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# The *Sinorhizobium medicae* WSM419 *lpiA* gene is transcriptionally activated by FsrR and required to enhance survival in lethal acid conditions

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*Sinorhizobium medicae* WR101 was identified as a mutant of WSM419 that contained a minitransposon-induced transcriptional *gusA* fusion activated at least 20-fold at pH 5·7. The expression of this fusion in moderately acid conditions was dependent on the calcium concentration; increasing the calcium concentration to enhance cell growth and survival in acid conditions decreased the expression of the fusion. A gene region containing the *gusA* fusion was sequenced, revealing five *S. medicae* genes: *tcsA*, *tcrA*, *fsrR*, *lpiA* and *acvB*. The *gusA* reporter in WR101 was fused to *lpiA*, which encodes a putative transmembrane protein also found in other *Alphaproteobacteria* such as *Sinorhizobium meliloti*, *Rhizobium tropici* and *Agrobacterium tumefaciens*. As LpiA has partial sequence similarity to the lysyl-phosphatidylglycerol (LPG) synthetase FmtC/MprF from *Staphylococcus aureus*, membrane lipid compositions of *S. medicae* strains were analysed. Cells cultured under neutral or acidic growth conditions did not induce any detectable LPG and therefore this lipid cannot be a major constituent of *S. medicae* membranes. Expression studies in *S. medicae* localized the acid-activated *lpiA* promoter within a 372 bp region upstream of the start codon. The acid-activated transcription of *lpiA* required the fused sensor–regulator product of the *fsrR* gene, because expression of *lpiA* was severely reduced in an *S. medicae fsrR* mutant. *S. meliloti* strain 1021 does not contain *fsrR* and acid-activated expression of the *lpiA-gusA* fusion did not occur in this species. Although acid-activated *lpiA* transcription was not required for cell growth, its expression was crucial in enhancing the viability of cells subsequently exposed to lethal acid (pH 4·5) conditions.

## INTRODUCTION

Legumes can establish symbiotic relationships with prokaryotic root nodule bacteria, resulting in atmospheric nitrogen being fixed to a form that can be utilized by the plant hosts. In the absence of inorganic nitrogen, legume productivity is largely dependent on the formation of an effective symbiosis. One constraint on the nodulation of legumes in low pH soils is the failure of the microsymbiont to survive between growing seasons (Graham *et al.*, 1982; Robson &

Abbreviations: CDD, Conserved Domain Database; CL, cardiolipin; DMPE, dimethylphosphatidylethanolamine; GUS, β-glucuronidase; LPG, lysyl-phosphatidylglycerol; MMPE, monomethylphosphatidylethanolamine; OL, ornithine-containing membrane lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; pNP, *p*-nitrophenol.

The GenBank/EMBL/DDBJ accession number for the *lpiA* gene region derived from *S. medicae* WSM419 reported in this paper is AF199025.

Loneragan, 1970). A number of research groups have been active in the identification of more acid-tolerant rhizobial genotypes from the natural genetic pool (Graham *et al.*, 1982; Howieson & Ewing, 1986; Priefer *et al.*, 2001). A successful application of identifying superior acid-tolerant inoculants has been to extend *Medicago* pastures onto previously unproductive land in Western Australia (Dilworth *et al.*, 2001). The adaptation of rhizobial strains to low pH is being investigated at the physiological and genetic levels (Dilworth *et al.*, 2001; Peick *et al.*, 1999; Priefer *et al.*, 2001; Vinuesa *et al.*, 2003) to reveal the mechanisms that enable acid-tolerant inoculants to outperform other strains in acidic soils.

A number of genes required for the growth of rhizobia in low pH conditions have been identified (Goss *et al.*, 1990; O'Hara *et al.*, 1989; Riccillo *et al.*, 2000; Tiwari *et al.*, 1992; Vinuesa *et al.*, 2003). Construction of mutants from the acid-tolerant strain *Sinorhizobium medicae* WSM419 has allowed the identification of some of the genes required in acidic conditions (Dilworth *et al.*, 2001). The protein products of these genes include ActA (an apolipoprotein acyl transferase; Tiwari *et al.*, 1996a), ActS (a histidine kinase 'sensor'; Tiwari *et al.*, 1996b), ActR (a response regulator; Tiwari *et al.*, 1996b) and ActP (a CPx heavy metal-transporting ATPase; Reeve *et al.*, 2002). The acid-sensitive mutants with lesions in *actA*, *actS* and *actR* can be restored to wild-type levels of acid-tolerance by addition of high concentrations of calcium (50 mM), but those with lesions in *actP* cannot be repaired by calcium (Reeve *et al.*, 2002). Increased concentrations of calcium also decrease the mean generation times for growth of both *S. medicae* WSM419 and *Rhizobium leguminosarum* bv. *viciae* WSM710, allow growth to occur at a lower pH (Howieson *et al.*, 1992; Reeve *et al.*, 1993; Tiwari *et al.*, 1992), increase the rate of exopolysaccharide production by *S. medicae* at low pH and markedly increase the survival of cells of *S. medicae* exposed to pH 4 (Dilworth *et al.*, 1999). These effects of calcium under acid conditions might be explained by calcium stabilization of various cellular components, or by direct or indirect calcium effects on gene expression.

Genes have also been identified that are not essential for growth in acidic conditions but which are transcriptionally up-regulated by acidity (Reeve *et al.*, 1999). The *S. medicae* WSM419 genes *phrR* (Reeve *et al.*, 1998) and *lpiA* (Reeve *et al.*, 1999) belong in this category and their transcriptional activation implies a significant role under acidic conditions. The *lpiA* gene is the most highly acid-activated gene in *S. medicae*, but its role and regulation have not been studied in detail.

In this paper we have investigated the expression of *lpiA* in *S. medicae* WSM419. The expression of *lpiA* was specifically triggered by acidity and was affected by the concentration of calcium in the medium. The activation of a plasmid-borne *lpiA-gusA* fusion was not subject to regulation by ActS/R or PhrR but was low-pH-activated via the fused sensor–regulator FsrR. Promoter localization studies pinpointed the position of the low-pH-responsive promoter to within a 372 bp region upstream of the *lpiA* start codon. Although expression of *lpiA* was dispensable for growth, acid-activation of *lpiA* was essential for enhanced cell viability under lethal acid conditions.

## METHODS

**Bacterial strains, plasmids and media.** Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured at 37 °C using LB or Antibiotic Medium 3 (Oxoid) when using gentamicin (Reeve *et al.*, 1999). Strains of *Sinorhizobium* were grown at 28 °C using TY, JMM or MJMM (O'Hara *et al.*, 1989; Reeve *et al.*, 2002) media. Minimal medium contained glutamate (3 mM) as nitrogen source. Media were supplemented with the following concentrations of antibiotics (μg $ml^{-1}$): ampicillin (100), chloramphenicol (20), gentamicin (70 for *Sinorhizobium*; 10 for *E. coli*), kanamycin (50) and tetracycline (20 for *Sinorhizobium*; 12·5 for *E. coli*). Agar was added at a concentration of 1·5 % (w/v) to solidify media.

