The transcriptional regulator gene \textit{phrR} in \textit{Sinorhizobium meliloti} WSM419 is regulated by low pH and other stresses

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The \textit{phrR} gene in \textit{Sinorhizobium meliloti} (previously known as \textit{Rhizobium meliloti}) WSM419, directly downstream from \textit{actA}, is induced by low pH or certain stresses (e.g. high concentrations of \textit{Zn}^{2+}, \textit{Cu}^{2+}, \textit{H}_2\textit{O}_2, or ethanol), but not in stationary phase or by other stresses (e.g. phosphate limitation, elevated temperature, high concentrations of sucrose or iron). A DNA fragment containing the wild-type \textit{phrR} gene could not be cloned and inverse PCR was therefore used to amplify a 3-5 kb \textit{BamH}I fragment containing \textit{phrR} from the mutant \textit{S. meliloti} TG2-6 (\textit{actA::Tn5}). DNA fragments from a \textit{BamH}I/\textit{SalI} digest of the amplified product were cloned into pUK2l and sequenced. The \textit{phrR} open reading frame contiguous to \textit{actA} appears to code for a 15.2 kDa protein showing significant identity with the proteins encoded by \textit{y4wC} and \textit{y4aM} in \textit{Rhizobium} sp. NGR234. All three proteins resemble transcriptional regulators in containing a DNA-binding helix-turn-helix motif similar to that reported for URF4 in \textit{Rhodospirillum rubrum} and repressors in coliphage.

\textbf{Keywords:} acidity, stress, acid-inducible gene, regulator, DNA-binding domain

\section*{INTRODUCTION}

The survival or growth of bacteria can be adversely affected by low pH and cells need to adapt to a changing environment to survive and be competitive. An understanding of the response of bacteria to low pH is particularly relevant to interactions between bacteria and their hosts. Bacteria of medical importance must survive harsh acidic environments such as those encountered in the stomach or macrophage phagolysosomes (Mekalanos, 1992; Foster, 1995) while agriculturally important bacteria like root nodule bacteria must first survive in acidic soils before they can successfully invade legume roots (Munns, 1986). The relatively tight regulation of the cytoplasmic pH (Booth, 1985) suggests that there are mechanisms allowing cells to combat proton influx. The mechanisms allowing cells to survive and grow at low pH are not yet defined, although a number of processes have been proposed to be involved for the enterics, including cytoplasmic buffering (Booth, 1985; Krulwich et al., 1985), DNA repair (Foster, 1995), ion cycling (Booth, 1985), pH amelioration (Huang et al., 1986; Slonczewski et al., 1987; Stim & Bennet, 1993) and proton translocation (Shibata et al., 1992; White et al., 1992).

Among the stress-inducible genetic systems is the ‘acid habituation’ or ‘acid-tolerance response’ (ATR) identified in \textit{Escherichia coli} (Goodson & Rowbury, 1989) and \textit{Saimonella typhimurium} (Foster, 1991), and more recently found in \textit{Aeromonas hydrophila} (Karem et al., 1994), \textit{Listeria monocytogenes} (Farber & Pagatto, 1992) and various species of root nodule bacteria (O’Hara & Glenn, 1994). The ATR is an important response that enables cells exposed to mildly acid pH to cope more effectively with subsequent more severe low-pH conditions than cells previously grown at neutral pH. In the enterobacteria, exposure to mildly acidic conditions induces the synthesis of 51 acid-shock proteins (ASPs) (Lee et al., 1995) and provides cross-protection to heat, osmotic and oxidative challenge (Leyer & Johnson, 1993; Lee et al., 1995).
Agriculture in Western Australia is heavily dependent upon the saprophytic competence of root nodule bacteria, the persistence of which between seasons is very much dependent on the pH of the soil and its rate of acidification. Production from medic-based pastures is markedly decreased in soils of low pH. The acid sensitivity of the (Sino)rhizobium—Medicago symbiosis results from the inability of many of the standard rhizobial inoculants to survive and grow in acid soils. The discovery of natural variants from the south Mediterranean which perform well in such moderately acid soils has allowed stable medic pastures to be developed on more than 1 million ha of soils that were previously unable to sustain them. The isolation of the superior Mediterranean strains raises questions as to why these strains are more acid tolerant in field conditions than others and about how such strains cope with acid stress.

