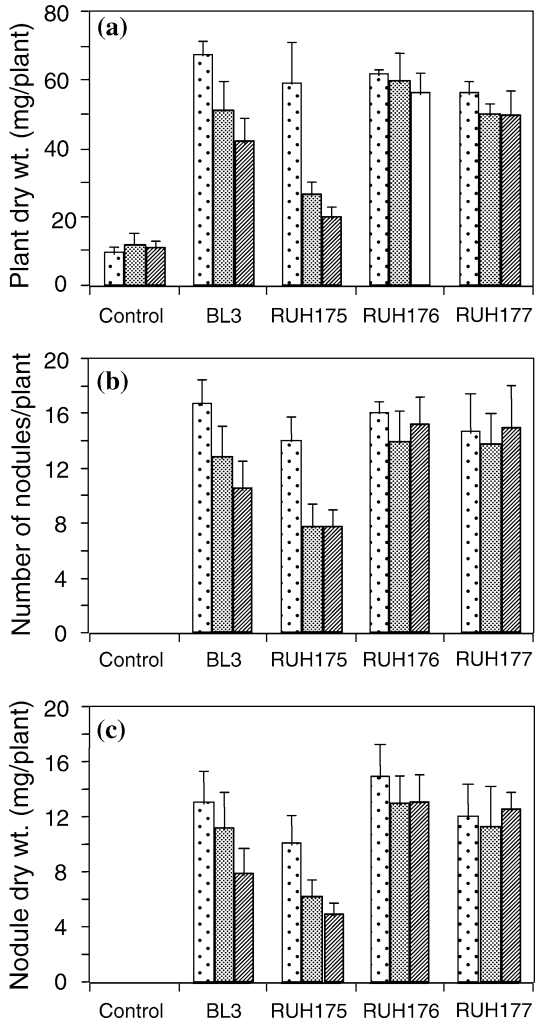

Figure 4. Plant dry weights (a), number of nodules (b), and nodule dry weights (c) of *P. lathyroides*, inoculated with BL3, the *actX* mutant RUH175, and its derivatives containing multiple copies of *actX* in plasmid pUHR326 (strain RUH176) and plasmid pUHR327 (strain RUH177). Plants were grown at pH 7.0 (white bar), 6.0 (shaded bar), and 5.0 (striped bar).

Discussion

We have identified a gene involved in acid tolerance by enhancing its expression through increase of its copy number in an otherwise acid sensitive strain. Previously, Mavingui et al. (1997) used a method of random DNA amplification of genomic DNA to select derivatives of a *Rhizobium tropici* strain that were more competitive for nodule formation. We increased the copy number of the acid tolerance gene by transferring the cloned gene in a pLAFR3-based multiple copy plasmid. The transconjugant BL3:pUHR324 showed acid tolerance, although BL3 is acid sensitive. Similarly, the transconjugants RUH175:pUHR326 and RU H175:pUHR327 containing a 4.4-kb fragment cloned into pRK404 showed acid tolerance. The copy numbers of both pLAFR3- and pRK404-based recombinant plasmids containing the RK2 replicon are known to be 4–7 per cell in *Rhizobium* (Figurski and Helinski 1979). Therefore, the combined expression of the acid tolerance gene in the transconjugant BL3:pUHR324 is expected to increase at least four times. Transposon–insertion

mutagenesis of pUHR324 resulted in the identification of a pUHR324::Tn*3*Ho*gus* derivative that had lost the ability to confer acid tolerance. Sequencing of the DNA fragment, disrupted by the Tn*3*Ho*gus* insertion led to the identification of *actX*, a gene involved in acid tolerance. The GUS activity due to the *actX:gus* fusion gene in mutant RUH175 and the transconjugant BL3:pUHR325 was induced at low pH condition, indicating that *actX* is involved in ATR. Subsequent experiments with strain BL3 and RUH175:pUHR327 indicated that the acid tolerance phenotype is associated with an ATR.

The *actX* gene encodes a putative histidine kinase sensor protein of a two-component regulatory system. Tiwari et al. (1996b) identified two *S. meliloloti* genes, *actS* and *actR*, involved in acid tolerance. They have shown that *actS* and *actR* encode the sensor and the response regulator, respectively, of a two-component regulatory system. However, *actX* identified in the present study does not have homology with *actS*. On the other hand, *actX* has 75% identity with a sensor protein of unknown function from *S. meliloti*. The *actX*-like sensor gene in *S. meliloti* is followed by a response regulator gene, which has 80% identity with the ORF2-encoded protein in the present study. Unlike the *S. meliloti* homolog, ORF2 is located upstream of *actX*. The ORF2-encoded response regulator may also be involved in the ATR. Since we have not isolated any ORF2 mutant of BL3, we do not know if ORF2 is really involved in ATR.

ATR may be common in diverse bacteria including rhizobia (O'Hara and Glenn 1994). In this work, the enhanced expression of the *actX* gene due to its presence in a multiple copy plasmid, may have resulted in higher expression of certain ATR genes. Acid tolerance genes, such as *actX*, may be present in both acid-sensitive and acid-tolerant strains of rhizobia. Sensitivity or tolerance to acid may result from low or high level expression of these genes. Accordingly, the acid-tolerant strains of rhizobia are expected to show high expression of both *actX* and *actR*. However, the expression of *actX* in acid-tolerant rhizobia remains to be determined. Disruption of *actX* in mutant RUH175 resulted in lower intracellular pH of the mutant compared to BL3 under low pH conditions. However, multiple copies of *actX* in the transconjugant BL3:pUHR324 did not increase its internal pH further than the level of BL3 at low pH conditions. These results suggest that the acid tolerance of BL3:pUHR324 may involve additional mechanisms besides maintenance of high intracellular pH under low external pH conditions. The data on cell growth under low pH conditions and symbiotic performance of mutants on *P. lathyroides* indicate that *actX* provides adaptive acid tolerance to rhizobia and facilitate symbiosis under acidic conditions. The method of gene identification through enhanced expression using a multiple copy plasmid may be used for the isolation of other genes involved in tolerance to adverse environmental factors.

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