**Nodule occupancy studies and nitrogen fixation.** Seeds of *Medicago murex* (L.), *Medicago sativa* (L.), *Medicago polymorpha* (L.) and *Medicago truncatula* (Gaertn) were surface-sterilized, germinated on water agar, sown and watered as described previously (Reeve *et al.*, 1999) in an axenic sand-based culture system (Howieson *et al.*, 1995). Immediately after planting, *Medicago* seedlings were inoculated with a culture of *Sinorhizobium*. Uninoculated controls received either no nitrogen or 50 mg $KNO_3$ per week. Nodule isolates were recovered as described previously (Reeve *et al.*, 1999). The competitiveness of WR101 against its parent WSM419 was determined by inoculating *M. murex* seedlings with a total of $10^4$ cells in 2 : 1, 1 : 1 and 1 : 2 ratio combinations. Nodule occupancy was assessed from the proportion of stained versus unstained nodules after incubation of the root system in buffer containing X-Glc (Wilson *et al.*, 1995). The amount of nitrogen fixed was determined using Kjeldahl digestion (Unkovich *et al.*, 1993).

**DNA manipulation and analysis.** Methods for plasmid or genomic DNA isolation, transformation, conjugation, DNA manipulation and DNA sequencing have been described previously (Reeve *et al.*, 1999, 2002). Potential proteins were identified using the BLASTP algorithm (Altschul *et al.*, 1997) using the NCBI (www.ncbi.nlm.nih.gov/blast/) or *S. meliloti* BLAST server (http://sequence.toulouse.inra.fr/rhime/public/Access/RhimeFormRA.html). Protein domains were identified from the Conserved Domain Database (CDD) and Search Service through the BLAST algorithm (Marchler-Bauer *et al.*, 2003).

**DNA hybridizations.** DNA labelling, Southern hybridization and probe detection were performed as described previously (Tiwari *et al.*, 1996a). DIG-labelled pCRS606 or DIG-labelled pCRS487 was hybridized to *Eco*RI- or *Hin*dIII-digested genomic DNA from WSM419 and WR101 to identify the number of copies of *lpiA* or the minitransposon.

**PCR amplification and cloning of the *mprF* homologue from *Bacillus subtilis* 168.** The oligonucleotide primers Bsub_MprF5 (5′-ACGTCCATGGAGAGACCATTGCTGATTAAAAAGAATGCTTTATC-3′) and Bsub_MprF3 (5′-ACGTGGATCCTTAGACGGAGTCTTTTTTGCTTTTGCCAATCAGACG-3′), introducing *Nco*I and *Bam*HI restriction sites (underlined), respectively, were used to amplify the *mprF* homologous gene from *Bacillus subtilis* (Staubitz & Peschel, 2002) using genomic DNA of *Bacillus subtilis* 168 as a template. After digestion with *Nco*I and *Bam*HI, the PCR product was cloned into pET16b (Novagen) to yield plasmid pCCS51.

**Table 1.** List of strains and plasmids used

Abbreviations: Acid$^s$, acid-sensitive; Acid$^t$, acid-tolerant; Ap$^r$, resistance to ampicillin; Cm$^r$, resistance to chloramphenicol; Gm$^r$, resistance to gentamicin; Km$^r$, resistance to kanamycin; Tc$^r$, resistance to tetracycline.

| Strain/plasmid | Relevant characteristics | Source/reference |
|---|---|---|
| **Strains** | | |
| *E. coli* | | |
| BW20767 | RP4-2-*tet*::Mu-1*kan*::Tn*7* integrant *leu-63*::IS*10 recA1 creC510 hsdR17 endA1 zbf-5 uidA*ΔΔ*Mlu*I):*pir*$^+$ *thi* | Metcalfe *et al.* (1996) |
| DH5α | F$^-$ *ϕ*80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^-$ $m_K^+$) *supE44 relA1 deoR* Δ(*lacZYA-argF*)U169 | Invitrogen |
| *S. meliloti* | | |
| 1021 | SU47 *str-21* | Meade *et al.* (1982) |
| *S. medicae* | | |
| MUR1973 | Plasmid integrant containing pCRS1083 inserted within *fsrR* in WSM419; Cm$^r$, Km$^r$ | This study |
| RT10 | *phrR10*:ΩKm interposon inserted into codon 10 of *phrR* in WSM419; Cm$^r$, Km$^r$ | This study |
| RT295G | *actS*:CAS-GGm mutant of WSM419; Cm$^r$, Gm$^r$ | Tiwari *et al.* (1996b) |
| TG5-46 | Acid$^s$ Tn*5*-induced mutant of WSM419; Km$^r$ | Goss *et al.* (1990) |
| WR101 | *lpiA*::mTn*5*-GNm mutant of WSM419; Cm$^r$, Km$^r$ | Reeve *et al.* (1999) |
| WSM419 | Acid$^t$ Sardinian isolate; Cm$^r$ | Howieson & Ewing (1986) |
| **Plasmids** | | |
| pBBR1MCS5 | Broad-host-range vector; Gm$^r$ | Kovach *et al.* (1995) |
| pCCS51 | *mprF* homologue from *Bacillus subtilis* cloned into pET16b; Ap$^r$ | This study |
| pCCS57 | pCCS51 cloned as *Bam*HI fragment into pBBR1MCS5; Ap$^r$, Gm$^r$ | This study |
| pCRS433 | Broad-host-range vector; Tc$^r$ | Reeve *et al.* (1999) |
| pCRS487 | pUT::mTn*5*-GNm; Ap$^r$, Km$^r$ | Reeve *et al.* (1999) |
| pCRS527 | pUC6S derivative with a *Hin*dIII fragment from WR101 containing *lpiA*::mTn*5*-GNm; Ap$^r$, Km$^r$ | This study |
| pCRS536 | pCRS433 derivative with a *Hin*dIII fragment from WR101 containing *lpiA*::mTn*5*-GNm; Km$^r$, Tc$^r$ | Reeve *et al.* (1999) |
| pCRS580 | *Bgl*II blunted fragment of pCRS527 cloned into a blunted *Hin*dIII site of pCRS433; Km$^r$, Tc$^r$ | This study |
| pCRS583 | Blunted *Eco*RI fragment containing mTn*5*-GNm cloned into a blunted *Hin*dIII site of pCRS433; Km$^r$, Tc$^r$ | This study |
| pCRS606 | *Hin*dIII–*Xho*I fragment containing mTn*5*-GNm in pCRS433; Km$^r$, Tc$^r$ | This study |
| pCRS679 | *Apa*I-blunted/*Eco*RI intragenic *fsrR* fragment cloned into *Kpn*I-blunted/*Eco*RI-digested pUC21; Ap$^r$ | This study |
| pCRS690 | *Eco*RI fragment containing partial *fsrR* in pFUS1; Tc$^r$ | This study |
| pCRS691 | An intragenic *fsrR Hin*dIII–*Pst*I fragment in pFUS1; Tc$^r$ | This study |
| pCRS725 | *Sma*I–*Xho*I fragment containing mTn*5*-GNm in pCRS433; Tc$^r$ | This study |
| pCRS1083 | *Not*I/*Bgl*II cassette containing *nptI*, RP4*oriT* and R6K cloned into *Not*I/*Bam*HI-digested pCRS679; Ap$^r$, Km$^r$ | This study |
| pET16b | Expression vector; Ap$^r$ | Studier *et al.* (1990) |
| pFUS1 | pCRS433 derivative containing a promoterless *gusA*; Tc$^r$ | Reeve *et al.* (1999) |