Several genes involved in acid tolerance in Sino-rhizobium meliloti (previously known as Rhizobium meliloti) WSM419 have been identified by Tn5 mutagenesis (O’Hara et al., 1989; Tiwari et al., 1992). The process by which this strain senses and/or responds to low pH appears to involve a signal transduction system (ActS, a histidine protein kinase ‘sensor’, and ActR, its cognate regulator); insertional inactivation of either component results in an acid-sensitive phenotype (Tiwari et al., 1996b). It is anticipated that signal processing via ActS-ActR will result in altered gene expression in response to low pH. Similarly, the presence of an intact actA gene, which may code for a lipid acyl transferase, has also been shown to be essential (Tiwari et al., 1996a). Studies using lacZ fusions have revealed that actA (Tiwari et al., 1996a), actR and actS (Tiwari et al., 1996b) are constitutively expressed. By analogy to the enteric organisms, the existence of an ATR system in root nodule bacteria (O’Hara & Glenn, 1994) suggests that synthesis of new proteins should be induced under acidic conditions.

This report presents the first finding of a pH-regulated gene (designated phrR, for pH regulated) in S. meliloti WSM419. This gene, located downstream from the actA gene, encodes a putative repressor protein PhrR and is induced by low pH and other environmental stresses.

**METHODS**

**Bacterial strains, plasmids and media.** Bacterial strains and plasmids are described in Table 1. Strains of Sinorhizobium were grown at 28 °C in JMM minimal medium (O’Hara et al., 1989). Escherichia coli strains were grown in Luria–Bertani (LB) medium at 37 °C. Media were supplemented with the following concentrations of antibiotics (μg ml⁻¹): ampicillin (100), chloramphenicol (20), gentamicin (40), kanamycin (50), or tetracycline (20).

**DNA preparation and manipulation.** The plasmid DNA isolation, transformation and manipulation techniques used have been described earlier (Sambrook et al., 1989; Inoue et al., 1990). Restriction and modification enzymes were purchased from either Life Technology or Boehringer Mannheim. Genomic DNA was isolated from Sinorhizobium cultures as described earlier (Reeve et al., 1997).

**DNA sequencing and analysis.** The techniques used have been described earlier (Tiwari et al., 1996a, b).

**Inverse PCR.** Genomic DNA from strain TG2-6 was restriction digested using BamHI and the mixture of fragments religated at low concentration (1 μg ml⁻¹). The ligated mixture was ethanol precipitated and the DNA dissolved to give a concentration of 10 μg ml⁻¹. The circular DNA molecules generated by ligation were linearized by HindIII. Two primers, one in the phrR region (PHR-187 primer; 5’ CGC AGA TAC TCA ATG TGC C 3’) and another from the Tn5 sequence (TN5-2770 primer; 5’ AGG TCA CGT GGA TGG AG 3’), were used to amplify a 3.5 kb fragment using the method of Rich & Willis (1990). The amplified DNA was run on a 1% gel and the 3.5 kb PCR product was purified using Agarase (Boehringer Mannheim).

**Reconstruction of phrR.** The BamHI fragment of pRT206-6 (the clone containing the reconstructed actA; Tiwari et al., 1996a) was cloned into the BamHI site of pUK21 to construct pRT206-18. Plasmid pRT206-16, containing the SalI clone from the PCR product of TG2-6 DNA, was partially cut by SalI, blunted and ligated to a BamHI-blunted digest of ϕ5m interposon to construct pRT206-23. The SalI-KpnI fragment from pRT206-23 was cloned into the SalI/KpnI sites of plasmid pRT206-18 to construct pRT206-24. This plasmid contains a reconstructed wild-type phrR gene that was verified by DNA sequence analysis. To facilitate mobilization of this plasmid into Sinorhizobium, the BamHI fragment of pRT206-24 was cloned into the BglII site of the broad-host-range vector pMP220 (Spaink et al., 1987) to construct pRT206-25.

