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A new small regulatory protein, HmuP, modulates haemin acquisition in *Sinorhizobium meliloti*

Vanesa Amarelle,[1] Uriel Koziol,[1,2] Federico Rosconi,[1] Francisco Noya,[1] Mark R. O'Brian[3] and Elena Fabiano[1]

Correspondence
Elena Fabiano
efabiano@iibce.edu.uy

[1]Laboratorio de Bioquímica y Genómica Microbianas, Instituto de Investigaciones Biológicas Clemente Estable, MEC, Unidad Asociada a la Facultad de Ciencias, Av. Italia 3318, Montevideo 11600, Uruguay

[2]Sección Bioquímica, Facultad de Ciencias, Iguá 4225, Montevideo 11400, Uruguay

[3]Department of Biochemistry, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, NY 14214, USA

Sinorhizobium meliloti has multiple systems for iron acquisition, including the use of haem as an iron source. Haem internalization involves the ShmR haem outer membrane receptor and the *hmuTUV* locus, which participates in haem transport across the cytoplasmic membrane. Previous studies have demonstrated that expression of the *shmR* gene is negatively regulated by iron through RirA. Here, we identify *hmuP* in a genetic screen for mutants that displayed aberrant control of *shmR*. The normal induction of *shmR* in response to iron limitation was lost in the *hmuP* mutant, showing that this gene positively affects *shmR* expression. Moreover, the HmuP protein is not part of the haemin transporter system. Analysis of gene expression and siderophore production indicates that disruption of *hmuP* does not affect other genes related to the iron-restriction response. Our results strongly indicate that the main function of HmuP is the transcriptional regulation of *shmR*. Sequence alignment of HmuP homologues and comparison with the NMR structure of *Rhodopseudomonas palustris* CGA009 HmuP protein revealed that certain amino acids localized within predicted β-sheets are well conserved. Our data indicate that at least one of the β-sheets is important for HmuP activity.

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INTRODUCTION

The alpha-proteobacterium *Sinorhizobium meliloti* has the ability to fix nitrogen in symbiotic association with certain legumes. It is also a free-living organism in the soil and rhizosphere, and adaptation to diverse ecological niches involves differential regulation of gene expression. Nitrogen-fixing bacteria have a high demand for iron during symbiosis, since nitrogenase and other iron-containing proteins are required for N_2 fixation (Georgiadis *et al.*, 1992; Hennecke, 1992; Rees & Howard, 2000; Sangwan & O'Brian, 1992). In soil, iron is mostly insoluble, and therefore scarce. Like other bacteria, *S. meliloti* possesses highly efficient iron-acquisition systems. These systems comprise the synthesis and transport of the di-hydroxamate siderophore rhizobactin 1021, the use of the xenosiderophores ferrichrome and ferrioxamine B, and iron acquisition from different iron-porphyrin compounds such as haemin, haemoglobin and leghaemoglobin (Cuiv *et al.*, 2008; Lynch *et al.*, 2001; Noya *et al.*, 1997; Persmark *et al.*, 1993). The haem transport mechanism involves ShmR, an outer membrane haem receptor, and the HmuTUV transport system (Amarelle *et al.*, 2008; Cuiv *et al.*, 2008). *Yersinia enterocolitica* HemTUV was among the first ABC transporters involved in haem uptake to be characterized, and is considered as a prototype for haem transport (Stojiljkovic & Hantke, 1994). Its homologue in *Yersinia pestis* is called HmuTUV (Hornung *et al.*, 1996). According to the model of Hem/Hmu transport, once the haem moiety is translocated into the periplasm it is bound by a periplasmic binding protein, HmuT, which in turn presents it to the inner membrane permease–ATP hydrolase complex HmuU/HmuV. Intact haem is then delivered to the bacterial cytoplasm (Hornung *et al.*, 1996; Stojiljkovic & Hantke, 1994).

Genes that encode rhizobactin 1021 biosynthesis and transport are located in a regulon that comprises the biosynthesis operon *rhbABCDEF*; *rhtA*, encoding the

Abbreviations: EDDHA, ethylenediamine-di-*o*-hydroxyphenylacetic acid; qPCR, quantitative real-time PCR; 5′-RACE, rapid amplification of 5′ complementary DNA ends.

A supplementary figure, showing an alignment of the last 36 amino acid residues (HemP domain) of some HmuP homologues from the alpha-, beta-, gamma-, delta- and epsilon-proteobacteria, and a supplementary table, showing primers used in the study, are available with the online version of this paper.

ferri-rhizobactin 1021 outer membrane receptor; *rhtX*, encoding a permease that belongs to a novel family of siderophore transporters; and *rhrA*, the AraC-like regulator of the receptor and biosynthetic genes (Cuiv *et al.*, 2004; Lynch *et al.*, 2001). Ferrichrome and ferrioxamine B transport systems consist of FhuA1 and FoxA, the respective outer membrane receptors; FhuP, a periplasmic binding protein for both siderophores; and the HmuUV complex, also involved in haem transport across the inner membrane (Cuiv *et al.*, 2008).

Studies of the control of bacterial iron homeostasis have focused largely on the ferric uptake regulator Fur. This protein senses the intracellular ferrous ion concentration, through the formation of a Fur–Fe^{2+} complex, which in turn interacts with specific DNA targets in the promoters of iron-repressed genes (Andrews *et al.*, 2003). Nonetheless, in *S. meliloti* and other alpha-proteobacteria, Fur homologues are manganese-responsive regulators (Chao *et al.*, 2004; Diaz-Mireles *et al.*, 2004; Hohle & O'Brian, 2009; Platero *et al.*, 2004). In the alpha-proteobacteria *S. meliloti*, *Rhizobium leguminosarum* and *Agrobacterium tumefaciens*, a different protein, RirA, which belongs to the Rrf2 superfamily of regulators, is responsible for the regulation of most genes involved in iron uptake (Chao *et al.*, 2005; Ngok-Ngam *et al.*, 2009; Todd *et al.*, 2002; Viguier *et al.*, 2005). In particular, under iron-replete conditions, RirA represses the expression of the rhizobactin biosynthesis and transport regulon, *shmR* gene expression and the *hmuPSTUV* locus.