Plasmid pCCS51 was linearized with *Bam*HI and cloned into the *Bam*HI site of pBBR1MCS5 (Kovach *et al.*, 1995) to yield plasmid pCCS57.

***In vivo* labelling of bacterial strains with [$^{14}$C]acetate and quantitative analysis of lipid extracts.** The lipid compositions of sinorhizobial strains were determined following labelling with [1-$^{14}$C]acetate. Cultures (1 ml) in minimal medium were inoculated from pre-cultures grown in the same medium. After addition of 1 μCi (37 kBq) [1-$^{14}$C]acetate (60 mCi mmol$^{-1}$) to each culture, they were incubated for 24 h at pH 7·0 or for 48 h at pH 5·7. Cells were harvested by centrifugation and washed once with 500 μl water. Lipids were extracted using a modified Bligh & Dyer (1959) extraction where water was substituted with 0·12 M sodium acetate, pH 4·8. The chloroform phase was used for lipid analysis on TLC plates and, after two-dimensional (Geiger *et al.*, 1999) separation, the individual lipids were quantified as described by de Rudder *et al.* (1997).

**Construction of an *fsrR* knockout mutant.** A 904 bp intragenic *fsrR* fragment was cloned into pCRS1083 and the resulting plasmid mobilized into WSM419. This plasmid is a pUC21 derivative containing a kanamycin resistance marker, RP4 *oriT* (required for plasmid transfer) and the R6K origin of replication. It cannot replicate in *Sinorhizobium* and therefore selection for kanamycin resistance following conjugal transfer into WSM419 enabled transconjugants to be generated that contained the plasmid integrated into *fsrR*. In knockout mutants, the *lacZ* and *nptI* promoters in the integrated plasmid diverge from the orientation of the *lpiA* promoter. Single cross-over insertion into *fsrR* was verified by PCR using the primers LpiA-1556R (5′-GACGGCGGTGAGATAGCTC-3′) and R6K-95R (5′-TAACGGCTGACATGGGGGGG-3′). The PCR reaction mixture contained 1 µl saturated cell suspension, 2·5 µl 10 mM $MgCl_2$, 5 µl 5× PCR Polymerization Buffer (Fisher-Biotech), 0·5 µl each 50 µM primer, 0·2 µl *Taq* DNA polymerase (5 U $\mu l^{-1}$; Invitrogen Life Technologies) and 15·3 µl PCR grade water. Cycling conditions were as follows: hold at 94 °C for 5 min, amplification for 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and polymerization at 72 °C for 2 min, followed by a hold at 14 °C. Following agarose gel electrophoresis, the successful amplification of a 1·6 kb product using the LpiA-1556R and R6K-95R primer pair verified an *fsrR* single-crossover knockout mutation.

**Stress tolerance studies.** Cultures of WSM419 and WR101 were grown in TY to late exponential phase. A stress sensitivity assay was performed by spotting 10 µl ($10^4$ cells) of a culture onto TY plates buffered to pH 7·0 or 5·7 and onto TY plates containing sodium azide (250 µM), cadmium chloride (100 µM), chromium chloride (500 µM), copper sulfate (1000 µM), sodium chloride (500 mM), sucrose (10 %) or zinc sulfate (500 µM) prior to incubation at 28 °C. To investigate the effect of temperature, late-exponential-phase cultures grown in JMM (pH 7·0) broth were subcultured into JMM (pH 7·0) broths (20 µl culture per 5 ml) and incubated at 37 or 41 °C. Turbidity measurements were recorded at $OD_{600}$ over 2 days.

**Cell viability at pH 4·5.** WSM419 or WR101 were cultured in 50 ml JMM (pH 7·0) in a 250 ml Erlenmeyer flask until the $OD_{600}$ reached 0·8. The cultures were centrifuged (10 000 ***g*** for 10 min) and the pellet was resuspended in JMM (pH 5·7) to an $OD_{600}$ of 0·25. The resuspended cultures were incubated for 24 h at 28 °C and then centrifuged at 10 000 ***g*** for 10 min. The pellet was resuspended in saline to an $OD_{600}$ of 0·1, 25 µl of which was inoculated into 50 ml JMM at pH 4·5 in a 250 ml Erlenmyer flask. Seeded flasks were incubated at 28 °C after which cell viability was determined by spread plating aliquots on TY plates at intervals over a 15 h period.

**Expression studies.** Cultures were inoculated into 5 ml MJMM (pH 7·0) and grown at 28 °C to an $OD_{600}$ of approximately 0·8. Tetracycline was added to a starter culture at pH 7·0 if the bacterial strain contained a broad-host-range plasmid. Suspensions were centrifuged (10 000 ***g*** for 10 min), concentrated and resuspended in MJMM (at pH 7·0 or 5·7) to obtain an $OD_{600}$ of approx. 0·25–0·5 after overnight incubation at 28 °C. For expression studies at elevated temperature, cells were incubated at 37 °C. Cells were incubated overnight in MJMM at 28 °C in the presence of chemical stressors at the concentrations indicated in the text. The concentration of $Ca^{2+}$ in MJMM was 1 mM, unless otherwise specified. β-Glucuronidase (GUS) specific activity was determined by using the microplate method as described previously (Reeve *et al.*, 2002) and expressed as nmol *p*-nitrophenol (pNP) produced $min^{-1}$ $(OD_{595}$ unit$)^{-1}$ at 28 °C. A minimum of three replicate assays per strain were used.