**Inactivation of phrR.** To facilitate marker exchange, the plasmid antibiotic-resistance marker of pRT206-24 was changed from kanamycin to ampicillin by subcloning the BamHI fragment of pRT206-24 into pGEM7zf(−) to construct pRT206-24G. The ΩKm resistance interposon was cloned into the ClaI site in the 0.7 kb EcoRI–SalI fragment to construct pRTES1. pRT206-24G was cut with SalI, blunted and then the DNA cleaved with EcoRI and ligated with pRTES1, which was cut with HindIII, blunted and then cut by EcoRI. The new clone pRT206-27 had the Ω interposon at the tenth codon of phrR.

pRT206-24G was cut with SalI, blunted and ligated with HindIII-blunted ΩKm interposon to construct pRT206-28, placing the interposon at codon 126 of phrR. A BamHI fragment of either pRT206-27 or pRT206-28 was cloned into the BglII site of pMP220 to construct pRT206-27M and pRT206-28M, respectively. The latter plasmids were mobilized into Sinorhizobium using pRK2013 as a helper. The incompatible plasmid pPH171 (Beringer et al., 1978) was mobilized into tetracycline- and kanamycin-resistant clones and transconjugants were selected on gentamicin and kanamycin. Genomic DNA was extracted from tetracycline-sensitive isolates and hybridized with DIG-labelled pES1 to confirm that marker exchange had occurred.

**Nodulation tests.** Cultures of S. meliloti WSM419 or RT10 were inoculated onto germinated seedlings of Medicago murex or M. sativa as described by Reeve et al. (1997). Nodules isolated from 4-week-old plants were sterilized, crushed in sterile saline and the suspension replica patched onto solid medium in the presence and absence of kanamycin.

**Reporter gene fusions.** Reporter gene fusions were constructed in wide-host-range IncP1 plasmids containing a
### Table 1. Strains and plasmids used