With the aim of finding novel transcriptional regulators involved in *shmR* expression, we performed a generalized mutagenesis approach in *S. meliloti*. In this work, we discover that the small protein HmuP is essential for *shmR* expression in iron-depleted conditions.

METHODS

Bacteria, plasmids and growth conditions. Bacteria and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown aerobically at 37 °C in Luria–Bertani (LB) medium. *S. meliloti* strains were grown at 30 °C either in tryptone-yeast extract (TY) medium (Beringer, 1974) or in defined minimal medium M9 (Sambrook *et al.*, 1989) supplemented with 6 mM glutamate, 200 μM methionine and 1 μM biotin. Low-iron conditions were obtained by supplementation with ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDHA). When required, 50 μg kanamycin ml^{-1}, 100 μg neomycin ml^{-1}, 100 μg streptomycin ml^{-1}, 10 μg gentamicin ml^{-1}, 50 μg ampicillin ml^{-1} or 1 μg tetracycline ml^{-1} was added to the media.

Tn*5*-1063a transposon mutagenesis and selection of transconjugants. Plasmid pRL1063a (Wolk *et al.*, 1991), containing the

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
***S. meliloti* strains**		
SM1021	Streptomycin derivative of SU47	Meade *et al.* (1982)
SHMR	SM1021 *shmR*::*lacZ-Gm*^{r}	Amarelle *et al.* (2008)
B20	SHMR hmuP::Tn*5*-1063a	This work
HMUP	SM1021 hmuP::Tn*5*-1063a	This work
SM1021::pRG1SMc02726	SM1021 with pRG1SMc02726 construct integrated into genome	This work
HMUP::pRG1SMc02726	HMUP with pRG1SMc02726 construct integrated into genome	This work
***E. coli* strains**		
DH5α	*sup*E44Δ*lac*U169(ϕ80*lacZ*ΔM15)*hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Hanahan (1983)
S17-1	*pro hsdR recA* [RP4-2(T_c::Mu)(K_m::Tn*7*)] mobilization strain for biparental matings	Simon *et al.* (1983)
S17-1 λ *pir*	λ *pir* lysogen of S17-1	Simon *et al.* (1983)
Plasmids		
pBluescript SK	Cloning vector, Am^R	Stratagene
pOT2	Promoter probe vector based on pBBR-1-MCS-5 replicon. Contains promoterless *gfpuv* and multiple cloning site (MCS) between two transcriptional terminators, Gm^R	Allaway *et al.* (2001)
pGEM-T	Cloning vector for PCR products, Am^R	Promega
pRK2013	ColE1 replicon with RK2 *tra* genes. Used for mobilizing incP and incQ plasmids, Km^R	Ditta *et al.* (1980)
pRL1063a	Tn*5* derivative; promoterless *luxAB*; *oriV* Km^R	Wolk *et al.* (1991)
pRG1SMc02726	pMK2030 with SMc02726 ORF inserted, Tet^R	Humann *et al.* (2008)
pOT-HmuP	*hmuP* under its native promoter and fused to *gfpuv*	This work
pOT-HmuPΔCt	*hmuP* lacking the region encoding the C-terminal GKLILNK residues, under its native promoter and fused to *gfpuv*	This work
pOT-HmuPΔK	*hmuP* lacking the codon encoding the C-terminal lysine residue, under its native promoter and fused to *gfpuv*	This work
pOT-HmuPY	*hmuP* with a codon encoding tyrosine inserted before the stop codon, under its native promoter and fused to *gfpuv*	This work
pOT-$HmuP_{pr}$	*hmuP* presumptive promoter region fused to *gfpuv*	This work

transposable element Tn*5*-1063a, was used to generate over 10 000 insertional mutants of *S. meliloti* strain SHMR (Amarelle *et al.*, 2008). The transposon was delivered to strain SHMR by triparental mating with *E. coli* DH5α (pRL1063a) and *E. coli* DH5α (pRK2013) (Ditta *et al.*, 1980) as donor and helper strains, respectively. Transconjugants were screened for changes in *shmR* expression using TY solid media supplemented with streptomycin, neomycin, 20 μg X-Gal ml^{-1} and either 37 μM $FeCl_3$ or 50 μM EDDHA. Differences in *shmR* expression were confirmed by measuring β-galactosidase activity, using *S. meliloti* strain SHMR as control. Cells were grown for 48 h in TY medium and diluted 100-fold in TY medium supplemented with either 37 μM $FeCl_3$ or 100 μM EDDHA. Cultures were grown to early stationary phase at 200 r.p.m. and 30 °C. The β-galactosidase assay was performed according to Miller (1972), with the modifications described by Poole *et al.* (1994).

Sequence analysis of *S. meliloti* SHMR Tn1063a-tagged locus and transposon transduction. To identify the location of the transposon insertion in the B20 mutant, genomic DNA was isolated using the UltraClean Microbial DNA kit (MoBio). Arbitrary PCR (Knobloch *et al.*, 2003) was used to amplify fragments containing transposon junctions using arbitrary primers ARB1-A or ARB2 (Griffitts & Long, 2008) and the transposon-specific primers *Tn*5-4 or *Tn*5-2 (Yurgel & Kahn, 2005). The DNA sequence was determined using the sequencing primer TZ*Tn*5 (Yurgel & Kahn, 2005) and the obtained nucleotide sequence was searched in the *S. meliloti* 1021 genome using the BLAST algorithm.

Lysates obtained from B20 mutant cells infected with the ΦM12 phage (Finan *et al.*, 1984) were used to transduce the *hmuP*::Tn*5*-1063a mutation to *S. meliloti* 1021, as described by Humann *et al.* (2009). Transductants were selected by three consecutive passages in LB medium supplemented with neomycin.

