Acid tolerance in *Rhizobium meliloti* strain WSM419 involves a two-component sensor-regulator system

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An acid-sensitive mutant, TG5-46, derived from *Rhizobium meliloti* WSM419 by Tn5 mutagenesis, fails to grow below pH 6.0 whereas the parent strain grows at pH 5.7. The DNA sequence of a 2.2 kb rhizobial DNA region flanking Tn5 in TG5-46 contains two open reading frames, ORF1 (designated *actS*) and ORF2 (designated *actR*), having high similarity to the sensor-regulator pairs of the two-component systems involved in signal transduction in prokaryotes. Insertion of an omega interposon into *actS* in *R. meliloti* WSM419 resulted in an acid-sensitive phenotype. A DNA fragment from the wild-type complemented the acid-sensitive phenotype of RT295 (*ActS*⁻) and TG5-46 (*ActR*⁻), while fragments containing only *actR* or *actS* complemented TG5-46 and RT295, respectively. The presence of multiple copies of *actR* complemented not only TG5-46 but also RT295. Cloning DNA upstream from *actR* and *actS* into a broad-host-range *lacZ* expression vector and measuring β -galactosidase activities showed that both genes are constitutively expressed regardless of the external pH. Genomic DNA from all strains of *R. meliloti*, but no other bacteria tested, hybridized with an *actRS* probe at high stringency. These data implicate a two-component sensor-regulator protein pair in acid tolerance in *R. meliloti* and suggest their involvement in pH sensing and/or response by these bacteria.

Keywords: *Rhizobium meliloti*, acid-tolerance genes, two-component sensor-regulator system, pH sensing

INTRODUCTION

Approximately a quarter of the world's agricultural soils are acidic and there is increasing concern about soil acidification (Munns, 1986). Soil acidity affects the growth and survival of soil bacteria like *Rhizobium* (Graham & Parker, 1964) and the nodulation of legumes (Munns, 1986; Penny *et al.*, 1977). This influences the amount of N₂ being fixed, and therefore the input of N into many agricultural systems. In some cases the root nodule bacteria represent the acid-sensitive component of the symbiosis (Robson & Loneragan, 1970; Munns, 1986); *Rhizobium meliloti* is especially sensitive to acidity. This raises questions as to how root nodule bacteria respond to acidity and why some, like *R. meliloti*, are acid-sensitive and others are more acid-tolerant.

Most bacteria that have been studied (Caldwell, 1956; Waddell & Bates, 1969; Thomas *et al.*, 1976; Padan *et al.*, 1982; Booth, 1985; Padan & Schuldiner, 1986) maintain a relatively constant internal pH (pH_i) over a wide range of external pH (pH_e). *R. meliloti* maintains a slightly alkaline pH_i even when the pH_e is acidic (O'Hara *et al.*, 1989). This control of pH presumably involves one or more mechanisms for regulating the influx and efflux of protons across the cytoplasmic membrane (Glenn & Dilworth, 1994). Little is known about such mechanisms in *Rhizobium*, whether they are inducible or constitutive, or how the cell 'senses' changes in the pH of the external environment.

Acid-inducible changes in cell behaviour occur in *Salmonella typhimurium* (Aliabadi *et al.*, 1988; Foster & Hall, 1990, 1991; Foster *et al.*, 1994; Lee *et al.*, 1994), *Escherichia coli* (Goodson & Rowbury, 1989a, b), *Aeromonas hydrophila* (Karem *et al.*, 1994), and *Rhizobium* and *Bradyrhizobium* (O'Hara & Glenn, 1994). Cells grown under

The GenBank accession number for the nucleotide sequence of the BamHI-SalI fragment containing the *actR* and *actS* genes is L39938.

Table 1. Strains and plasmids used

Strain/plasmid	Relevant characteristics*	Source/reference
<i>A. tumefaciens</i>		
GMI9023	C58C1, Sm ^r Rf ^r , cured of pATC58	T. Finan†
<i>E. coli</i>		
DH5α	F ⁻ ϕ 80dlacZΔM15 <i>recA1 endA1 gyrA96 thi-1</i> <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1 deoR</i> Δ (<i>lacZYA-argF</i>)U169	BRL
DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80dlacZΔM15 Δ <i>lacX74 endA1 recA1 deoR</i> Δ (<i>ara, leu</i>)7697 <i>araD139 galU galK nupG rpsL</i>	BRL
HB101	F ⁻ <i>thi-1 hsdS20</i> (r _B ⁻ m _B ⁻) <i>supE44 recA13 ara-14</i> <i>leuB6 proA2 lacY1 rpsL20</i> (Sm ^r) <i>xyl-5 mtl-1</i>	Boyer & Roulland-Dussoix (1969)
<i>R. leguminosarum</i> bv. <i>trifolii</i>		
NA3001	Acid ^t	J. Howieson‡
TA1	Acid ^s ; commercial strain for white clover	J. Howieson
<i>R. leguminosarum</i> bv. <i>viceae</i>		
WSM710	Acid ^t strain from <i>Vicia</i> spp. in Japan	J. Howieson
WSM937	Acid ^t strain from <i>Vicia</i> spp. in Greece	J. Howieson
<i>R. meliloti</i>		
CC169	Commercial inoculant from South Australia	
Rm1021	SU47 <i>str-21</i>	T. Finan
RT295	<i>actS</i> : :ΩKm mutant of WSM419	This study
TG5-46	<i>actR</i> : :Tn5 mutant of WSM419	Goss <i>et al.</i> (1990)
U45	Commercial inoculum from Uruguay	
WSM232	Acid ^s isolate from Iraq	J. Howieson
WSM244	Acid ^s isolate from Iraq	J. Howieson
WSM419	Acid ^t Sardinian isolate, Cm ^r	J. Howieson
WSM540	Acid ^t commercial strain	J. Howieson
<i>R. tropici</i>		
5024	Acid ^t	J. Howieson
Plasmids		
pGEM-7Zf(+)	Cloning vector; Ap ^r	Promega
pJQ200KS	Gene replacement vector; Gm ^r Suc ^s	Quandt & Hynes (1993)
pMP220	Broad-host-range <i>lacZ</i> fusion vector; Tc ^r	Spaink <i>et al.</i> (1987)
pMP220-Km2	pMP220 derivative with Km ^r interposon; Km ^r Tc ^r	This study
pPH1J1	Broad-host-range IncP plasmid; Gm ^r Sm ^r Sp ^r	
pHP45ΩKm	pHP45Ω derivative with Km ^r interposon; Ap ^r Km ^r	Fellay <i>et al.</i> (1987)
pRK2013	Helper plasmid; Km ^r	Ditta <i>et al.</i> (1980)
pRT546Cla	pGEM-7Zf(+) containing Km ^r <i>Cla</i> I fragment of TG5-46	Tiwari <i>et al.</i> (1992)
pRT546-1	A 25 kb <i>Sau</i> 3AI fragment of WSM419 genomic DNA cloned into the <i>Bam</i> HI site of pSW213 which hybridized with the 0.5 kb <i>Sma</i> I- <i>Hpa</i> I probe	This study
pRT546-6	pSW213 with the 3.8 kb <i>Bgl</i> II <i>actS</i> fragment	This study
pRT546-11	pSW213 with the 3.7 kb <i>Eco</i> RI <i>actR</i> fragment	This study
pRT546-20	pMP220 with the 4.0 kb <i>Bgl</i> II- <i>Kpn</i> I <i>actS/actR</i> fragment	This study
pRT546-22	pRT546-20 derivative, <i>actS</i> : :ΩKm; insertion site: <i>Eco</i> RI	This study
pRT546-29	pMP220-Km2 derivative, with the potential <i>actS</i> promoter	This study