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics*</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F− ϕ80lacZΔM15 recA1 endA1 gyrA96 thi-1 hsdR17 (rK− mK+*) supE44 relA1 deoR Δ(lacZYA-argF)U169</td>
<td>Bethesda Research Laboratories (1986)</td>
</tr>
<tr>
<td>S. meliloti</td>
<td></td>
<td></td>
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<tr>
<td>RT10</td>
<td>pbrR10; QKm interposon inserted into codon 10 of pbrR in WSM419; Cm' Km'</td>
<td>This study</td>
</tr>
<tr>
<td>RT126</td>
<td>pbrR126; QKm interposon inserted into codon 126 of pbrR in WSM419; Cm' Km'</td>
<td>This study</td>
</tr>
<tr>
<td>TG2-6</td>
<td>Tn5-induced Acid&lt;sup&gt;d&lt;/sup&gt; mutant of WSM419; Km'</td>
<td>Goss et al. (1990)</td>
</tr>
<tr>
<td>TG5-46</td>
<td>Tn5-induced Acid&lt;sup&gt;d&lt;/sup&gt; mutant of WSM419; Km'</td>
<td>Goss et al. (1990)</td>
</tr>
<tr>
<td>WSM419</td>
<td>Acid&lt;sup&gt;d&lt;/sup&gt; Sardinian isolate</td>
<td>J. Howieson†</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
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<tr>
<td>pES1</td>
<td>0.7 kb EcoRI–SalI fragment from pTG2-6S cloned into pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM7Zf(−)</td>
<td>Cloning vector; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Promega</td>
</tr>
<tr>
<td>pHP45ΩKm</td>
<td>pHp45Ω derivative with Km' interposon; Ap&lt;sup&gt;+&lt;/sup&gt;, Km'</td>
<td>Fellay et al. (1987)</td>
</tr>
<tr>
<td>pMP220</td>
<td>Broad-host-range lacZ fusion vector; Tc'</td>
<td>Spaink et al. (1987)</td>
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<tr>
<td>pFUS1</td>
<td>pMP220 in which lacZ has been replaced with promoterless gusA</td>
<td>This study</td>
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<td>pFUS1-1</td>
<td>pFUS1 containing a blunted 0.7 kb EcoRI–SalI fragment from pTG2-6S; pbrR transcribed towards gusA</td>
<td>This study</td>
</tr>
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<td>pPH1J1</td>
<td>Broad-host-range IncP plasmid; Gm&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Beringer et al. (1978)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid; Km&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Ditta et al. (1980)</td>
</tr>
<tr>
<td>pRTES1</td>
<td>ΩKm interposon inserted into the ClaI site in pES1</td>
<td>This study</td>
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<td>pRT206-6</td>
<td>pLAFR3 containing a reconstructed actA gene</td>
<td>Tiwari et al. (1996a)</td>
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<tr>
<td>pRT206-8</td>
<td>The blunted 0.7 kb EcoRI–SalI fragment from pTG2-6S cloned into pMP220; pbrR transcribed towards lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pRT206-16</td>
<td>1.5 kb SalI fragment (Fig. 1d) cloned into the SalI site of pUC18</td>
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<tr>
<td>pRT206-18</td>
<td>pUK21 containing reconstructed actA gene</td>
<td>This study</td>
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<tr>
<td>pRT206-23</td>
<td>See text for details</td>
<td>This study</td>
</tr>
<tr>
<td>pRT206-24</td>
<td>pUK21 containing reconstructed pbrR gene</td>
<td>This study</td>
</tr>
<tr>
<td>pRT206-24G</td>
<td>pGEM7Zf(−) containing reconstructed pbrR gene</td>
<td>This study</td>
</tr>
<tr>
<td>pRT206-25</td>
<td>pGEM7Zf(−) containing reconstructed pbrR gene</td>
<td>This study</td>
</tr>
<tr>
<td>pRT206-27</td>
<td>ΩKm interposon inserted into the 10th codon of pbrR (pbrR10-ΩKm) in pGEM7Zf(−)</td>
<td>This study</td>
</tr>
<tr>
<td>pRT206-28</td>
<td>pbrR126-ΩKm in pGEM7Zf(−)</td>
<td>This study</td>
</tr>
<tr>
<td>pRT206-27M</td>
<td>pbrR10-ΩKm in pMP220</td>
<td>This study</td>
</tr>
<tr>
<td>pRT206-28M</td>
<td>pbrR126-ΩKm in pMP220</td>
<td>This study</td>
</tr>
<tr>
<td>pTG2-6S</td>
<td>pUC18 containing Km' SalI fragment of TG2-6</td>
<td>Goss et al. (1990)</td>
</tr>
<tr>
<td>pUC18</td>
<td>Cloning vector; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUK21</td>
<td>Cloning vector; Km'</td>
<td>Vieira &amp; Messing (1991)</td>
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</tbody>
</table>

* Acid<sup>d</sup>, acid-sensitive; Acid<sup>t</sup>, acid-tolerant; Ap<sup>+</sup>, Cm<sup>+</sup>, Gm<sup>+</sup>, Km<sup>+</sup>, Sm<sup>+</sup>, Tc', resistant to ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, tetracycline.

† Centre for Rhizobium Studies.
promoterless lacZ (pMP220) or gusA (pFUS1). The 0.7 kb EcoRI-SalI fragment from S. meliloti TG2-6 (containing the phrR promoter) was cloned from pTG2-6S (Goss et al., 1990) as an EcoRI fragment into the EcoRI site of pMP220 (Spink et al., 1987) or pFUS1 to construct pRT206-8 or pFUS1-1, respectively.