Integration of pRG1SMc02726 in the *S. meliloti* genome and β-glucuronidase activity assays. The pRG1SMc02726 (Humann *et al.*, 2008) construction was transferred to *S. meliloti* 1021 and HMUP (SM1021 *hmuP*::Tn*5*-1063a) strains by biparental conjugation with strain *E. coli* S17-1 λ *pir* (pRG1SMc02726), and transconjugants were selected in Min-succinate-NH_4 medium (Yurgel & Kahn, 2005) supplemented with tetracycline. Transconjugants with the plasmid integrated by homologous recombination were confirmed by colony PCR using primers shmRforward (Amarelle *et al.*, 2008) and 2030F (Humann *et al.*, 2008).

Differences in *shmR*::*gusA* expression were assessed by measuring β-glucuronidase activity. Cells were grown for 48 h in M9 minimal medium and diluted 100-fold in M9 medium supplemented with 300 μM EDDHA. Cultures were grown to early stationary phase at 200 r.p.m. and 30 °C. β-Glucuronidase activity assays were performed as described by Jefferson *et al.* (1986), and β-glucuronidase arbitrary units were defined as 1000 A_{415} units min^{-1} ml^{-1} $(OD_{620}$ unit$)^{-1}$ (Jefferson *et al.*, 1986).

Construction of plasmids containing the *hmuP* gene or the *hmuP* mutated versions, and *in vivo* complementation of the HMUP mutant strain. PCRs were carried out with Tli Polymerase (Promega) and *S. meliloti* 1021 genomic DNA as template. Primers used are listed in Supplementary Table S1. The *hmuP* gene and its presumptive promoter region were amplified using primers HSF and HSR. The resulting 1952 bp fragment was cloned in the *Sma*I site of pBluescript SK (Promega), generating plasmid pBSK-HmuPS. Plasmid pBSK-HmuPS was subsequently digested with *Xho*I, and the 620 bp fragment obtained was subcloned in the *Xho*I site of pBluescript SK to obtain the pBSK-HmuP plasmid. This plasmid was digested with *Pst*I, and a 650 bp fragment was cloned into the *Pst*I site of pOT2 (Allaway *et al.*, 2001), creating pOT-HmuP.

For the construction of pOT-HmuPY, pOT-HmuPΔK and pOT-HmuPΔCt, primer HPprF was used as a forward primer and HPY, HPK or HPCt as reverse primer, respectively. The amplicons were cloned into the *Eco*RV site of pBluescript SK, giving rise to plasmids pBSK-HmuPY, pBSK-HmuPΔK and pBSK-HmuPΔCt. The *Bam*HI–*Hin*dIII fragments obtained from these plasmids were subcloned in pOT2, creating plasmids pOT-HmuPY, pOT-HmuPΔK and pOT-HmuPΔCt. These constructions were confirmed by sequencing.

For the construction of the pOT-$HmuP_{pr}$ plasmid (*hmuP* presumptive promoter region fused to *gfpuv*), a 464 bp fragment was amplified using the primers HPprF and HPprR. This amplicon was cloned into the *Eco*RV site of pBluescript SK, giving rise to plasmid pBSK-$HmuP_{pr}$. Finally, pBSK-$HmuP_{pr}$ was digested with *Hin*dIII/*Xba*I and subcloned into pOT2, resulting in plasmid pOT-$HmuP_{pr}$.

Plasmids were introduced into *S. meliloti* strains by triparental mating as described above.

Bioassays and growth assays. Bioassay experiments were carried out as previously described (Noya *et al.*, 1997). Briefly, 10 μl of the stock solutions to be tested were added to wells in solid TY medium (15 g agar l^{-1}) supplemented with 300 μM EDDHA and containing about 10^6 c.f.u. ml^{-1}. Stock solutions of the following compounds were used as iron sources: 37 mM $FeCl_3$, 0.15 mM haemoglobin, 1 mM haemin, 0.3 mM ferrichrome and 46 mM Desferal.

For colony size experiments, appropriate dilutions of mid-exponential cultures grown in TY media were made. Dilutions were spotted with a replica plater in TY solid media or TY solid medium supplemented with 300 μM EDDHA and 10 μM haemin. Cells were grown for 5 days at 30 °C and colony sizes were recorded.

RNA purification. Wild-type and *hmuP* mutant strains were grown in M9 minimal medium supplemented with 300 μM EDDHA. At mid-exponential phase (OD_{620} 0.8–1.0), 20 ml of culture was treated with 4 ml RNAprotect (Qiagen) and harvested at 7000 r.p.m. at 4 °C for 10 min. Pellets were frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated using a hot-phenol procedure, as described elsewhere (Yang *et al.*, 2006). RNA samples were treated with RQ1 RNase-free DNase I (Promega) and purified using the clean-up procedure of the RNeasy Bacterial RNA Purification kit (Qiagen).

Determination of the transcription start site and RT-PCR. The transcription start site of the *hmuP* gene was determined by rapid amplification of 5′ complementary DNA ends (5′-RACE) using a kit from Invitrogen according to the manufacturer's instructions. We used total RNA purified from cells grown in iron-limited media. A 5′-RACE amplification product of 465 bp was obtained. Conventional cloning methods were used to clone the fragment in the pGEM-T vector (Promega). The plasmid was sequenced and the transcriptional start site was determined by comparison with the *S. meliloti* 1021 published genome using the BLAST algorithm.

Co-transcription of *hmuPST* was assessed by RT-PCR using the primer sets HmuPF/GSP1 and HmuSF/HmuTR shown in Supplementary Table S1. cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad) with total RNA obtained from iron-starved cells as a template. A negative control in which no reverse transcriptase was included in the reverse transcription reaction was used as a template in order to evaluate genomic DNA contamination.