Table 1 (cont.)

Strain/plasmid	Relevant characteristics*	Source/reference
pRT546-32	pMP220-Km2 derivative, with the potential <i>actR</i> promoter	This study
pSW213	Broad-host-range cloning vector; Tc ^r	Chen & Winans (1991)
pUC18	Cloning vector; Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pWR546	The Km ^r <i>EcoRI</i> fragment of pTG5-46 cloned into <i>EcoRI</i> -digested pJQ200; Tc ^r , Km ^r	This study

* Abbreviations: acid-sensitive (Acid^s) or tolerant (Acid^t); ability to maintain alkaline intracellular pH (pH_i⁺) or not (pH_i⁻); sucrose sensitive (Suc^s). Resistance to ampicillin (Ap^r), chloramphenicol (Cm^r), gentamicin (Gm^r), kanamycin (Km^r), rifampicin (Rf^r), spectinomycin (Sp^r), streptomycin (Sm^r) and tetracycline (Tc^r).

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mildly acidic conditions are more resistant to a subsequent severe pH shock than cells grown at neutral pH: the so-called adaptive acid tolerance response (ATR) (Foster & Hall, 1990; O'Hara & Glenn, 1994) or acid habituation (Goodson & Rowbury, 1989a, b; Raja *et al.*, 1991a, b). Some genes involved in the cellular response to pH are inducible (Foster & Hall, 1991; Foster, 1992) whereas others are constitutive (Foster & Hall, 1991; Tiwari *et al.*, 1996).

The isolation from Sardinia of strains of *R. meliloti* like WSM419 and WSM540 with superior performance in nodulating medic species in acid soils (Howieson & Ewing, 1986) raised the question why such strains were more acid-tolerant under field conditions than others. Tn5-induced acid-sensitive mutants have been isolated from *R. meliloti* WSM419 (O'Hara *et al.*, 1989; Goss *et al.*, 1990; Tiwari *et al.*, 1992). These mutants grow as well as the wild-type at neutral or alkaline pH and effectively nodulate *Medicago polymorpha*. One of these acid-sensitive mutants of *R. meliloti*, strain TG5-46, fails to grow below pH 6.0 at 0.3 mM calcium (Goss *et al.*, 1990; Tiwari *et al.*, 1992; Reeve *et al.*, 1993), but will grow at pH 5.6 in the presence of 10 mM calcium (Reeve *et al.*, 1993). It is defective in the maintenance of pH_i; as pH_e drops below 7.0, pH_i begins to fall (O'Hara *et al.*, 1989).

It has been proposed (Glenn & Dilworth, 1994) that root nodule bacteria, and other bacteria, may have some form of sensing mechanism which enables cells to respond to acidic environments. Such systems for environmental sensing and response in bacteria commonly have two components – a sensor to detect the environmental change and a regulator to control the transcriptional response (Albright *et al.*, 1989; Stock *et al.*, 1989; Parkinson & Kofoid, 1992).

We now report that the acid-sensitive mutant strain *R. meliloti* TG5-46 is defective in a regulator gene and that a sensor-regulator pair is essential for acid-tolerance in the wild-type *R. meliloti* WSM419.

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids are described in Table 1. Strains of *Rhizobium* were grown at 28 °C in JMM minimal medium or TY (O'Hara *et al.*, 1989). *Escherichia coli* and *Agrobacterium* strains were grown in Luria-Bertani (LB) medium at 37 °C and 28 °C, respectively. Media were supplemented with the following concentrations of antibiotics (µg ml⁻¹): ampicillin (100), chloramphenicol (20), gentamicin (50), kanamycin (50) or tetracycline (20).