**Expression studies.** Exponential cultures, grown in JMM broth at pH 7.0 containing chloramphenicol (for WSM419) or tetracycline (for WSM419[pFUS1, pFUS1-1, pMP220 or pRT206-24]), were centrifuged (3024g, 5 min; Beckman Avanti J251 centrifuge) and the cell pellet resuspended in normal saline (0.89%, w/v, NaCl). An aliquot of cells was used to inoculate flasks containing JMM at a range of pH values (5.8-7.0), and JMM at pH 5.8 or 7.0 with or without addition of 20-100 μM CuSO4, 0.8% (v/v) ethanol, 0.1-100 μM FeCl3, 200-800 μM H2O2, 0-300 μM potassium phosphate, 12.5% (w/v) sucrose and 10-40 μM ZnSO4. The cultures were then incubated at 28 °C (unless otherwise indicated) on a gyratory shaker set at 250 r.p.m. and harvested at an OD600 between 0.2 and 0.5.

Stationary-phase cells that had grown to a constant density were harvested after 5 d incubation in JMM at pH 7.0. For the time-course experiment, cells grown in JMM at pH 7.0 were centrifuged and resuspended in JMM at pH 5-8 to provide an initial OD600 of 0.3.

**Enzyme assays.** Exponential-phase cultures (OD600 0.2-0.5) were centrifuged and resuspended into cold normal saline to an OD600 of approximately 0.6. Three replicates per strain were performed. Protein concentration was measured using a Bio-Rad protein assay kit.

β-Galactosidase activity. β-Galactosidase was quantified as described by Miller (1972) and the specific activity was expressed as nmol p-nitrophenol (pNP) produced min-1 (mg protein)-1 at 28 °C.

β-Glucuronidase activity. A 50-200 μl aliquot of cells was mixed with 790 μl buffer (50 mM sodium phosphate, 50 mM DTT and 1 mM EDTA; pH 7) and saline was added if required to produce a final volume of 990 μl. For a blank, 200 μl saline was used instead of cells. One drop of toluidine blue was added to each tube, the mixture vortexed and the tubes then incubated at 37 °C for 30 min with the lids off to remove toluidine blue. Tubes were equilibrated at 28 °C for 5 min before the assay was started. A 10 μl aliquot of p-nitrophenyl β-D-glucuronide (pNPG; 35 mg ml-1) was added to start the reaction. To terminate the reaction, a 200 μl aliquot from each tube was removed and added to 700 μl 0.46 M Na2CO3 at three different time points. These tubes were centrifuged for 1 min at room temperature to pellet cell debris. The absorbance of the supernatant was read at 405 nm. β-Glucuronidase specific activity was expressed as nmol p-nitrophenol (pNP) produced min-1 (mg protein)-1 at 28 °C.

**RESULTS AND DISCUSSION**

**Studies on the actA region**

Previous studies of the actA gene in S. meliloti WSM419 showed that it was essential for acid tolerance and constitutively expressed (Tiwari et al., 1996a). However, when a 0.7 kb EcoRI–SalI fragment containing an incomplete ORF downstream from actA (Tiwari et al., 1996a) was cloned into the broad-host-range vector pMP220, a fivefold induction of this gene occurred in cells grown at pH 5.8 relative to pH 7.0 (see Table 2). It was therefore termed a phr (pH regulated) gene.

Further studies on this low-pH-regulated gene were hindered initially by our inability to clone the complete gene. The DNA region containing actA was extensively restriction mapped (Fig. 1a) using information derived from Southern hybridization studies (Tiwari et al., 1992), and clones isolated from either TG2-6 (Goss et al., 1990), or WSM419 (Tiwari et al., 1996a). The restriction map located a BamHI site 4.5 kb from the site of Tn5 insertion in TG2-6 (Fig. 1a). Attempts to clone the 6.3 kb BamHI fragment (containing actA and the phr gene) from strain WSM419 using a subgenomic library were unsuccessful (Tiwari et al., 1992), suggesting that a gene located on this fragment may be toxic to E. coli. A 7.5 kb BamHI fragment from TG2-6 (Fig. 1a) containing the phr gene could not be cloned into either pUC18 or pBR322 using the kanamycin-resistance marker of Tn5 as a positive selection for incorporation of the insert. DNA downstream of actA could not be identified from a library of WSM419, which is known to overrepresent other genes (Tiwari et al., 1996a). These studies, together with the finding that actA could be reconstructed (Tiwari et al., 1996a), narrow the toxic region to within a 4.2 kb EcoRI–BamHI fragment (Fig. 1a).