Quantitative real-time PCR (qPCR). Expression of the *shmR*, *rhrA*, *rhbE*, *hmuS*, *hmuT* and *SMc01515* genes was assessed by qPCR. Reverse transcription was carried out with total RNA using the iScript cDNA Synthesis kit (Bio-Rad). For real-time PCR, 0.02 μg cDNA and 0.25 μM of each primer (IDT DNA Technologies) were used in a 20 μl reaction, using the iQ SYBR Green Supermix (Bio-Rad).

Primers used are listed in Supplementary Table S1. PCRs were run on an iCycler thermal cycler (Bio-Rad) using a 3 min hot start at 95 °C, and then 40 cycles with steps of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. The generation of specific PCR products was confirmed by melting curve analysis. *gapA* (*SMc03979*) was used as a housekeeping gene control. Samples in which the reverse transcriptase was omitted in reverse-transcriptase reactions were used as negative controls. For relative quantification, the standard curve method was used. *S. meliloti* 1021 genomic DNA was used as the PCR template for standard curves for each gene. Relative starting quantities of mRNAs for each gene were calculated from the corresponding standard curve and were normalized to *gapA*. Every reaction was done in triplicate.

Bioinformatics analysis. Using the *S. meliloti* 1021 HmuP protein as query, the translated nucleotide database of NCBI was searched (TBLASTN). An expect value of 100 was used. Results were selected based on the following criteria: similarity along the HemP domain (from residues 10 to 46 in *S. meliloti* 1021); absence of gaps larger than two amino acids in region 20–46 (where the β-sheets are localized); GKLILTK motif conserved in at least four to five residues; Y22 and T27 conserved.

Plant assays. *Medicago sativa* cv. Creola was used for screening the symbiotic phenotype of the *hmuP* mutant. The wild-type strain was used as a control. Plant assays were done in nitrogen-free Jensen medium, as previously described (Platero *et al.*, 2004). Nitrogen-fixation efficiencies were estimated by determining plant dry weights 60 days after planting.

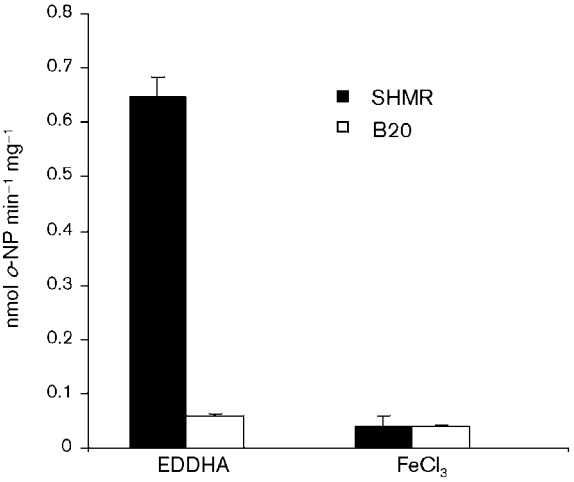

Fig. 1. β-Galactosidase activity in the SM1021 *shmR*::*lacZ* mutant (SHMR) and in its derivative mutant (B20). Cells were grown to early stationary phase in TY broth. One hundred-fold dilutions were made in TY supplemented with either 150 μM EDDHA or 37 μM $FeCl_3$ and grown for 48 h. β-Galactosidase activity is expressed as nmol *o*-nitrophenol min^{-1} (mg protein)$^{-1}$. The data shown are the mean of two independent experiments done in triplicate. Error bars, 1 SD.

RESULTS

Identification of *hmuP* in a genetic screen for mutants deregulated in *shmR* gene expression

We previously demonstrated the iron responsiveness of the *shmR* promoter using a chromosomal transcriptional fusion *shmR*::*lacZ* in the *S. meliloti* SHMR mutant strain (Amarelle *et al.*, 2008). Colonies of *S. meliloti* SHMR are white when they are grown in TY solid medium supplemented with X-Gal and $FeCl_3$, while the colonies are blue in TY supplemented with X-Gal and EDDHA, as a result of the repression or induction of *shmR*::*lacZ* expression, respectively. Here, we used this construction as a tool to search for mutants that show deregulation of iron-dependent *shmR* expression. *S. meliloti* SHMR was mutagenized with Tn*5*-1063a and transconjugants were screened for loss of metal regulation in TY solid medium supplemented with $FeCl_3$ or EDDHA. From over 10 000 transconjugants analysed, one mutant named B20 was selected for further analyses. Colonies of B20 were white in TY medium supplemented with EDDHA and X-Gal, and this mutant presented a reduced β-galactosidase activity in TY EDDHA broth when compared with the reporter strain, indicating a loss in *shmR*::*lacZ* expression (Fig. 1).

The DNA sequence of the transposon-flanking region in the B20 mutant was determined by arbitrary PCR. The PCR fragment was sequenced and the results obtained from BLAST searches in the *S. meliloti* 1021 genome indicated that the tagged locus was *SMc01747*. The deduced product of *SMc01747* is a small protein (5.3 kDa) that belongs to the HemP superfamily. An *hmuP/hemP* homologue has been shown to be involved in the utilization of haem as an iron source in *R. leguminosarum* (Wexler *et al.*, 2001), but the function of this protein is not known. Based on this homology, we designate *SMc01747* as *hmuP*. In the *S. meliloti* genome, *hmuP* is localized together with the *hmuS* gene and upstream of the *hmuTUV* cluster (Fig. 2). The function of the putative *hmuS* gene has not yet been described in *S. meliloti*. The *hmuTUV* cluster has recently been described as encoding an ABC transport system involved in haem, ferrichrome and ferrioxamine B transport in *S. meliloti* 2011 (Cuiv *et al.*, 2008).