Tn5/interposon marker exchange. The vector pJQ200SK (Quandt & Hynes, 1993) containing a p15A replicon was used as a suicide delivery vehicle to re-insert Tn5 into *actR*. The *EcoRI* fragment containing Tn5 was cut from pTG5-46, end-filled, and cloned into the *SmaI* site of pJQ200SK to create pWR546. This construct was then mobilized into *R. meliloti* WSM419 and transconjugants were plated on JMM pH 7.0 containing sucrose (5%, w/v) and kanamycin. Kanamycin-resistant colonies which were gentamicin-sensitive were selected for acid-sensitivity tests.

To inactivate *actS* the 2.3 kb kanamycin-resistance interposon from the plasmid pHP45Ω-km (Fellay *et al.*, 1987) was cut out by *HindIII*, end-filled and then cloned into pRT546-20 at the end-filled *EcoRI* site. This new clone pRT546-22, having the interposon inserted at the 295th codon of *actS*, was mobilized into *R. meliloti* WSM419 by triparental mating using the helper plasmid pRK2013. The transconjugants of WSM419 having pRT546-22 were selected on TY plates containing chloramphenicol, kanamycin and tetracycline. The incompatible plasmid pPH1J1 was mobilized into WSM419(pRT546-22) and transconjugants were selected on TY plates containing chloramphenicol, kanamycin and gentamicin. Kanamycin-resistant colonies which were tetracycline-sensitive were selected for acid-sensitivity tests.

Acid sensitivity was established by patching transconjugants onto JMM (pH 5.7). The acid-sensitive phenotype was subsequently confirmed by spotting 10⁴ cells onto JMM minimal medium (pH 7.0 or 5.7) containing various concentrations of calcium (Reeve *et al.*, 1993).

DNA preparation and manipulation. Plasmid DNA was extracted by the alkaline lysis technique and purified by CsCl/ethidium bromide isopycnic centrifugation (Sambrook *et*

al., 1989). Genomic DNA was isolated using a procedure essentially similar to that of Goss *et al.* (1990) except that after phenol/chloroform extraction, the aqueous phase was dialysed against TE buffer at room temperature overnight followed by five changes at 4 °C over 3 d.

Construction of a DNA probe. A 0.5 kb *Sma*I–*Hpa*I fragment (Fig. 1a) was used as a probe in hybridization studies. The 0.5 kb fragment was purified by electrophoresis on a 1% (w/v) agarose gel and transferred into a 0.4% SeaPlaque GTG agarose gel (FMC Corporation). DNA was recovered using Agarase (Boehringer Mannheim) and ethanol precipitation, resuspended and then labelled with digoxigenin-11-dUTP (Boehringer Mannheim) as described by the manufacturer.

Southern hybridization. *Eco*RI-digested genomic DNA from different rhizobial strains was separated on 0.7% agarose, denatured, blotted and UV crosslinked onto nylon membranes (Boehringer Mannheim). Prehybridization and hybridization were performed at 42 °C; high-stringency washing conditions were as described by Tiwari *et al.* (1992). The chemiluminescent substrate AMPPD (Boehringer Mannheim) was used to detect the hybridized digoxigenin-labelled probe.

DNA sequencing and analysis. DNA sequencing was carried out using an Applied Biosystems Prism Ready Reaction kit and automated DNA sequencer. The pUC/M13 Forward (–47; 24-mer) and Reverse (22-mer) primers were purchased from Promega Corporation. Custom-made oligonucleotides were chemically synthesized and purified by Bresatec. The Tn5 primer was a 25-mer oligonucleotide containing the sequence 5'-TAC GAG GTC ACA TGG AAG TCA GAT C-3'. One custom primer was used which contained the sequence 5'-CGT AGA CGC GCT GGA TAT GC-3' (positions 2110–2091 in Fig. 2). DNA sequences were analysed using the MacVector 3.5 sequence analysis programs from IBI. The FASTA (Pearson & Lipman, 1988) and CLUSTAL W (Thompson *et al.*, 1994) analysis programs were accessed via The Australian National Genomic Information Service (ANGIS) located at the University of Sydney.

lacZ-fusion studies. The wide-host-range vector pMP220 (Spaak *et al.*, 1987), containing a promoterless *lacZ*, was modified and used for fusion studies. β -Galactosidase was assayed as described by Miller (1972) and protein concentration was measured using a Bio-Rad protein assay kit. β -Galactosidase specific activity was expressed as nmol *o*-nitrophenol produced min^{–1} (mg protein)^{–1} at 28 °C.

RESULTS AND DISCUSSION

TG5-46 is an acid-sensitive mutant

The acid-sensitive mutant TG5-46 was isolated by Tn5 mutagenesis of *R. meliloti* WSM419. Unlike its parent strain, which could grow down to pH 5.6, TG5-46 was unable to grow below pH 6.0 in JMM (O'Hara *et al.*, 1989). Southern hybridization revealed that a single copy of Tn5 was present in the genome of TG5-46 (Goss *et al.*, 1990). The *Eco*RI fragment containing Tn5 from TG5-46 was cloned into pUC18 and pJQ200SK to construct pTG5-46 and pWR546, respectively. Tn5 marker exchange, in which pWR546 was mobilized into *R. meliloti* WSM419, generated kanamycin- and sucrose-resistant transconjugants. These were also acid-sensitive, showing that the acid-sensitive phenotype in TG5-46 is directly linked to the presence of Tn5.

Fig. 1. Restriction mapping of the *actR* gene region in *R. meliloti* WSM419. (a) Amended restriction map of pTG5-46 as determined in this study. The region of DNA used as a probe in hybridization studies is presented as a hatched box. (b) Restriction map of pRT5-46Cla. This construct contains the Km^r *Cla*I fragment from TG5-46 cloned into the *Cla*I site of pGEM-7Zf(+). Restriction sites are as follows: B, *Bam*HI; Bgl, *Bgl*II; Cla, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hind*III; Hpa, *Hpa*I; K, *Kpn*I; S, *Sal*I; Sm, *Sma*I; X, *Xho*I.