**Cloning of the phr gene**

An inverse-PCR strategy was used to amplify a DNA fragment containing the phr gene (Fig. 1b) from TG2-6 genomic DNA using PHR-187 and TNS-2770 primers. The 3.5 kb PCR product was subsequently purified from an agarose gel and used directly for sequencing (Fig. 1c). Restriction digestion with Sall showed three sites in this fragment (Fig. 1d) and the two SalI fragments were cloned into the SalI site of pUC18. Sequencing of the PCR product using PHR-187 primer and sequencing of the two SalI clones consolidated the order of SalI fragments in this 3.5 kb fragment. Restriction mapping of the 1.5 kb SalI fragment identified two SphI sites (Fig. 1d), which were used to obtain double-stranded DNA sequence of the phr gene.

**Nucleotide sequence data**

Analysis of 988 bp of DNA sequence derived from an EcoRI–SphI DNA fragment revealed an open reading frame (phrR) that started 140 bases downstream of the stop codon of actA (Tiwari et al., 1996a). The region upstream of the start codon of phrR contains a putative RBS. The gene phrR encodes a predicted 152 kDa protein product containing 139 amino acids. The complete nucleotide and annotated protein sequence is available from GenBank (accession number L13845).

A database search with PhrR detected 50.7% identity over a 138 amino acid overlap with the hypothetical 16.5 kDa Y4wC protein of Rhizobium sp. NGR234. Another match of 49.6% identity over a 131 amino acid overlap occurred with the hypothetical Y4aM protein from the same organism. Although the latter two proteins are encoded by genes located on the symbiotic megaplasmid, the phrR locus is positioned on the
Low-pH-regulated gene \textit{phrR}

\begin{align*}
\text{TG2-6 genomic DNA} & \quad \text{Cut by BamHI} \\
\text{Religated product cut by HindIII and amplified by inverse PCR using TNS-2770 and PHR-187 primers.} & \quad \downarrow \\
\text{The 3.5 kb PCR product was used for sequencing and cloning.} & \\
\end{align*}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{inverse-PCR-strategy}
\caption{Inverse-PCR strategy to reconstruct the \textit{phrR} gene. The \textit{actA} gene region (a) of \textit{S. meliloti TG2-6} (Tiwari \textit{et al.}, 1996a) was digested with \textit{BamHI} (b), ligated and cut by \textit{HindIII} (c). The 3-5 kb DNA fragment (d) was amplified, restriction mapped and used for sequencing. The 1-0 and 1-5 kb \textit{SalI} fragments were cloned into pGEM7Zf(--) to construct pRT206-15 and pRT206-16, respectively. Restriction sites are as follows: B, \textit{BamHI}; Bg, \textit{BglII}; Bg, \textit{BgIII}; E, \textit{EcoRI}; Ev, \textit{EcoRV}; H, \textit{HindIII}; S, \textit{SalI}; Sm, \textit{SmaI}; Sp, \textit{SphI}. The \textit{SphI} sites have been partially restriction mapped and therefore marked with an asterisk (*).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{alignment}
\caption{Alignment of PhrR with three other DNA-binding proteins as compared earlier by Falk & Wagner (1978). The underlined region corresponds to the DNA-binding domain. Sm, \textit{Sinorhizobium meliloti} WSM419; Rr, \textit{Rhodospirillum rubrum}; 434, bacteriophage 434; P22, bacteriophage P22.}
\end{figure}

\section*{Inactivation of \textit{phrR}}

The \textit{phrR} gene was inactivated by inserting the \textit{\Omega}Km interposon at codon 10 or codon 126. The two mutants (RT10 and RT126) created by the inactivation of \textit{phrR}

\section*{Expression of \textit{phrR}}

The \textit{phrR–lacZ} fusion (pRT206-8) or the \textit{phrR–gusA} fusion (pFUS1-1) was mobilized into \textit{S. meliloti} WSM419 to study factors controlling the expression of \textit{phrR}.