The *hmuP* and *hmuS* genes are part of the *hmuPSTUV* transcriptional unit

The small intergenic space between the *hmuP* and *hmuS* genes suggested that both genes are part of the same transcriptional unit. On the other hand, a putative *rho*-independent transcriptional terminator downstream of the *hmuS* gene is indicated in the published *S. meliloti* 1021 genome, suggesting that *hmuPS* could be a transcriptional unit independent of the *hmuTUV* cluster. Here, we wanted to establish whether *hmuPSTUV* are independently transcribed. First, we performed a 5′-RACE, and we determined the transcription start site for *hmuP* as located 115 bp upstream of the initiation codon (Fig. 2). However, we were unable to determine the transcription start site for *hmuT*, suggesting that the *hmuT* gene could be cotranscribed together with the *hmuP* and *hmuS* genes. To assess this possibility, we employed an RT-PCR approach using

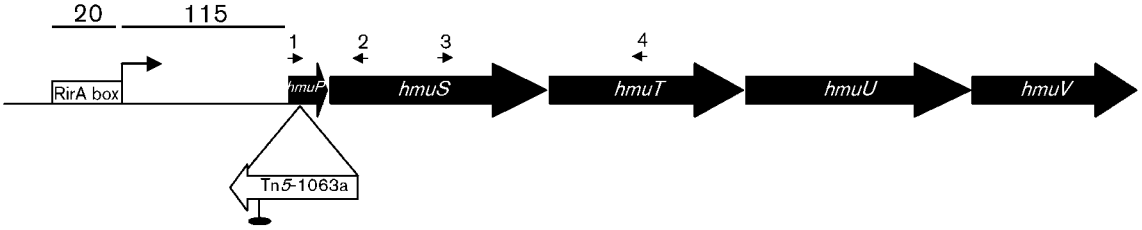

Fig. 2. Genetic organization of the *hmuP* gene in *S. meliloti* 1021. A black oval indicates a *rho*-independent transcriptional terminator. The Tn*5*-1063a insertion site and orientation are illustrated with a white arrow not to scale. The small numbered arrows indicate the positions of primers used for RT-PCR: HmuPF (1), GSP1 (2), HmuSF (3), HmuTR (4). The transcription start site of *hmuP* was determined by 5′-RACE. The bent arrow indicates the transcription start site and the numbers show the distance (bp) from it to the initiation codon (GTG) and to the putative RirA box.

different sets of primers. The locations of the primers used are shown in Fig. 2. The length of the products obtained indicates that the *hmuP* and *hmuS* genes as well as the *hmuS* and *hmuT* genes are transcribed in the same mRNA unit. No products could be detected when we used as a template a mock control in which no reverse transcriptase was included in the reverse transcription reaction (data not shown). The results obtained indicate that *hmuP*, *hmuS* and *hmuTUV* are part of the same transcriptional unit.

HmuP is essential for *shmR* expression and iron acquisition from haem compounds

Since the B20 mutant was obtained from a generalized mutagenesis in an *shmR* mutant context, the *hmuP*::Tn*5*-1063a mutation was transduced to the *S. meliloti* 1021 wild-type strain in order to obtain a mutant with a single transposon insertion in an *shmR*$^+$ context. This mutant was named HMUP. To investigate *shmR* expression, we used the transcriptional gene fusion system reported by Humann *et al.* (2008) with the pRG1SMc02726 plasmid kindly provided by Michael L. Kahn, Washington State University, Pullman, WA, USA. With this approach, after integration of pRG1SMc02726 by homologous recombination, two functional copies of *shmR* are present; one is under the control of the native *shmR* promoter, which also drives the expression of the *gusA* and *gfp* reporter genes, and the other is expressed at a low and constitutive level from the tetracycline (*tet*) promoter located in pRG1SMc02726 (Fig. 3a). To evaluate the effect of *hmuP* disruption on *shmR* expression, this construction was integrated into *S. meliloti* 1021 wild-type and HMUP mutant strains.

As shown in Fig. 3(b), expression of the *shmR*::*gusA* reporter in iron-restricted media was drastically reduced in the HMUP::pRG1SMc02726 (pOT2) strain. In order to confirm that the lack of *shmR* expression was due to the lack of HmuP, we expressed *hmuP in trans*. Complementation of the HMUP::pRG1SMc02726 strain with the pOT-HmuP plasmid completely restored the expression of the *shmR*::*gusA* reporter. These results clearly demonstrate that HmuP is required for *shmR* gene expression.

Furthermore, the presence of multiple copies of *hmuP* in the SM1021::pRG1SMc02726 (pOT-HmuP) strain resulted in an 81 % increase of *shmR*::*gusA* expression with respect to 1021::pRG1SMc02726 (pOT2) (Fig. 3b),

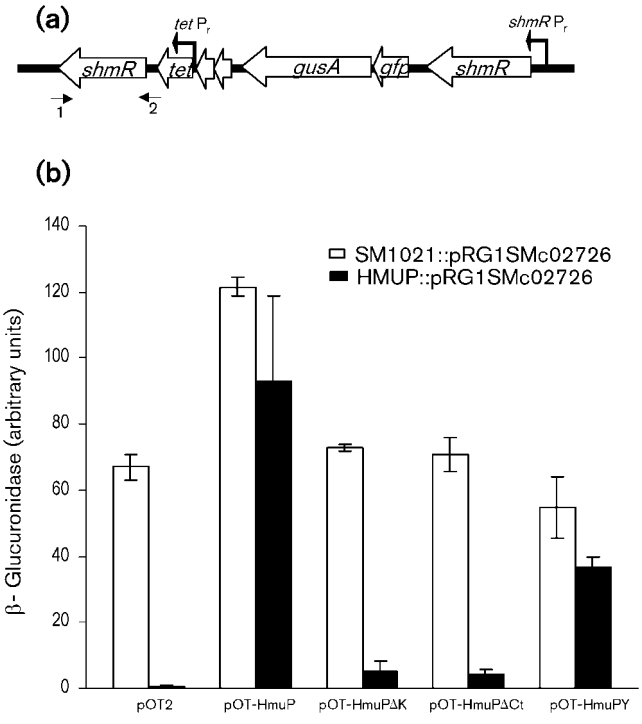

Fig. 3. *In vivo* effect of *hmuP* mutation on *shmR*::*gusA* activity. (a) The pRG1SMc02726 construction integrated into the SM1021 or HMUP genome. Small numbered arrows indicate the position of primers shmRforward (1) and 2030F (2). (b) Strains SM1021::pRG1SMc02726 and HMUP::pRG1SMc02726 containing the indicated pOT2 derivatives were grown until early stationary phase in M9 broth. One hundred-fold dilutions were made in M9 supplemented with 300 μM EDDHA and grown for 48 h. β-Glucuronidase activity is expressed as β-glucuronidase arbitrary units. The data shown are the mean of two independent experiments done in triplicate. Error bars, 1 SD.

indicating that HmuP may act as a positive regulator of *shmR* gene expression.