Restriction mapping of DNA

The rhizobial DNA fragment flanking the IS50R of pTG5-46 ended at the *Eco*RI site only 0.6 kb from Tn5 and therefore another genomic DNA fragment from TG5-46 was cloned in order to obtain more sequence information. A *Cla*I fragment from TG5-46 genomic DNA, carrying the kanamycin resistance determinant, was cloned into the *Cla*I site of pGEM-7Zf(+) to construct pRT546Cla, which provided approximately 2.5 kb of DNA after the *Eco*RI site on the right-hand flanking sequence (Fig. 1).

Sequencing and analysis

Rhizobial DNA subcloned from pTG5-46 and pRT546Cla into either pGEM7Zf(+) or pUC18 was sequenced. The DNA around the Tn5 insertion has been fully sequenced on both strands (Fig. 2) from the *Bam*HI₂ site of the rhizobial DNA flanking the IS50R (Fig. 1b) to the *Eco*RV₂₂₆₃ site flanking the IS50L (Fig. 1a).

During the subcloning of DNA from pTG5-46, it was established that the *Kpn*I and *Hpa*I sites originally reported in the rhizobial DNA flanking the IS50R of Tn5 by Goss *et al.* (1990) were incorrect; the restriction map of pTG5-46 has therefore been amended. The sequencing data confirmed the amended restriction map and also

BamHI	-GG ATC CCG CCA ACG CCA TGC CGA CAA CGC GCG CCA AGC CAC GCG GCG CTT AAC CCC ATT TGA TAA TTG CCG GGT TCG GCA TAA AGT TCG	90
GTA GGA CGA ACT GAT GCG GCC GCA TGC GGC CGC ATC GCG GAA TGG AAA	CGC ATG GCA GCC TCA ACG CTC TAC AAT GAC CTG AAC AGC AAT	180
CGC AGC CTC AGA CTT CAG ACG CTC GTG CGG CTG CGC TGG CTG GCG GTG GGC GGA CAA TTG GCG CTA GCC GTT ATC GTC ACG GCG CTT TGG CTG	ORF1 (actS) M A A S T L Y N D L N S N>	270
CAG TTC CCC CTG CCG ATC GTC CCC TGT TCG GTT CTG ATT GCC TGC CTG GCA CTC GTC AAC GTC ATT CTC ACA TTG CGG TTT CCG CCT ACG	Q F P L P I V P C S V L I A C L A L V N V I L T L R F P P T>	360
CAG CGG CTG ACG CCG CCG GCC GCT TTC GCC CTG CTC GGC CTG GAT CTG GCG CAA CTC ACC GCC TTG CTC TTC ATC ACC GGC GGG CTT GCC	Q R L T P P A A F A L L G L D CTG GCA CTC ACC T A T T G L F I T G A C C G G G L A>	450
AAC CCC TTT GCG CCG CTT CTC TGC GTT CCG GTC ATC ATC TCT TCG GCC TCG CAG CCG AAG CCT CAC AGC ATC GTG CTG GCC GTG TTT GCC	N P F A P L L C V P V I I S S A S Q P K P H S I V L A V F A>	540
GTC GTG GGC GTC ACC GCG CTC GCC TTC TCG CCC TTC CAG CTG CCC TGG TAT CCG GGG ACC GTT CTG CTG ATC CCG CAC GTG CTG ACG GCC	V V G V T A L A F S P F Q L P W Y P G T V L L I P H V L T A>	630
GCG ATC TGG TTC GCG ATC GTC TCG ATG ACG GCG TTC GCA GCA TTC TAC ACC TAC CGT GTA TCG CTC GAG GCA AGC GAA CTG TCA GAA CGC	G I W F A I V T S M T G C A F A A T T Y A C T Y C R V S L E A S E L T S E A>	720
CTG ACC GCC ACC GAA CTC GTG CTC CAG CGC GAA AAG CAC CTG TCG CAA CTC GAC GGG CTC GCA GCC GCC GCC GCG CAC GAA CTC GGA ACG	L T A T E L V L Q R E K H L S Q L D G L A A A A A H E L G T>	810
CCG CTT GCG ACG ATC AGC GTC GTA GCC AAG GAG ATG GAA CCG GAG CTC GGC GAC GAT CCT CGT TTC GGC GAA GAC GTC CAC CTC CTG CGC	P L A T I S V A K E M E R G L G D G A T C P R F G G A A C V C A C L L R>	900
AGC CAG AGC GAG CGC TGC CGA GAC ATA CTG AGG CGG CTC ACG ACG CTT TCT TCG GAG AGC GAG GAA CAT ATG CGA CTT CTG CCG CTC TCG	S Q S E R C R D I L R R L T T L S S E S E E H M R L L P L S>	990
TCC CTT ATC GAG GAG GTG ATG GCG CCG CAT CGC GAA TTC GGA ATC GAA ATC GAG CTG AAG GAG CAG GGC GAA CGC GCC ACG GAG CCC GTC	S L I E E V M A P H R E F G I E I E L K E Q G E R A T E P V>	1080
GGC ATC CGC AAT GCA GGC ATT CTC TAT GGC CTG GGA AAC CTG CTG GAA AAC GCG GTC GAT TAT GCG CGC AAG AAA GTG ACG GTC ACG ACG	G I R N A G I L Y G L G N L L G A A C G C V D Y A R K K V T V T T>	1170
GAG CAC ACG CCC GAG CGC GTA CGG GTG ACG ATC GAA GAC GAC GGC GAA GGT TTT TCG CCG GAT ATC CTG GCG CGG ATC GGC GAA CCC TAT	E H T P E R V R V T I E D D G E G F S P D I L A R I G E P Y>	1260
GTG ACG CGG CGT CAG AAG GAC GAC AGT GCC GGC GGG CTC GGG CTC GGG CTC TTC ATC GCC AAG ACG CTG CTC GAG CGT TCG GGT GCC CGG	V T R R Q K D D S A G G L G L G L F I A K T L L E R S G A R>	1350
CTG CGC TTC GAG AAC GGT GGA GCC AAA CAC CCG GGT GCC CGG GTC AGC GTC GAA YGG CCG CGC GCG CTG ATG GAC TCG AAA CTG GCG AAA	L R F T E N G A A A K H P G R V S V E W P R A L M D S K L A K>	1440
TGA CTT TTG GCA GTT TAT TGA AGA CAA GCG TGG GAA AAC CCA CAT GAC TGC CCG CAA AAC AGC TTG CCG ACT GCA GTT CCT TTG GTC		1530
CCA CCG TTC AAA GGA CAT GCG GAA ACT GTT ACA GGT TAC AGA AGC CAT TGC GCG TCC GGC TGG ACG CGC GGC GCT GTA GGG GTT TGA GAA		1620
ATG ATC GAA AAG TCG ACG CCT GCG CCA ATC ACC CAC GCC GCG GAT GCG GAC CTG ATC GGA CCC GAC AAG AGC CTT CTG ATC GTC GAT GAC	M I E K S T P A P I T H A A D A D L I G P D K S L L I V D D>	1710
GAC ACC GCT TTT CTG CCG CGC CTC GCC CGC GCG ATG GAA GCG CGC GGC TTC GCG GTA GAG ATC GCC GAG TCG GTT GCC GAA GGC ATA GCC	ORF2 (actR) D T A F L R R L A C R A M E A R G F A V E I A E S V A E G I A>	1800
AAA GAC AAG ACT CGG CCA CCG AAA CAT GCA GTC ATC GAT CTC CGC CTT GGC GAC GGC AGC GGT CTC GAC GTG ATC GAG GCA ATC CGC GGG	ClaI K D K A T R A G P K H A V I D L G G D G S G S G L D V I E A I R G>	1890
CGT CGC GAC GAC ACG CGG ATG ATC GTT CTA ACG GGT TAT GGT AAT ATC GCG ACC GCG GTC AAC GCC GTG AAG CTC GGA GCG CTC GAC TAT	R R D D T R M I V L T G Y G N I A T A V N A V K L G A L D Y>	1980
CTG GCA AAA CCG GCG GAC GCC GAT GAC ATT CTC GCA GCA TTG ATT CAG CGC CCG GGC GAA CGG GTA GAG CCC CCG GAG AAC CCC ATG TCG	L A K P A D A D D I L A T I Q C R P G E R V E P P E N P M S>	2070
GCG GAC CGC GTC CGA TGG GAG CAT ATC CAG CGC GTC TAC GAA ATG TGC GAA CGC AAT GTT TCG GAA ACG GCG CGC CGG CTA AAC ATG CAC	A D R V R W E H I C Q R V Y E M C E R N V S E T A C C R R L N M H>	2160
CGG CGG ACC TTG CAG CGC ATT CTG GCG AAA CGG GCC CCG AAA TAG GCC GGC GGC GGA TGA GGG GCA AAT CTC GGG AGC GCT CTA GTC CCG	EcoRV R R T L Q R I L A K R A P K>	2250
CCC CCC GCT TAC GAT ATC		