\section*{Regulation by pH}

The \textit{phrR–lacZ} fusion in \textit{S. meliloti} WSM419 was expressed at low levels [approx. 500 nmol min$^{-1}$ (mg protein)$^{-1}$] between pH 7-0 and pH 6-2, but increased significantly as the pH fell to 5-8

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textit{Sm} & \textit{PhrR} & PDDHVGS & R & IRL & RR & TM & L & QMS & E & BGLGE & L & TFOQ & T & QRY & E & KTG & R
\hline
\textit{Rr} & \textit{URF4} & HVDAVQG & R & VRQ & TR & TN & L & ILO & E & TRG & G & SPO & N & CRY & E & RENR & IAS
\hline
\hline
\textit{434} & \textit{Cro} & MQTSL & R & LKK & TR & LA & L & KMT & O & TEL & TAKA & G & KQG & I & QLI & E & AGTV & RKPRF
\hline
\end{tabular}
\caption{DNA binding domain}
\end{table}

chromosome of \textit{S. meliloti} WSM419 (Tiwari \textit{et al.}, 1992, 1996a). The proteins encoded by \textit{y4wC} and \textit{y4aM} have been classified as putative transcriptional regulators (Freiberg \textit{et al.}, 1997) on the basis of their identity with the DNA-binding domain of a protein encoded by a gene \textit{URF4} in the ATPase region of \textit{Rhodospirillum rubrum} (Falk & Walker, 1988). The \textit{PhrR} protein has 53.5\% identity in a 71 amino acid overlap which spans the DNA-binding domain of the \textit{URF4} protein. The \textit{PhrR} and \textit{URF4} DNA-binding domains are similar to those reported for the phase 434 \textit{Cro} and \textit{P22} C2 repressor proteins (Fig. 2). The \textit{phr} gene thus appears to code for a repressor protein that binds to DNA. For this reason this gene has been termed \textit{phrR} for \textit{pH}-regulated Regulator.

were as acid tolerant as the parental strain WSM419, suggesting that, in contrast to \textit{actA}, the \textit{phrR} gene is not essential for growth at low pH. The \textit{phrR} gene is not required for successful invasion of \textit{Medicago} since the mutant RT10 produced normal nodules on both \textit{Medicago murex} and \textit{M. sativa}. Kanamycin-resistant cells were recovered from the nodules of plants inoculated with RT10. No nodules were found on uninoculated seedlings.

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\section*{Regulation by pH}

The \textit{phrR–lacZ} fusion in \textit{S. meliloti} WSM419 was expressed at low levels [approx. 500 nmol min$^{-1}$ (mg protein)$^{-1}$] between pH 7-0 and pH 6-2, but increased significantly as the pH fell to 5-8.
Fig. 3. (a) Expression of the phrR-lacZ fusion (pRT206-8) in S. melliloti WSM419 cultured in JMM minimal medium at different pH values. (b) Time course of the expression of the phrR-lacZ fusion (pRT206-8) in S. melliloti WSM419 transferred from JMM minimal medium at pH 7.0 to 5.8. Error bars represent the standard error of the mean, which was calculated from the results of three independent experiments.

The maximum expression occurred at pH 5.8, representing a fivefold increase in specific activity from that recorded at pH 7.0. The time course for β-galactosidase expression from the phrR-lacZ fusion at pH 5.8 indicated that near-maximum induction occurred approximately 8 h after transfer of the cells from neutral to acidic conditions (Fig. 3b).

**Regulation by other factors.** One stress can regulate the expression of genes belonging to different regulons, while a gene of one regulon may be regulated by different agents (VanBogelen et al., 1987; Foster, 1995). Agents known to induce oxidative stress (heavy metals, hydrogen peroxide), heat shock (heat, ethanol and heavy metals) or SOS responses (heavy metals, hydrogen peroxide) were investigated for their effect on the expression of the phrR-gusA fusion in a wild-type WSM419 background. A maximum fivefold induction of the phrR-gusA fusion in WSM419 was observed at low pH (5.8) or at neutral pH in a medium containing 400 μM hydrogen peroxide or 40 μM Zn2+ (Table 2).