We previously demonstrated that the *shmR* gene is necessary for iron acquisition from haem compounds (Amarelle *et al.*, 2008). Here, we wanted to evaluate whether *hmuP* disruption affects iron nutrition from haem and haem compounds. The *hmuP* mutant was unable to use either haem or haemoglobin as the sole iron source, but retained the ability to grown on $FeCl_3$ (Table 2, Fig. 4a). Complementation with the *hmuP* gene *in trans* completely restored the ability to use haem iron sources (Table 2, Fig. 4a), indicating that the observed phenotype was due to *hmuP* disruption. These results clearly demonstrate that HmuP is necessary for haem-mediated iron acquisition in *S. meliloti*.

Since *hmuP* is part of the *hmuPSTUV* transcriptional unit, the inability to use haemin as an iron source could be due in part to polar effects. By using qPCR we observed that mRNA levels for *hmuS* and *hmuT* were similar in the wild-type and the *hmuP* mutant strains, indicating that the transposon insertion had no detectable effect on the expression of downstream genes (Fig. 5). The absence of polar effects on downstream genes can be explained by the direction in which the transposon was inserted. In the HMUP mutant strain, the transcriptional terminator present in the Tn*5*-1063a construction is localized in the opposite strand of the *hmuP* transcriptional promoter, as illustrated in Fig. 2.

The observed impairment of the *hmuP* mutant strain in growth with haemin as sole iron source could be due to the loss not only of *shmR* expression but also of other HmuP-dependent factors involved in haemin utilization. To test this possibility we assessed the ability to use haemin as an

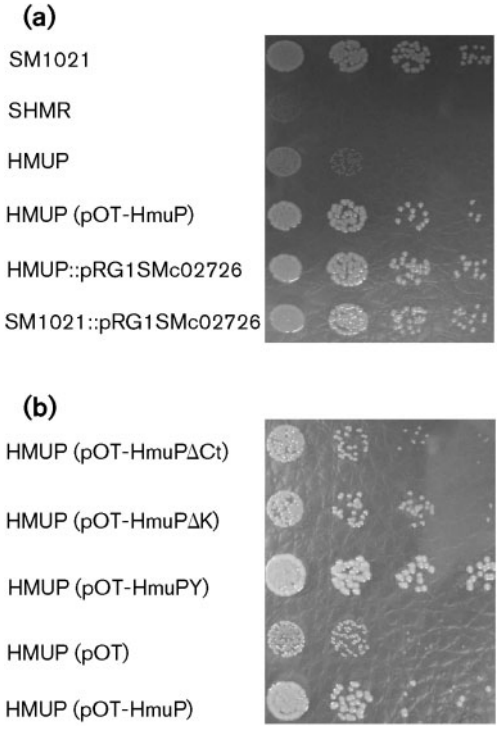

Fig. 4. Growth assays. Cells were grown in TY broth until early stationary phase. Appropriate dilutions were made in TY broth and then plated in TY solid media supplemented with 300 μM EDDHA and 10 μM haemin. Dilutions were also spotted in TY media, and all the strains displayed a similar growth pattern (data not shown). Cells were grown for 5 days and colony sizes were determined. The experiments were done in triplicate with similar results. (a) Effect of HmuP on the ability of *S. meliloti* 1021 to use haemin as an iron source. (b) Effect of *hmuP* mutations on HmuP activity.

Table 2. Assessment of the ability of *S. meliloti* strains to use different compounds as sole iron sources

Stock solutions were added to wells in TY medium supplemented with 300 μM EDDHA, containing about 10^6 c.f.u. ml^{-1}.

S. meliloti strain	Bacterial growth around wells containing different iron sources [mean diameter ± SD (cm)]*			
	370 nmol $FeCl_3$	5 nmol Hb†	20 nmol Hm†	456 nmol Desferal
SM1021	0.55 ± 0.05	0.7 ± 0.1	0.8 ± 0.1	2 ± 0.2
SHMR	0.5 ± 0.1	0	0	ND‡
HMUP	0.5 ± 0.1	0	0	2.2 ± 0.1
HMUP (pOT-HmuP)	0.5 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	2 ± 0.1
HMUP (pOT-HmuPΔCt)	0.55 ± 0.05	0	0	2 ± 0.2
HMUP (pOT-HmuPΔK)	0.8 ± 0.2	0.75 ± 0.1§	0.8 ± 0.1§	2 ± 0.1
HMUP (pOT-HmuPY)	0.45 ± 0.05	0.8 ± 0.1	0.9 ± 0.1	1.9 ± 0.1
SM1021::pRGSMc02726	0.45 ± 0.05	0.7 ± 0.1	0.7 ± 0.1	ND
HMUP::pRGSMc02726	0.5 ± 0.1	0.7 ± 0.1	0.75 ± 0.05	ND

*Results are the mean ± SD of three independent experiments.
†Hb, haemoglobin; Hm, haemin.
‡ND, Not determined.
§Haloes were fainter than the rest.