# RESULTS

## Sequence analysis of the *lpiA* gene region

Strain WR101 was isolated as an mTn*5*-GNm-induced mutant containing a promoterless *gusA* gene fused to an acid-activated promoter. Colonies of this mutant stained pale blue at pH 7·0 but intense blue at pH 5·7 in the presence of the chromogenic substrate X-Glc (Reeve *et al.*, 1999). A single *Eco*RI or *Hin*dIII genomic DNA fragment hybridized to DIG-labelled pCRS487, revealing a single copy of the minitransposon in this mutant (data not shown). DNA (8 kb) flanking the site of mTn*5*-GNm insertion was restriction-mapped and sequenced. ORF analysis identified the presence of five genes in this DNA fragment (Fig. 1a): *tcsA* (two-component sensor protein A), *tcrA* (two-component regulator gene A), *fsrR* (fused sensor–regulator gene R), *lpiA* (low pH-inducible gene A) and *acvB* (acid virulence gene B). The mutant WR101 contained the mTn*5*-GNm insertion within *lpiA* (Fig. 1a).

**The *tcsA* gene.** The *tcsA* gene encodes a protein containing the protein domain COG4251 (cluster of orthologous groups; Tatusov *et al.*, 2001) belonging to the histidine kinase family, indicating that TcsA could be the sensor component of a two-component signal transduction system in WSM419. This protein aligned to histidine kinases from *A. tumefaciens* strain C58 (NP_354959 and NP_532665; 40 % identity), *Bradyrhizobium japonicum* (NP_770929; 55 % identity) and *Mesorhizobium loti* (NP_106342; 44 % identity).

**The *tcrA* gene.** Downstream from *tcsA* is the *tcrA* gene (Fig. 1a) which encodes a protein containing the Pfam00072 (protein families database; Bateman *et al.*, 2002) response regulator receiver domain. This motif receives the signal from the sensor partner in bacterial two-component systems and points to TcrA as the regulatory component of a two-component signal transduction system in WSM419. This protein aligned to two-component response regulators from *A. tumefaciens* strain C58 (NP_354958 and NP_532664; 64 % identity), *Bradyrhizobium japonicum* (NP_770928; 55 % identity) and *Mesorhizobium loti* (NP_106343; 74 % identity). The *tcrA* start codon was located 266 bp from the stop codon of *tcsA* and the size of this intergenic spacer suggested that a promoter for *tcrA* could reside in this region. It is noteworthy that we found a similar gene arrangement for *tcsA*/*tcrA* orthologues in *A. tumefaciens* (Fig. 1b), *Bradyrhizobium japonicum* and *Mesorhizobium loti*. It therefore seems likely that *tcsA*/*tcrA* constitute a two-component signal transduction system in these organisms.

**The *fsrR* gene.** Coupled to the *tcrA* stop codon is the start codon for *fsrR*, suggesting that *fsrR* is under transcriptional control from the upstream *tcrA* promoter. The *fsrR* gene (Fig. 1a) encodes a protein that contains both an N-terminal region Pfam00072 response regulator receiver

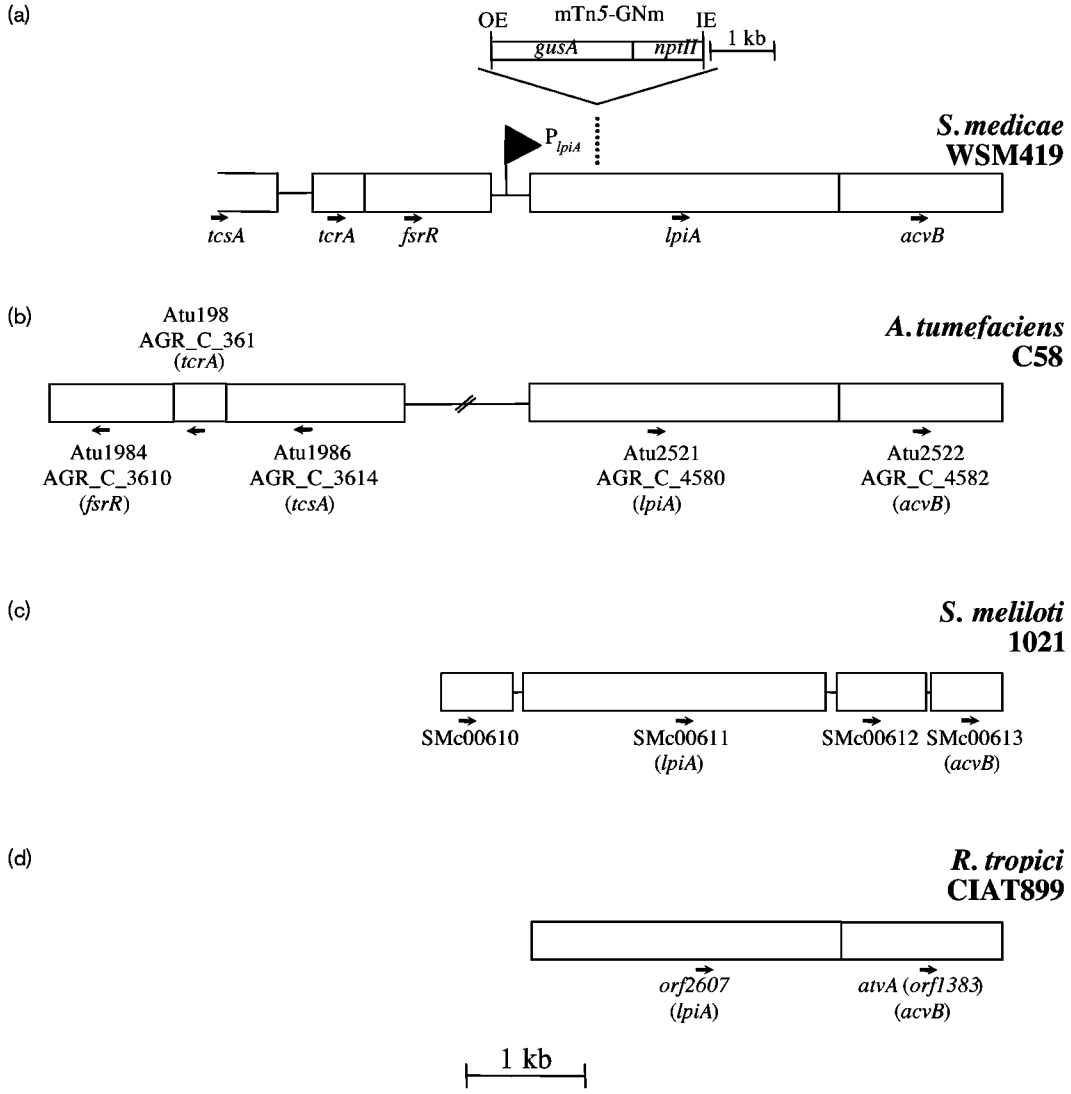


**Fig. 1.** (a) Genetic map of the *lpiA* gene region in *S. medicae* WSM419. The black triangle denotes the direction of transcription from the promoter of *lpiA* ($P_{lpiA}$). The ORF for *tcsA* is incomplete (incomplete box). The site of mTn*5*-GNm insertion within the *lpiA* gene in the mutant WR101 is shown by a dotted line. (b) Genetic map of the *lpiA* and *fsrR* gene regions in *A. tumefaciens* C58. Atu and AGR prefixes refer to ORFs designated by the University of Washington and Cereon Genomics, respectively. (c) Genetic map of the *lpiA* gene region in *S. meliloti* strain 1021. (d) Genetic map of the *lpiA* gene region in *R. tropici* CIAT899.

domain and a C-terminal region COG3920 signal transduction histidine kinase site. FsrR is similar to proteins from *A. tumefaciens* (Fig. 1b) (NP_354957 and NP_532663; 44 % identity), *Bradyrhizobium japonicum* (NP_770927; 53 % identity) and *Mesorhizobium loti* (NP_106344; 48 % identity) that contain signal transduction histidine kinase and response regulator receiver domains. In contrast, *fsrR* is absent from the genome sequence of *S. meliloti* strain 1021.