Fig. 2. Nucleotide sequence of the cloned rhizobial DNA flanking the site of Tn5 insertion in *R. meliloti* TG5-46. The boxed region represents the 9 bp which were duplicated by Tn5 insertion in TG5-46. Potential ribosome-binding sites are underlined. The predicted amino acid sequence of the ActS and ActR proteins is presented in single-letter code below the nucleotide sequence. Restriction sites of the DNA sequence are shown in bold.

established the presence of an additional *SmaI* site on the rhizobial DNA flanking the IS50R separated by only two bases from the previously identified site (Fig. 1a).

Analysis of the DNA sequence using the MacVector program revealed the presence of two open reading frames: ORF2 spanned the site of Tn5 insertion; ORF1 started at nucleotide position 142 and ended at 1440 in the DNA sequence presented in Fig. 2. A putative ribosome-binding site (5'-GGAAA-3') was located 3 bp upstream from the ATG initiation codon of ORF1. ORF2, which

started at position 1621, continued through the site of Tn5 insertion at 1683 and ended at 2202, had a potential ribosome-binding site (5'-GAGAAA-3') starting 5 bp upstream from the start codon (Fig. 2).

actR

The computer-predicted protein from ORF2 contains 193 amino acids and has an estimated molecular mass of 21 463 Da and a pI of 8.3. A search of databases using the

Fig. 3. CLUSTAL W alignment of *R. meliloti* WSM419 ActR with the amino acid sequences of five other response regulators. The numbers on the top line indicate the amino acid residue numbering of ActR. Residues conserved among all the response regulators are highlighted with an asterisk. Similar residues are marked with a dot. The highly conserved amino acids in the response regulators are boxed. An explanation of abbreviations used for bacterial strains is provided in the legend of Table 2.

FASTA program indicated that the protein encoded by ORF2 showed significant similarity with proteins such as PrrA, RegA, NtrC, FixJ and PilR, which are the regulator components of sensor-regulator protein pairs involved in

signal transduction in prokaryotes (Stock *et al.*, 1989; Parkinson & Kofoid, 1992). ORF2, tentatively identified as coding for a regulatory protein, has been designated as *actR*.

The proteins showing similarity with the ActR sequence are involved in responding to a diverse range of environmental stimuli. These proteins show similarity in their N-terminal portions. The program CLUSTAL W was used to align five selected sequences with ActR (Fig. 3). The invariant aspartate and lysine residues conserved between regulatory proteins are also found in ActR and have been boxed. The Asp-30, Asp-73 and Lys-123 residues of ActR appear to correspond to residues Asp-13, Asp-57 and Lys-109 in CheY. These N-terminal amino acid residues of the regulators are clustered at the end of what is thought to be a β -sheet, as found in CheY (Stock *et al.*, 1989). This region of the response-regulator is thought to correspond to an active site in these proteins, consistent with the finding that Asp-57 is phosphorylated in CheY (Stock *et al.*, 1989).