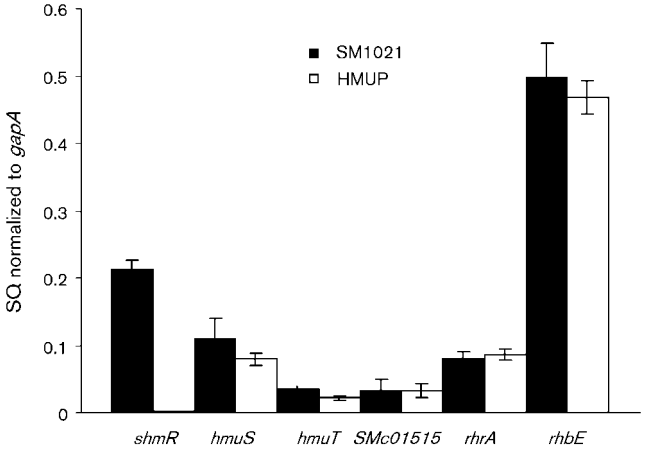

Fig. 5. Effect of HmuP on the expression of iron-regulated genes. mRNAs from wild-type (black bars) and *hmuP* mutant (white bars) cells grown in M9 minimal media supplemented with 300 μM EDDHA were analysed by qPCR. The genes assessed are indicated below the bars. The data are expressed as the relative starting quantity (SQ) of mRNA normalized to the housekeeping gene *gapA*. The data are expressed as the mean of three replicates; error bars, 1 SD.

iron source of strain HMUP::pRG1SMc02726, which expresses the *shmR* gene at a low constitutive level from the *tet* promoter (Table 2, Fig. 4a). The results obtained demonstrate that constitutive expression of *shmR* completely restores the ability to use iron from haemin in an *hmuP* mutant context, indicating that no other defects in haemin transport and utilization exist in the *hmuP* mutant except the lack of ShmR.

HmuP is not required for the global response to iron limitation

In order to evaluate a possible effect of the *hmuP* mutation on other genes involved in iron uptake and metabolism, we compared by qPCR the expression of *shmR*, *rhrA*, *rhbE* and *SMc01515* genes in the wild-type and HMUP mutant strains grown in iron-restricted media. The *gapA* housekeeping gene was used as control. As shown in Fig. 5, expression of the *rhrA* and *rhbE* genes was not modified by the *hmuP* disruption. Siderophore quantification assays reinforced these results, as the siderophore production in iron-restricted media was similar in the wild-type and the *hmuP* mutant strain (data not shown). These observations suggest that HmuP is not involved in this iron-restriction response.

The expression of *SMc01515*, a putative gene encoding a TonB homologue, was similar in the wild-type and mutant strains (Fig. 5). *SMc01515* is adjacent to and transcribed in the opposite direction from *hmuP*. These results indicate that HmuP is not involved in *SMc01515* expression.

The *hmuTUV* gene cluster has recently been described as the ABC transport system involved in the utilization not only of haem but also of ferrichrome and ferrioxamine B in *S. meliloti* 2011 (Cuiv *et al.*, 2008). The utilization of Desferal for iron nutrition was not affected in the *hmuP* mutant strain (Table 2). Similar results were obtained when ferrichrome was included as the sole iron source (data not shown). These results indicate that the transposon insertion does not affect xenosiderophore internalization and corroborate our previous data showing that the *hmuP* mutation has no polar effect on *hmuUV* expression.

Wild-type and HMUP strains containing the pOT-$HmuP_{pr}$ plasmid (*hmuP* presumptive promoter region fused to *gfpuv*) showed no GFP fluorescence difference when bacteria were grown in low-iron conditions (data not shown). This result agrees with our previous findings which show that the *hmuS* and *hmuT* genes, which are co-transcribed with *hmuP*, are expressed in the *hmuP* mutant (Fig. 5). These data demonstrate that HmuP is not involved in its own transcriptional regulation.

In sum, these results suggest that HmuP is not required for the global response to iron limitation.

HmuP is well conserved among proteobacteria

Using the BLAST algorithm with the deduced amino acid sequence of *S. meliloti* 1021 HmuP as a query, HmuP homologues were found mainly in the alpha-, beta- and gamma-proteobacteria, although some examples were also found in the delta- and epsilon-proteobacteria. As expected, HmuP from *S. meliloti* is more similar to other HmuP homologues from the alpha-proteobacteria, mainly among the Rhizobiales, having 81 % identity with *R. leguminosarum* bv. *viciae* strain 3841, 66 % with *Mesorhizobium loti* MAFF303099 and 44 % with *Rhodopseudomonas palustris* CGA009. The alignment of the last 36 amino acid residues of the C terminus indicates a high conservation in this region (Supplementary Fig. S1). The presence of a KLILXK motif at the end of the region is highly conserved. The structure of the HmuP homologue in *Rhodopseudomonas palustris* CGA009 has been determined by NMR (PDB ID: 2jra), showing that this protein is a dimer with four β-sheets and one small α-helix per monomer. According to the SWISS-MODEL SIB Service (Arnold *et al.*, 2006; Guex & Peitsch, 1997; Schwede *et al.*, 2003), the *S. meliloti* HmuP protein is structured in three β-sheets and one small α-helix. The C-terminal β-sheet has a well-conserved KLILXK motif, suggesting that this region is important for protein function (Supplementary Fig. S1).

The analysis of the genomic context of the *hmuP* homologues using GenCont (Hinrichs *et al.*, 2006) revealed that the *hmuP* gene is usually located near other genes related to iron or haem uptake and metabolism. Usually, it is located adjacent to *hmuTUV* in the alpha-proteobacteria, and also to *hmuS*, *tonB*, *exbB/exbD*, haem outer membrane receptors and bacterioferritin (data not shown). This

localization suggests that HmuP has a conserved role in haem and/or iron metabolism.