**The *lpiA* gene.** The start codon of *lpiA* is located 372 bp downstream from the *fsrR* stop codon. The *lpiA* gene (Fig. 1a) encodes a putative membrane-spanning protein that contains the following CDDs (Marchler-Bauer *et al.*, 2003): COG0392 (indicative of an integral membrane protein) and DUF470, DUF471 and DUF472 (domains of unknown function). LpiA matched to hypothetical transmembrane proteins from *S. meliloti* strain 1021 (NP_385286; 83 % identity) (Fig. 1c), *R. tropici* (NP_AF433669; 62 % identity) (Fig. 1d) and *A. tumefaciens* strain C58 (NP_355467 and NP_533192; 58 % identity) (Fig. 1b). The function of these proteins remains to be determined, but the three domains DUF470, DUF471 and DUF472 together make up the C-terminal portion of *Staphylococcus aureus* FmtC/MprF. The latter proteins are required for lysinylation of the membrane phospholipid

phosphatidylglycerol (PG), providing resistance to defensins (Oku *et al.*, 2004; Staubitz *et al.*, 2004).

**The *acvB* gene.** The *acvB* gene is directly downstream from *lpiA* (Fig. 1a). The arrangement of *lpiA* and *acvB* genes is similar in *A. tumefaciens*, *Ralstonia solanacearum*, *R. tropici*, *S. medicae* and *S. meliloti* 1021. In the last case, a frameshift in the *acvB* sequence replaces the full-length ORF with two ORFs. The *S. medicae acvB* protein product contains the COG3946 VirJ component (93·2 % aligned). It shared identity with the *A. tumefaciens* C58 chromosomal virulence protein B (NP_355468 and NP_533193; 54 % identity), AtvA (AcvB orthologue) from *R. tropici* (AF433669; 51 % identity) (Fig. 1d) and to two hypothetical proteins (NP_385287; 74 % identity and NP_385288; 85 % identity) from *S. meliloti* strain 1021 (Fig. 1c).

## Phenotypic analysis of the mutant *S. medicae* WR101

Although the expression of *lpiA* was induced at least 20-fold at pH 5·7 relative to that at pH 7·0 (Reeve *et al.*, 1999), a lesion in this gene did not affect the ability of this organism to grow in minimal medium (MJMM) at pH values as low as pH 5·7. To determine if the inactivation of *lpiA* imparts any growth defect following cell exposure to stress, the tolerance of WR101 to azide, temperature, heavy metals and osmotic stress was examined. In response to these stresses, the growth of the mutant was not significantly different to that of the wild-type (data not shown).

The effect of the *lpiA* mutation on cell viability at lethal pH was then determined by exposing WSM419 and WR101 to a pH of 4·5 in JMM. Cells grown in neutral-buffered JMM were exposed to the moderately acidic condition of pH 5·7 for 24 h to induce genes required for an acid tolerance response (O'Hara & Glenn, 1994). The cells were subsequently transferred to pH 4·5 to examine viability (Fig. 2). After 15 h the number of mutant viable cells was reduced to only 2 % of the number of wild-type cells. This implied a significant role for *lpiA* for cell survival at lethal pH.

## Nodule occupancy of *Medicago* species and nitrogen fixation

Strain WR101 was able to nodulate and fix nitrogen effectively in association with *Medicago murex*, *Medicago polymorpha* and *Medicago truncatula*, revealing that *lpiA* is not required to establish a fully functional symbiosis (data not shown). In these experiments, kanamycin-resistant cells were recovered from the nodules of plants originally inoculated with WR101. Moreover, all nodules containing WR101 stained intense blue following incubation of the root system in the presence of the chromogenic substrate X-Glc, revealing that the *lpiA* mutation was maintained *in vivo*. The amount of nitrogen fixed in plants inoculated with WR101 was comparable to that in plants inoculated with WSM419. In addition, the *lpiA*::mTn*5*-GNm mutation did not disrupt the ability of the mutant WR101 to compete against

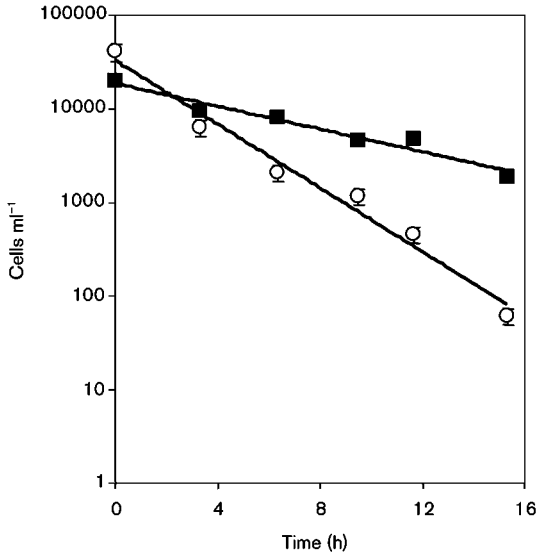


**Fig. 2.** Survival of WSM419 (■) and WR101 (○) exposed to pH 4·5 in JMM following transfer from pH 5·7. Values are shown as means ± SEM ($n = 3$).

the wild-type for nodule occupancy. The mutant occupied 200 of 329, 162 of 280 or 138 of 399 nodules of *Medicago murex* co-inoculated with mutant and wild-type at 2 : 1, 1 : 1 or 1 : 2 ratios, respectively (for equal competitiveness, theoretical values would be 219, 140 and 133 for the 2 : 1, 1 : 1 or 1 : 2 ratios, respectively).