Although the majority of these regulator sequences align only to the N-terminal portions of ActR, RegA from *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* and PrrA from *R. sphaeroides* showed a high degree of similarity to ActR over the entire amino acid sequence. The ActR protein showed 71.2% and 69.8% identity to the RegA protein from *R. capsulatus* and *R. sphaeroides*, respectively, and 87.0% similarity to RegA from either species. PrrA, which is closely related to RegA, showed 70.1% identity and 87.0% similarity with ActR (Table 2).

The specificity of the CLUSTAL W analysis was increased by aligning the PrrA and RegA proteins to ActR as shown in Fig. 3. In this case, identical residues were found to align throughout the amino acid sequence of ActR, with the greatest similarity occurring in the C-terminus. The high

Table 2. Similarity of ActS/ActR to other sensor-regulator pairs involved in signal transduction

Sensor-regulator pair*	Protein match			System†	Reference
	Over no. of amino acids	% identity	% similarity		
Rs PrrB	428	42.1	71.0	Photosynthesis	Eraso & Kaplan (1995)
Rs PrrA	177	70.1	87.0	(Oxygen)	Eraso & Kaplan (1994)
Rs RegB	427	41.7	71.0	Photosynthesis	Qian & Tabita (1996)
Rs RegA	172	69.8	87.3	(Oxygen)	Phillips-Jones & Hunter (1994)
Rc RegB	410	38.5	65.6	Photosynthesis	Mosley <i>et al.</i> (1994)
Rc RegA	177	71.2	87.0	(Oxygen)	Sganga & Bauer (1992)
Ac FixL	273	31.9	58.9	Nitrogen fixation	Kaminski & Elmerich (1991)
Ac FixJ	119	36.1	61.3	(Oxygen)	Kaminski & Elmerich (1991)
Pa PilS	234	26.5	55.1	Pilin synthesis	Boyd <i>et al.</i> (1994)
Pa PilR	122	36.1	66.4	(?)	Ishimoto & Lory (1992)

* Proteins are from the following sources: Ac, *Azorhizobium caulinodans*; Pa, *Pseudomonas aeruginosa*; Rc, *Rhodobacter capsulatus*; Rs, *Rhodobacter sphaeroides*.

† Proposed signals for these systems are given in parentheses. The question mark designates an undetermined signal.

Fig. 4. CLUSTAL W alignment of *R. melioli* WSM419 ActS with the amino acid sequence of five other histidine protein kinases. Conserved residues are highlighted with an asterisk and similar residues are marked with a dot. The numbers on the top line indicate the amino acid residue numbering of ActS. The highly conserved amino acids in the sensors of two-component sensor-regulator systems (histidine, asparagine, aspartic acid and glycine) are boxed. An explanation of abbreviations used for bacterial strains is provided in the legend of Table 2. Only the kinase domains of Ac FixL and Pa PilS are shown. Introduction of a frameshift change to the sequence of *regB* from *R. capsulatus* for the first 40 codons would generate a reading frame yielding a protein with a higher degree of similarity between Rc RegB and Rs PrrB.

degree of similarity between the normally variable C-terminal domains suggests that these proteins might have a similar mode of action.

The environmental response-regulators have been sub-grouped into several classes on the basis of the similarity of their C termini. The main classes include the NtrC subclass, the OmpR subclass and the FixJ subclass (Stock *et al.*, 1989; Bourret *et al.*, 1991). Using a different selection criterion, Parkinson & Kofoed (1992) have designated these as the ROII, ROIII and ROIV classes. Eraso & Kaplan (1994) have suggested that PrrA and RegA be placed into a new subgroup since their C termini are different from those of other regulators. We propose that ActR should be included in this subgroup. Although these proteins lack a known DNA-binding motif it is still possible that they bind to DNA sequences. Such is the case for OmpR (a member of the ROII family), which does not have a helix-turn-helix motif (Nara *et al.*, 1986) but binds to promoter sequences upstream of *ompC* and *ompF* (Tate *et al.*, 1988). Alternatively, RegA, PrrA or ActR could act as intermediaries (as seen with CheY, SpoOF and CheB) by interacting with other proteins in more complex regulatory cascades (Bourret *et al.*, 1991).

acts

Response-regulatory proteins interact, directly or indirectly, with a sensor protein, forming two-component sensor-regulator protein pairs. The sensor component detects an environmental stimulus through its N-terminal domain and activates the C-terminal kinase activity of the sensor protein (Stock *et al.*, 1989). Activated sensor protein phosphorylates the N-terminal domain of the regulatory protein, which then executes the response. Different sensors show homology at the C-terminal region since this is the domain that contains kinase activity. The gene encoding the sensor protein component is often located near the regulator gene (Wanner, 1992). It was therefore plausible that a sensor gene could be located next to *actR*.

The other ORF (ORF1 in Fig. 2) was located on the same strand and potentially transcribed in the same direction as *actR*. Conversion of this ORF into the corresponding amino acid sequence revealed that this protein would contain 433 amino acids, and have an estimated molecular mass of 47 208 Da and a pI of 6.2. From a search of protein databanks using the FASTA program the top ten scores obtained were to sensor histidine kinase proteins of two-component sensor-regulatory systems. The FASTA analysis revealed that the greatest similarity occurred in the C-terminal region of these proteins. ORF1, tentatively identified as coding for a sensor protein, has therefore been designated as *actS*.