The C-terminal motif is essential for HmuP function

The high conservation of the KLILXK C-terminal motif in HmuP proteins suggests that it is important for HmuP function. To investigate this hypothesis, we mutagenized this motif by site-directed mutagenesis, and the mutated *hmuP* gene was used to complement the HMUP mutant *in trans*. When the *S. meliloti* HmuP GKLILNK motif or the C-terminal lysine was deleted (in pOT-HmuPΔCt and pOT-HmuPΔK, respectively), no significant *shmR*::*gusA* expression was observed (Fig. 3b). Furthermore, when an extra tyrosine was present at the C terminus (pOT-HmuPY), a partial complementation was obtained (Fig. 3b). These results show that the integrity of the C-terminal GKLILNK motif of HmuP is essential for transcriptional activation of the *shmR* gene. It is worth mentioning that the presence of mutagenized versions of *hmuP in trans* in the wild-type context did not affect expression of *shmR*::*gusA*, which means that these mutations are recessive with respect to the wild-type allele (Fig. 3b).

Subsequently, we evaluated by bioassays and growth assays the ability of the different mutagenized versions of HmuP to complement the *hmuP* mutant strain phenotype. The results obtained showed that the presence of pOT-HmuPY *in trans* was able to fully complement the *hmuP* mutant phenotype, restoring the ability to use haemin as iron source. The presence of pOT-HmuPΔK showed only a minor effect, while the presence of pOT-HmuPΔCt was unable to restore the use of haem-iron sources (Table 2, Fig. 4b). These observations were corroborated by using the toxic haemin analogue gallium protoporphyrin-IX (Ga-PPIX). Ga-PPIX was highly toxic for HMUP (pOT-HmuP) and for HMUP (pOT-HmuPY), but non-toxic for HMUP (pOT-HmuPΔCt). Toxicity was moderate for HMUP (pOT-HmuPΔK) (data not shown). A plausible explanation for the results obtained for the HMUP (pOT-HmuPΔK) strain could be that the small activation of *shmR* gene expression produced by the HmuP mutated protein results in a small but active haemin transport, although the expression is not enough to produce a detectable level of β-glucuronidase activity.

Altogether, these results support the conclusion that the GKLILNK C-terminal motif is required for HmuP-dependent expression of the haem receptor.

HmuP is not essential for nitrogen fixation

The *hmuP* mutant strain was able to develop nitrogen-fixing nodules in *Medicago sativa* cv. Creola plants, indicating that HmuP is not essential for symbiosis or nitrogen fixation in alfalfa under the conditions assayed (data not shown). These results agree with previously reported data which show that the *shmR* gene is not necessary for nitrogen fixation (Amarelle *et al.*, 2008).

DISCUSSION

In this work, *hmuP* was found to be essential for transcriptional expression of the haemin receptor gene *shmR* in *S. meliloti* 1021. We found that an *S. meliloti hmuP* mutant was unable to express *shmR* by using transcriptional fusion analyses and qPCR (Figs 3 and 5). Not only does disruption of *hmuP* prevent transcriptional activation of *shmR* under iron-limited conditions, but multiple copies of *hmuP* result in increased transcriptional activation. In addition, we found that *shmR* expression was recovered by the presence of *hmuP in trans* (Fig. 3). These results were corroborated by growth analysis that showed that the *hmuP* mutant was unable to use haem and haem compounds as iron sources and that this phenotype was recovered by the presence of HmuP (Fig. 4, Table 2). Altogether, these results demonstrate that HmuP works under iron limitation to activate transcription of *shmR*. It has been demonstrated that the *shmR* gene is negatively regulated by iron via RirA (Chao *et al.*, 2005; Viguier *et al.*, 2005). RirA is the major iron-responsive regulator in *S. meliloti*, and the RirA regulon comprises numerous genes involved in iron transport. In particular, besides the *shmR* gene, different genes involved in rhizobactin synthesis and transport are also repressed by RirA when cells are grown in iron-sufficient conditions (Chao *et al.*, 2005; Viguier *et al.*, 2005). Interestingly, the rhizobactin synthesis and transport system responds to a second level of regulation, requiring the presence of the RhrA transcriptional activator under iron-limiting conditions. It has been established that RhrA belongs to the AraC-like family of transcriptional activators, although the induction signal and the mechanism of action of RhrA are still unknown (Lynch *et al.*, 2001). In addition, it seems to be a general rule that xenosiderophore receptors require different types of positive regulators for their expression. These results, together with our observations, suggest that the expression of genes involved in high-affinity iron transport requires not only the relief of RirA iron repression but also the positive action of a transcriptional regulator.

Furthermore, analysis of gene expression and siderophore production indicates that disruption of *hmuP* does not affect other genes related to the iron-restriction response (Fig. 5, Table 2). It is possible, however, that another role for *hmuP* exists but is masked by redundancy with other components. The fact that constitutive expression of *shmR* restores the ability to use haemin of an *hmuP* mutant indicates that it is not required for transcriptional activation of other haemin-related genes, and that it has no direct role as an effector in haemin-transport mechanisms. Our results strongly indicate that the main function of HmuP in *S. meliloti* 1021 is the regulation of *shmR* transcription.

In numerous bacterial genomes, *hmuP* homologues are located next to genes which are predicted to be involved in haemin transport, e.g. in *Y. pestis* the *hmuP* homologue is present in the *hmuPRSTUV* gene cluster, while in *Ralstonia solanacearum* it is in the *tonBexbB1exbD1hemP* genetic organization. Co-localization of *hmuP* with genes related to the iron-restriction response, together with the results obtained in this work, suggests that HmuP has a conserved role in haem and/or iron metabolism in bacteria.

Sequence alignment of HmuP homologues, as well as comparison with the NMR solution structure of the *Rhodopseudomonas palustris* CGA009 HmuP protein, revealed that certain amino acids localized within the predicted β-sheets are well conserved. Moreover, the C-terminal β-sheet presents a KLILXK motif highly conserved among the C termini of the HmuP homologues (Supplementary Fig. S1). Here, we demonstrate that the deletion of the conserved GKLILNK motif produces a non-functional HmuP protein, indicating that this motif is important for HmuP activity.

It is not possible from the data obtained here to conclude that HmuP functions as a direct positive transcriptional regulator, or whether it is an indirect regulator. The wide distribution of HmuP homologues among bacteria makes this regulator an interesting subject for further studies.

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