## Lysyl-phosphatidylglycerol (LPG) is not a major lipid in *S. medicae* membranes under neutral or acidic conditions of growth

Based on similarities, it has been proposed that *lpiA* might be a functional homologue of *mprF* from *Staphylococcus aureus* which is known to encode LPG synthetase forming the membrane phospholipid LPG (Oku *et al.*, 2004; Staubitz *et al.*, 2004). We therefore investigated whether LPG was present in membranes of *S. medicae* and, as the transcription of *lpiA* was much increased under acidic conditions, whether more LPG was present after growth under acidic conditions. The lipid pattern of *S. medicae* WSM419 after growth on JMM at pH 7·0 (Fig. 3a) was nearly identical to the lipid pattern described for *S. meliloti* 1021 after growth on minimal medium (Geiger *et al.*, 1999) and revealed the presence of the membrane phospholipids PG, cardiolipin (CL), phosphatidylethanolamine (PE), monomethylphosphatidylethanolamine (MMPE), dimethylphosphatidylethanolamine (DMPE) and phosphatidylcholine (PC) as well as the phosphorus-free ornithine-containing membrane lipids (OL). As found for other rhizobia, PC was the most abundant membrane lipid (Table 2) and usually composed more than half of the total lipids. When *S. medicae* WSM419 was grown on JMM at pH 5·7 (Fig. 3b), the membrane lipid pattern resembled that after growth at neutral pH. Quantitative lipid analysis (Table 2) revealed an

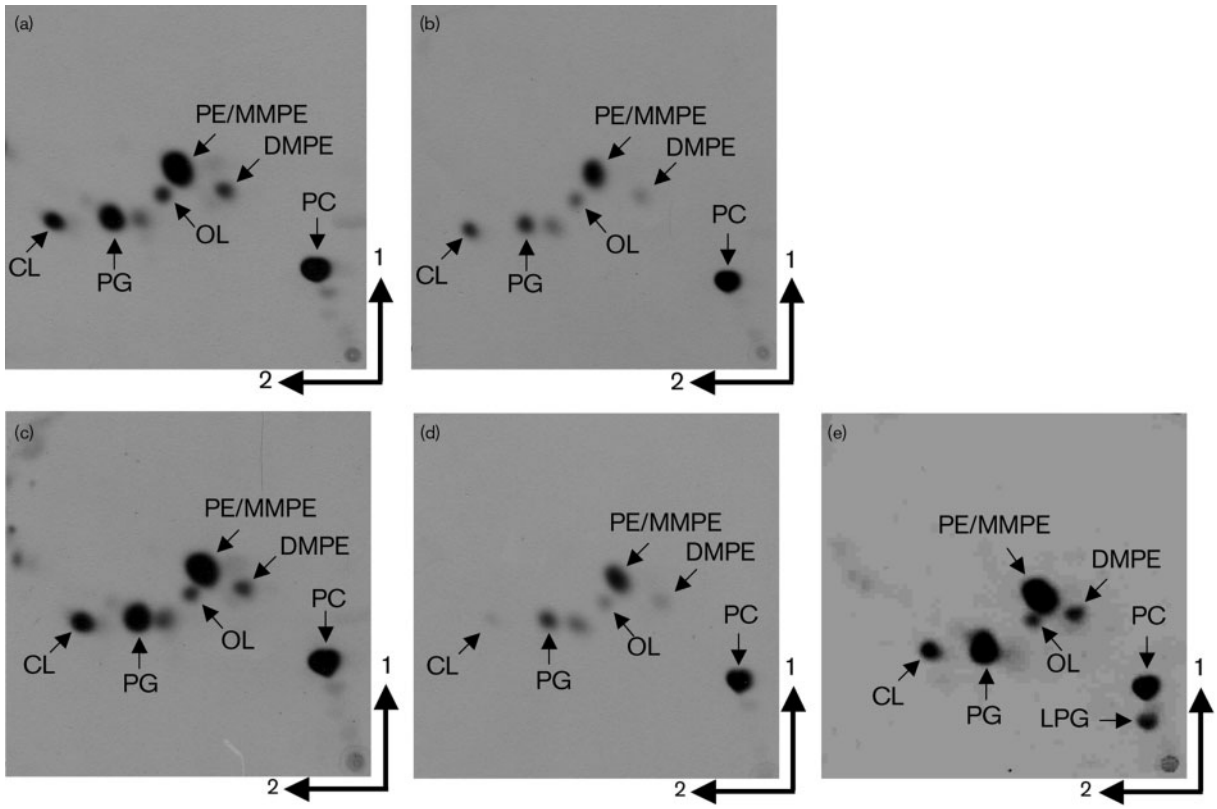


**Fig. 3.** Separation of [$^{14}$C]acetate-labelled lipids from *S. medicae* WSM419 wild-type (a, b) and *lpiA*-deficient-mutant WR101 (c, d) after growth on JMM at pH 7·0 (a, c) or 5·7 (b, d). [$^{14}$C]Acetate-labelled lipids were also separated from *S. meliloti* 1021(pCCS57) expressing the MprF homologue from *Bacillus subtilis* after growth on TY medium at pH 7·1 (e). The lipids PC, DMPE, MMPE, PE, PG, CL and OL are indicated.

increase in PC and a small but statistically significant decrease in PG under low pH conditions when compared to bacteria grown at neutral pH. LPG was not detectable under either condition. In a *S. meliloti* strain that harbours plasmid pCCS57, and therefore expressing the *mprF* homologue from *Bacillus subtilis*, an extra compound was formed that migrated like LPG in two-dimensional chromatography (Fig. 3e); since it was ninhydrin-positive (data not shown) it must contain a primary amino group. We therefore concluded that this extra compound formed by *S. meliloti* (pCCS57) was most probably LPG, demonstrating that if it is present in rhizobial membranes, it can be extracted and detected by the methods employed here. With our standard autoradiography method of thin-layer chromatograms we can easily detect individual lipid species that compose 1 % of total lipids. As LPG was not detectable in WSM419 after

**Table 2.** Membrane lipid composition (% of total $^{14}$C) of *S. medicae* WSM419 wild-type and *lpiA*-deficient mutant WR101 after growth on JMM at pH 7·0 or 5·7

The values shown are mean values ± SD derived from three independent experiments. ND, Not detected.

| Lipid | WSM419 JMM (pH 7·0) | WSM419 JMM (pH 5·7) | WR101 JMM (pH 7·0) | WR101 JMM (pH 5·7) |
|---|---|---|---|---|
| PE/MMPE | 22·4 ± 2·6 | 17·9 ± 2·2 | 23·1 ± 4·7 | 18·0 ± 2·0 |
| DMPE | 1·8 ± 0·3 | 1·2 ± 0·1 | 1·9 ± 0·3 | 1·5 ± 0·3 |
| OL | 1·9 ± 0·3 | 2·3 ± 0·2 | 2·0 ± 0·2 | 1·8 ± 0·5 |
| PG | 11·5 ± 1·1 | 8·8 ± 1·0 | 14·5 ± 0·7 | 10·6 ± 0·1 |
| CL | 3·1 ± 0·6 | 2·2 ± 1·4 | 4·6 ± 0·1 | 1·4 ± 1·0 |
| PC | 59·2 ± 3·8 | 67·6 ± 2·1 | 53·9 ± 5·0 | 66·6 ± 1·5 |
| LPG | ND | ND | ND | ND |

growth on neutral or acidic media (Fig. 3, Table 2), we concluded that if LPG was present it could certainly not amount to more than 1 % of the total membrane lipids and therefore did not constitute a major membrane lipid of *S. medicae* when grown in neutral or acidic conditions. When the *lpiA*-deficient mutant WR101 was grown on JMM at pH 7·0 or 5·7, the lipid pattern was practically identical to the lipid pattern observed for the wild-type WSM419 under the same growth conditions (Fig. 3).