CLUSTAL W analysis was used to align ActS with five other sensors (Fig. 4). ActS contains the three C-terminal regions possessing the invariant residues (boxed in Fig. 4) found in all proteins of the histidine kinase family. The first of these contains a histidine (normally the site of autophosphorylation) with a space of around 100 amino acids to an asparagine found in the second region. In ActS, these amino acid residues correspond to His-219 and Asn-326, separated by 107 amino acids. The third conserved region contains two glycine-rich regions

Fig. 5. Hydrophilicity analysis of the predicted amino acid sequence of the ActS protein using the MacVector analysis program and a window size of 7. Positive values indicate hydrophilic regions; negative values indicate hydrophobic regions.

separated by 20–50 amino acids. In ActS, these include Asp-Asp-Gly-Glu-Gly-356 (Asp-X-Gly-X-Gly region), separated by 26 amino acids from Gly-Leu-Gly-387 (Gly-X-Gly region). Parallel to the high homology of ActR with RegA and PrrA, ActS also showed very high similarity with RegB from *R. capsulatus* (38.5% identity and 65.6% similarity) and *R. sphaeroides* (41.7% identity and 71.0% similarity) and PrrB from *R. sphaeroides* (42.1% identity and 71.0% similarity), the sensor partners of RegA and PrrA, respectively (Table 2, Fig. 4). Although the *prrA/B* and *regA/B* pairs showed very high similarity with the *actR/S* pair, they differed in gene organization. *actR/S* are contiguous genes transcribed in the same direction (Fig. 2), while *regA/B* (Mosely *et al.*, 1994) and *prrA/B* (Eraso & Kaplan, 1995) are each separated by one gene and transcribed divergently.

PrrB, RegB and FixL belong to sensory systems that respond to oxygen (Mosley *et al.*, 1994; Eraso & Kaplan, 1995; Monson *et al.*, 1995; Qian & Tabita, 1996) and it is possible that ActS also responds to the level of oxygen in the environment. However, the role of oxygen in acid tolerance in *Rhizobium* is unknown and currently under investigation. Both RegA and PrrA are responsible for *trans*-activating expression of the photosynthetic apparatus under oxygen-deprivation conditions. RegA functions to induce the *puc*, *puf* or *pub* operons under anaerobic conditions (Sganga & Bauer, 1992). A *regA* mutant forms photosynthetic spectral complexes, but is unable to photosynthesize under conditions of low light. PrrA is thought to be a global regulator of photosynthetic gene expression which functions to induce the cytoplasmic membrane invagination required to encapsulate the photosynthetic spectral complexes under anaerobic conditions (Eraso & Kaplan, 1994). Under appropriate conditions, PrrA positively regulates the transcription of *cycA*, *puc*, *puf* and *pubA*. Unlike *regA* mutants, a mutation in *prrA* completely abolishes the ability to photosynthesize. The high degree of similarity between RegA/B, PrrA/B and ActR/S implies a similar mechanism of regulating gene expression.

The majority of sensors are membrane-associated molecules containing at least two membrane-spanning regions with a periplasmic loop which makes up a postulated receptor domain (Stock *et al.*, 1989). Determination of the hydrophilicity of the peptide sequence using the Kyte & Doolittle algorithm revealed that the N-terminal half of ActS contained five strongly hydrophobic domains (Fig. 5), implying that this portion of the protein is embedded in the cytoplasmic membrane. There are at least three

major hydrophilic domains (Fig. 5) which could protrude from the membrane and play a role in sensory input.

Inactivation of sensor

The DNA sequence data suggest that a two-component sensor-regulator system is involved in the response of *R. meliloti* to acidity. Inactivation of *actR* results in acid sensitivity and therefore the phenotype of an *actS* mutant was of interest. Out of fifty kanamycin-resistant colonies obtained by interposon marker exchange, six showed tetracycline sensitivity, indicating the loss of pMP220. All six clones were acid-sensitive, failing to grow below pH 6.0, thus demonstrating that inactivation of *actS* leads to a loss of acid tolerance. One of these mutants, RT295, was used in further studies.

Complementation

A DNA library of the acid-tolerant strain *R. meliloti* WSM419 (Tiwari *et al.*, 1996) was screened using a digoxigenin-labelled 0.5 kb *SmaI-HpaI* fragment as a probe. One clone, pRT546-1, containing a 25 kb rhizobial DNA insert, hybridized with the probe and complemented the acid-sensitive defect of TG5-46. pRT546-1 was subcloned to create clones pRT546-20, containing both *actR* and *actS*, and pRT546-11, containing *actR* only. Clone pRT546-6, which contained only *actS*, was subcloned from pRT546Cla. Clone pRT546-20, carrying both *actS* and *actR*, complemented the acid-sensitive defect in both TG5-46 (*actR*) and RT295 (*actS*); pRT546-6, containing only *actS*, complemented RT295, but not TG5-46; and pRT546-11, which carried only *actR*, complemented both mutants (Fig. 6). These results indicate that *actR* in multiple copies can complement the defect in *actS* in strain RT295. In other sensor-regulator systems, it has been suggested that multiple copies of the regulator can either bypass the need for phosphorylation or allow cross-talk phosphorylation by other sensors (Stock *et al.*, 1989).