Addition of 0.8% (v/v) ethanol or 100 μM Cu2+ to JMM minimal medium at neutral pH resulted in a three- or twofold increase, respectively, in β-glucuronidase activity of the phrR-gusA fusion in WSM419 (Table 2). The maximum expression was achieved at the stress levels presented in Table 2; under these conditions the growth rate was significantly decreased and a further increase in the stress level resulted in a significant loss in cell viability.

It was possible that altered solubility of metal ions at different pH values could have resulted in the induction of the phrR-gusA fusion at low pH. For example, it is known that the availability of Fe3+, Mn2+, Cu2+ and Zn2+ increases, whereas that of Mo2+ decreases, at low pH (Lindsay, 1991). The expression of the phrR fusion was therefore examined at different concentrations of metal ions. The expression of phrR remained unaffected at low pH if CuSO4, MnSO4 and ZnSO4 were omitted from JMM minimal medium. Similarly, increasing the concentration of CuSO4 and ZnSO4 did not alter the expression of phrR at low pH (data not shown). These results suggested that the induction of phrR at acidic

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**Table 2. Effect of different stresses on the expression of the phrR-gusA fusion**

<table>
<thead>
<tr>
<th>pH</th>
<th>Treatment*</th>
<th>β-Glucuronidase activity [nmol pNP produced min⁻¹ (mg protein)⁻¹]†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WSM419 (wild-type)</td>
<td>TG5-46 (WSM419 actR::Tn5)</td>
</tr>
<tr>
<td>7.0</td>
<td>Standard JMM</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Cu²⁺ (100 μM)</td>
<td>251</td>
</tr>
<tr>
<td></td>
<td>Zn²⁺ (40 μM)</td>
<td>554</td>
</tr>
<tr>
<td></td>
<td>Ethanol (0.8%, v/v)</td>
<td>331</td>
</tr>
<tr>
<td></td>
<td>Hydrogen peroxide (400 μM)</td>
<td>590</td>
</tr>
<tr>
<td>5.8</td>
<td>Standard JMM</td>
<td>580</td>
</tr>
</tbody>
</table>

* Cells were grown in JMM minimal medium at 28 °C unless otherwise indicated.
† The background β-glucuronidase activity, contributed by pFUS1 in WSM419 grown in similar conditions, has been subtracted. ND, Not determined.
environmental stress can be coordinately expressed by a number of different stimuli. The demonstration that acid-inducible expression of other environmental stresses also cause some genes like phrR, which, while not essential for growth, appear to be regulated not only in response to heat but also to ethanol (VanBogelen et al., 1987). The DNA sequence that includes the phrR promoter does not contain the consensus heat-shock promoter sequence [−35 TCN-CCTTGGAA (13−15 bp) CCCCATTTA −10 region]; this is consistent with the lack of up-regulation of phrR by heat.

The significant increase in β-glucuronidase activity by low pH and certain other environmental factors suggests the involvement of a regulatory system controlling expression of phrR. One possible regulatory circuit could involve the ActS/R two-component signal transduction system (Tiwari et al., 1996b) in controlling the expression of phrR in response to pH. Expression of the latter gene was therefore examined in both the wild-type (WSM419) and an actR (regulator) mutant (TG5-46). Since there was no significant difference in phrR expression between an actR and wild-type background, it appears that the regulatory system for phrR expression does not require actR.

These data show that, in addition to the genes like actA, actS and actR that are absolutely essential for growth of S. meliloti at low pH, there is another class of genes which, while not essential for growth, appear to be induced by exposure to low pH. In the case of the phrR gene, other environmental stresses also cause some degree of induction. Regulons responsive to a particular environmental stress can be coordinately expressed by a common regulatory gene (Gottesman, 1984). Expression of phrR is up-regulated by exposure of cells to different stresses. PhrR is a putative repressor that may be part of a cascade that regulates gene expression in response to different stimuli. The demonstration that acid-inducible proteins exist in this strain opens the way for further studies on the nature and role of such proteins in the response of WSM419 to acid stress.

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