### Expression of *lpiA* is regulated specifically by pH

The expression of *lpiA* in the mutant WR101 was examined following exposure to a variety of stress conditions. Transcriptional activation of *lpiA* occurred in exponential- or stationary-phase cells specifically exposed to low pH in minimal (Reeve *et al.*, 1999) or rich (buffered TY) media (data not shown). Because elevated levels of calcium enhance cell viability of *S. medicae* at low pH (Reeve *et al.*, 1993), the expression of *lpiA* was also examined in response to calcium; increasing the calcium concentration in the growth medium from 0·3 to 10 mM increased cell viability and decreased the pH required to induce transcription of *lpiA* (Fig. 4). However, stress imposed by cadmium chloride (50 μM), copper sulfate (100 μM), chromium chloride (500 μM), ferric chloride (100 μM), sodium azide (50 μM), zinc sulfate (75 μM), ethanol (0·85 %), hydrogen peroxide (1 mM), sodium chloride (500 mM), sucrose (12·5 %) and elevated temperature (37 °C) did not activate *lpiA* expression. These stress levels were chosen since they significantly affect the growth of WSM419 in broth.

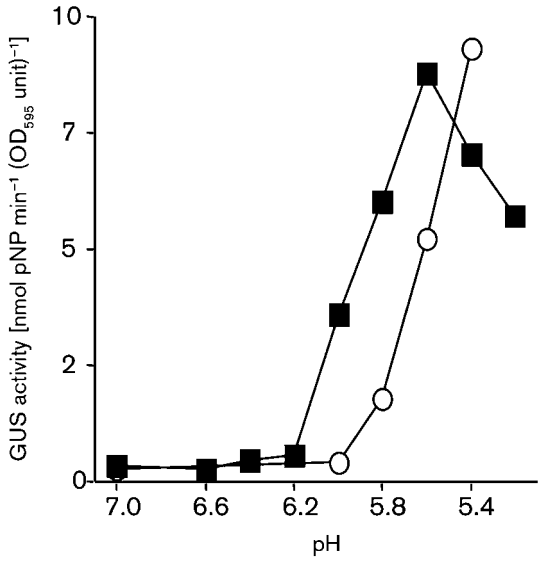


**Fig. 4.** Expression of *lpiA-gusA* in *S. medicae* WR101 as a function of pH and calcium concentration. ■, MJMM; ○, MJMM plus 10 mM $CaCl_2$.

### Promoter mapping

To identify the location of the *lpiA* promoter, expression studies were performed in WSM419 using various plasmid subclones of pCRS536 (Fig. 5a). The level of induction was similar to that measured for the mutant WR101 and demonstrates that this plasmid contains the necessary *lpiA* operator and promoter sites for the complete regulation of expression of this gene. Deletion of the region downstream from the insertion of mTn*5*-GNm did not affect activation of *lpiA* in WSM419 (pCRS606; Fig. 5b). In contrast, the *lpiA-gusA* fusion was no longer inducible in WSM419 if the sequence between *Hin*dIII$_1$ and *Bgl*II was removed (clone pCRS580). If the 1·1 kb *Hin*dIII$_1$/*Eco*RI$_2$ fragment was cloned upstream of *gusA* (clone pCRS690), the fusion was induced 27-fold at low pH. These data suggested that the *lpiA* promoter resides upstream from the *Eco*RI$_2$ site.

Within the 1·1 kb *Hin*dIII$_1$/*Eco*RI$_2$ fragment of clone pCRS690, an intragenic *fsrR* *Hin*dIII/*Pst*I fragment did not provide low pH-inducible expression if cloned upstream of *gusA* (plasmid pCRS691). The addition of a *Sma*I–*Eco*RI$_2$ sequence to pCRS580 (to construct pCRS725) restored low pH-inducible activity. These studies demonstrated that the rhizobial sequence upstream from the *Sma*I site was not required for low pH-induction and that the promoter resides within a 372 bp region located between the *Sma*I site and the *lpiA* start codon (Fig. 5c).

### Expression of *lpiA* is regulated by FsrR

To identify the regulator required for the acid-activated expression of *lpiA*, plasmid pCRS536 was mobilized into WSM419 (wild-type), RT10 (*phrR*$^-$), RT295G (*actS*$^-$), TG5-46 (*actR*$^-$) and MUR1973 (*fsrR*$^-$), and GUS activity of cells incubated at pH 7·0 or 5·7 was quantified. In the *phrR*, *actS* or *actR* knockout mutant backgrounds, induction of the *lpiA-gusA* fusion still occurred at low pH (Table 3). In contrast, there was only threefold acid-activation of *lpiA* transcription in the *fsrR* mutant in comparison to more than 20-fold induction in the wild-type (Table 3), indicating that *fsrR* plays a major role as a positive regulator of *lpiA* transcription at low pH.

## DISCUSSION

By creating a minitransposon in *lpiA* in *S. medicae* WSM419, we revealed that this gene was transcriptionally activated at least 20-fold in cells shifted to acid (pH 5·7) conditions (Reeve *et al.*, 1999). There is only a single copy of *lpiA* in the genome of WSM419 (this study, hybridization not shown) and in *S. meliloti* 1021 (Galibert *et al.*, 2001). The minitransposon insertion disrupting *lpiA* in WR101 did not perturb growth *in vitro* at pH 7·0 or 5·7, nor did it affect *Medicago* spp. nodulation or nitrogen fixation (Reeve *et al.*, 1999). Vinuesa *et al.* (2003) also found that the growth rate of an *R. tropici lpiA* mutant was not affected by pH. However, the authors suggested *lpiA* could be required for optimal performance of CIAT899 in symbiosis with