Distribution of *actR/actS*

Since strains of *R. meliloti* differ in their sensitivity to soil acidity, it is important to know whether *actR/actS*-like genes are found only in the acid-tolerant strains, or whether they also occur in acid-sensitive strains. Genomic DNA from a range of acid-tolerant and acid-sensitive strains was therefore digested with *EcoRI* and the fragments were separated electrophoretically and probed

Fig. 6. (a) Restriction map of the 25 kb *R. meliloti* WSM419 DNA insert in pSW213 clone pRT546-1. The filled box represents *actS*; the open box represents *actR*. Complementation of the acid-sensitive defect of TG5-46 or RT295 by pRT546-1, pRT546-11 or pRT546-6 is indicated. The bold dashed lines represent incompletely restriction mapped regions. (b) Restriction maps of the subclones pRT546-11 and pRT546-6. The cross-hatched box represents the DNA used for constructing the clone pRT546-29. Restriction sites are as follows: B, *Bam*HI; Bgl, *Bgl*II; Cla, *Clal*; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Sau, *Sau*3AI; Sm, *Sma*I.

with the 0.5 kb *Sma*I–*Hpa*I fragment (data not shown). The acid-tolerant strains of *R. meliloti* (WSM419 and WSM540) and the acid-sensitive strains (WSM232, WSM244, CC169, Rm1021 and U45) all had a single *Eco*RI fragment that hybridized with the probe. This indicates that this sensor-regulator system is present in all seven *R. meliloti* strains tested irrespective of their acid sensitivity or tolerance. It will be interesting to see if the acid-sensitive and acid-tolerant strains of *R. meliloti* differ because of *actR/S* or the genes regulated by this pair. At high stringency there was no hybridization of the probe to genomic DNA from *R. leguminosarum* bv. *viciae* WSM710 or WSM937, *R. leguminosarum* bv. *trifolii* NA3001 or TA1, *R. tropici* 5024, *Agrobacterium tumefaciens* GMI9023 or *E. coli* DH5 α (data not shown).

Expression of *actR* and *actS*

The inactivation of *actR* or *actS* in *R. meliloti* WSM419 results in an acid-sensitive phenotype. Complementation analysis has shown that pRT546-20 repaired the *actS* defect in RT295, whereas pRT546-11 complemented the *actR* defect in TG5-46. This suggests that there are promoters upstream of each of these genes. Expression from these potential promoters was analysed in pMP220-Km2. This latter vehicle was constructed by inserting a 2.3 kb blunted *Hind*III kanamycin-resistant omega interposon from pPH45 Ω -Km (Fellay *et al.*, 1987) into a blunted *Bgl*II site of the broad-host-range *lacZ* expression vector pMP220 (Spaink *et al.*, 1987). The kanamycin-resistance gene flanked by T4 translation/transcription

termination signals was orientated in such a way that its own transcription was in the opposite direction to that of *lacZ*. This modified vector had three advantages over using pMP220: (a) termination signals before the start codon of the *lacZ* gene should prevent any readthrough transcription originating upstream, (b) kanamycin resistance can be used as a selection pressure for the construct since tetracycline appears to be antagonistic to cells at low pH (Goss *et al.*, 1990), and (c) the 2.3 kb omega interposon insert aids in the visualization of any small insert cloned into this vector when the DNA is cut by the appropriate enzyme.

The clone pRT546-29 had a 1.2 kb *Bgl*II–*Pst*I fragment (Fig. 6) and pRT546-32 had a 0.24 kb *Sma*I₁₃₈₉–*Sal*I₁₆₃₁ fragment (Fig. 2) in pMP220-Km2, which should contain the *actS* and *actR* promoters, respectively. These clones were mobilized into *R. meliloti* WSM419 and β -galactosidase activity was measured in cells cultured at neutral and acidic pH (Table 3). The results showed that the β -galactosidase expression originating from the rhizobial DNA inserts was constitutive irrespective of the pH at which the cells were grown.

The marked similarity between ActR and RegA and PrrA, coupled with the equally marked similarity between ActS and RegB, raises the question of why sensor-regulator systems from such apparently diverse organisms as *R. meliloti* and the photosynthetic bacteria *R. sphaeroides* and *R. capsulatus* should be similar. The suggestion that root nodule bacteria originated via a common photosynthetic ancestor (Sprenst, 1990) and the finding that *R. meliloti* falls

Table 3. Specific activity of β -galactosidase from cells grown in JMM minimal medium at different pH

Clone	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]		
	pH 7.0	pH 6.0	pH 5.8
WSM419/pRT546-29 (<i>PactS</i>)	1071	759	1258
WSM419/pRT546-32 (<i>PactR</i>)	5856	5950	7153

within the alpha subdivision of the *Proteobacteria* on the basis of 16S rRNA phylogeny (Young *et al.*, 1991) offer a possible explanation in direct relatedness.

However, the ActS/ActR system in *R. meliloti* appears to be concerned with response to low pH, whereas the RegA/RegB and PrrA/PrrB systems in the photosynthetic bacteria appear to respond to decreases in O₂ concentration to switch on genes for photosynthetic activity (Mosley *et al.*, 1994; Eraso & Kaplan, 1995). An important question is why such similar proteins should have such apparently different functions in the two organisms. One obvious answer to the paradox is that despite their overall similarity their differences are great enough to allow one to sense and respond to O₂ and the other to react to low pH. In the photosynthetic bacteria, a connection between falling pO₂ and pH is not obvious, so that this system being only responsive to pO₂ raises no problems. However, the two conditions – low pO₂ and low pH – may well be physiologically linked for heterotrophic bacteria like *R. meliloti*; fermentative metabolism at high cell density and low pO₂ will produce acidic end-products and a low pH. A response to low pO₂ which anticipated a fall in pH would be biologically sensible for *R. meliloti*. Another important, though not easy question to answer would then be whether the same sensor-regulator system can detect and respond to both environmental signals, or whether such a system might perhaps respond to some parameter influenced by both. The simplest situation, however, is still that the RegB/RegA and PrrB/PrrA systems react to O₂ concentration and the ActS/ActR system to low pH.

Low pH induces synthesis of proteins not normally produced at neutral pH in enteric bacteria (Aliabadi *et al.*, 1988; Olson, 1993) and root nodule bacteria (Glenn *et al.*, 1986; Aarons & Graham, 1991). How the actS/actR sensor-regulator system may control expression of genes involved in resistance to low pH remains to be determined. Identification of genes which are regulated by the actS/actR system is in progress.

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