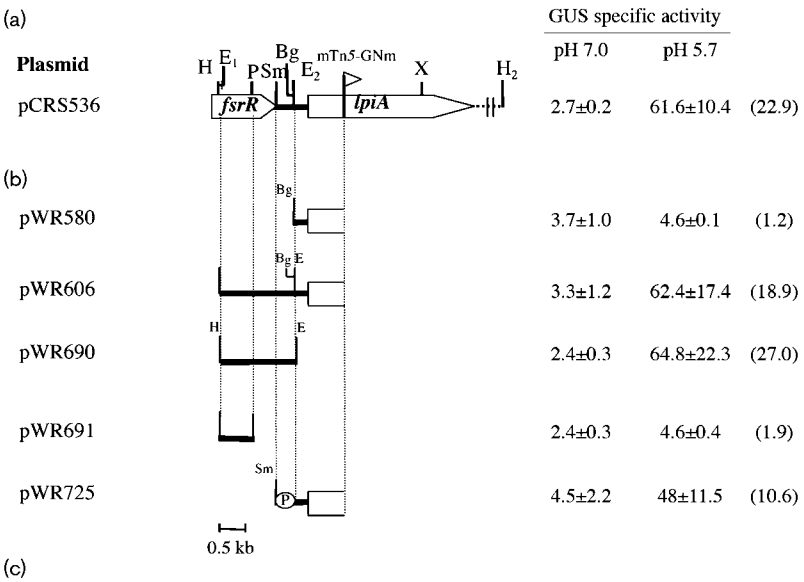


**Fig. 5.** (a) Restriction maps of the *S. medicae* WSM419 *Hin*dIII fragment containing the site of mTn*5*-GNm insertion. Restriction sites: Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sm, *Sma*I; X, *Xho*I. The discontinuous line extending between the *Xho*I and *Hin*dIII$_2$ sites represents a region that has only been partially restriction-mapped and is not to scale. The flag denotes the position of the minitransposon. Activities shown on the right are nmol pNP min$^{-1}$ (OD$_{595nm}$ unit)$^{-1}$ ± SEM ($n$=3); -fold induction of GUS activity at pH 5·7/pH 7·0 is shown in parentheses. (b) Promoter localization using transcriptional fusions to a promoterless *gusA*. Plasmid pCRS536 and subclones were used to identify the location of the *lpiA* promoter. Plasmids pCRS690 and pCRS691 contain rhizobial fragments fused to the promoterless *gusA* in pFUS1. (c) Nucleotide sequence (372 bp) of the DNA region containing the *lpiA* promoter deduced from (b). Restriction sites and a putative ribosome-binding site (RBS) have been underlined. The stop signal of the *fsrR* gene is in bold.

*Phaseolus vulgaris* based on the fact that the mutant failed to compete for nodule occupancy against a *gusA*-marked wild-type. However, it remains to be shown whether competitiveness of the *R. tropici lpiA* mutant can be restored by complementation with the *lpiA* gene. In contrast, the *S. medicae lpiA* mutant described in this paper was not compromised in its competitive ability to occupy *Medicago murex* nodules at neutral pH. Instead, we showed that the *lpiA* mutant was compromised in its ability to survive in lethal acid conditions, providing evidence for the first time that LpiA has an essential role in cell adaptation in *S. medicae*.

Sequence analysis of LpiA revealed significant sequence similarity with the FmtC/MprF family of proteins, initially suggesting a putative role in lipid metabolism. In particular, MprF is required for the synthesis of LPG, which has been proposed to increase the membrane net positive charge, preventing damage via cationic and host-defensive peptides (Peschel *et al.*, 2001). Based on our analysis (Fig. 3, Table 2), LPG was not a major lipid in *S. medicae* membranes under neutral or acidic conditions of growth. It was remarkable, however, that levels of PG were higher in the *lpiA*-deficient mutant WR101 than in the wild-type, under both neutral and acidic conditions of growth (Table 2), possibly indicating that PG was not consumed in an *lpiA*-dependent reaction. If LpiA was indeed involved in LPG formation, LPG must have been either formed in very small amounts or degraded as rapidly as it was produced. It is worth noting that the *S. medicae* AcvB protein, encoded by *acvB*

**Table 3.** Regulation of a plasmid borne *lpiA-gusA* fusion (pCRS536) in different *S. medicae* genetic backgrounds

| pH of medium* | GUS activity [nmol pNP min$^{-1}$ (OD$_{595}$ unit)$^{-1}$ ± SEM ($n$=3)] | | | | |
|---|---|---|---|---|---|
| | **WSM419 (wild-type)** | **RT10 (*phrR*:ΩKm)** | **RT295G (*actS*:CAS-GNm)** | **TG5-46 (*actR*::Tn*5*)** | **MUE1973 (*fsrR*:pMUE1083)** |
| 7·0 | 2·7±0·4 | 2·9±0·4 | 5·1±1 | 2·1±0·1 | 2·8±0·3 |
| 5·7 | 70±7 | 87·0±7·5 | 70±2·5 | 80±1 | 9·2±0·8 |

*MJMM minimal medium.

downstream from *lpiA*, contained a LIPASE_SER (PS00120) motif that suggested a role in lipid metabolism (Vinuesa *et al.*, 2003). The serine residue in this motif is required for the acid tolerance of *R. tropici* and expression of *acvB* has also been shown to be transcriptionally up-regulated by acidity (Vinuesa *et al.*, 2003). Moreover, that study also found an acid-responsive promoter in a 469 bp intergenic region located upstream of *lpiA* in *R. tropici*. The promoter for the acid up-regulation of *lpiA* in *S. medicae* was similarly located within a 372 bp region upstream of the start codon of *lpiA*.

To identify the regulator required for acid-activated expression, the expression of a plasmid borne *lpiA-gusA* fusion was investigated in various genetic backgrounds of *S. medicae* WSM419. The finding that PhrR was not required for acid activation of *lpiA* is consistent with the observation that sequence homology indicated PhrR to be a putative repressor. Furthermore, stresses other than pH that up-regulated the expression of *phrR* (Reeve *et al.*, 1998) did not activate the expression of *lpiA*. The ActSR signal transduction system required for cell growth below pH 6·0 (Tiwari *et al.*, 1996b) also did not regulate *lpiA* under these experimental conditions. However, FsrR was required for acid-activated transcription of *lpiA* in WSM419. This protein contained a sensory domain that may sense a cytoplasmic signal and a regulatory domain that could potentially control *lpiA* expression.

The fact that the mutation in *fsrR* failed to completely abolish acid-activation of *lpiA* expression suggested that there are still other elements in the pH-responsive circuit that regulated *lpiA* expression. The two-component signal transduction pair TcsA (histidine kinase) and TcrA (regulator) encoded by the genes immediately upstream from *fsrR* are likely regulatory candidates. Two lines of preliminary evidence suggested this to be the case. First, the *fsrR* start codon was coupled to the upstream *tcrA* stop codon in *S. medicae*, suggesting that transcription of *fsrR* occurred from a promoter upstream of *tcrA*. Second, the completed genome sequence of *S. meliloti* 1021 (Galibert *et al.*, 2001) did not contain *fsrR*, *tcsA* or *tcrA*, or their encoded protein products, providing a probable explanation for the failure to obtain any acid-activated transcription of *lpiA* in this background. Efforts are therefore currently underway to determine the role of *tcsA* and *tcrA* and to explore the regulation of *lpiA* expression in a diverse range of isolates to determine if acid-activation of this gene is specific to *S. medicae*.

## ACKNOWLEDGEMENTS

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