HmuS and HmuQ of Ensifer/Sinorhizobium meliloti *degrade heme in vitro and participate in heme metabolism in vivo*

Vanesa Amarelle, Federico Rosconi, Juan Manuel Lázaro-Martínez, Graciela Buldain, Francisco Noya, Mark R. O'Brian & Elena Fabiano

BioMetals
An International Journal on the Role of Metal Ions in Biology, Biochemistry and Medicine

ISSN 0966-0844
Volume 29
Number 2

Biometals (2016) 29:333-347
DOI 10.1007/s10534-016-9919-3

Springer

Your article is protected by copyright and all rights are held exclusively by Springer Science +Business Media New York. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at link.springer.com".

Author's personal copy

Biometals (2016) 29:333–347
DOI 10.1007/s10534-016-9919-3

HmuS and HmuQ of *Ensifer/Sinorhizobium meliloti* degrade heme in vitro and participate in heme metabolism in vivo

Vanesa Amarelle · Federico Rosconi · Juan Manuel Lázaro-Martínez · Graciela Buldain · Francisco Noya · Mark R. O'Brian · Elena Fabiano

Received: 17 February 2016 / Accepted: 19 February 2016 / Published online: 23 February 2016
© Springer Science+Business Media New York 2016

Abstract *Ensifer meliloti* is a nitrogen-fixing symbiont of the alfalfa legume able to use heme as an iron source. The transport mechanism involved in heme acquisition in *E. meliloti* has been identified and characterized, but the fate of heme once inside the cell is not known. *In silico* analysis of *E. meliloti* 1021 genome revealed no canonical heme oxygenases although two genes encoding putative heme degrading enzymes, *smc01518* and *hmuS*, were identified. SMc01518 is similar to HmuQ of *Bradyrhizobium japonicum,* which is weakly homologous to the *Staphylococcus aureus* IsdG heme-degrading monooxygenase, whereas HmuS is homolog to *Pseudomonas aeruginosa* PhuS, a protein reported as a heme chaperone and as a heme degrading enzyme. Recombinant HmuQ and HmuS were able to bind hemin with a 1:1 stoichiometry and displayed a K_d value of 5 and 4 μM, respectively. HmuS degrades heme in vitro to the biliverdin isomers IX-β and IX-δ in an equimolar ratio. The HmuQ recombinant protein degrades heme to biliverdin IX-δ only. Additionally, in this work we demonstrate that *humS* and *hmuQ* gene expression is regulated by iron and heme in a RirA dependent manner and that both proteins are involved in heme metabolism in *E. meliloti* in vivo.

Keywords Rhizobia · Heme-oxygenase · Iron metabolism · Heme · *E. meliloti* · Heme-degradation

Introduction

Ensifer meliloti belongs to the group of rhizobia, whose most conspicuous characteristic is the ability to establish symbiotic association with legumes and carry out nitrogen fixation. Rhizobia are normally present in different habitats (e.g. soil, rhizosphere, plant host) where free iron is usually mostly inaccessible due to low solubility, organic binding or immobilization. *E. meliloti* and many other bacteria possess different high affinity iron acquisition systems that share common traits: are maximally expressed when iron is scarce and, involve the presence of TonB-dependent outer membrane receptors and ABC transporters (O'Brian and Fabiano 2010). In *E. meliloti* strain 1021, these systems include the production and

Electronic supplementary material The online version of this article (doi:10.1007/s10534-016-9919-3) contains supplementary material, which is available to authorized users.

V. Amarelle · F. Rosconi · F. Noya · E. Fabiano (✉)
Biochemical and Microbial Genomics Department, Instituto de Investigaciones Biológicas Clemente Estable, MEC, Montevideo, Uruguay
e-mail: efabiano@iibce.edu.uy

J. M. Lázaro-Martínez · G. Buldain
CONICET and Faculty of Pharmacy and Biochemistry, Universidad de Buenos Aires, Buenos Aires, Argentina

M. R. O'Brian
State University of New York at Buffalo, New York, USA

Author's personal copy

secretion of the rhizobactin 1021 siderophore, the hijacking of xenosiderophores (ferrichrome and ferrioxamine B) and the acquisition of iron from heme compounds (Cuiv et al. 2008; Noya et al. 1997; Persmark et al. 1993). For many years, transport and degradation of heme as a mechanism to acquire iron was thought to be exclusive to pathogens. However, our group discovered that rhizobia and other non-pathogenic bacteria can use heme or hemoglobin as iron sources (Noya et al. 1997). Subsequently, genes involved in heme transport were identified in *Bradyrhizobium japonicum*, *Rhizobium leguminosarum* and *E. meliloti* (Amarelle et al. 2008; Battistoni et al. 2002; Nienaber et al. 2001; Wexler et al. 2001). Notably, while the use of xenosiderophores was found to be a variable trait, heme transport systems are present in all the rhizobia strains studied so far. Moreover, rhizobia heme transport systems are present in the chromosome and not in plasmids, suggesting its importance for the metabolism of these bacteria (O'Brian and Fabiano 2010).

The outer membrane heme-receptor involved in the internalization of heme, hemoglobin and leghemoglobin in *E. meliloti* is ShmR (Amarelle et al. 2008; Battistoni et al. 2002) and HmuTUV is the ABC transporter required, among other functions, for heme uptake (Cuiv et al. 2008). Expression of the *E. meliloti shmR* gene under iron limitation involves the loss of the repression exerted by RirA (Rhizobial iron regulator) (Chao et al. 2005; Viguier et al. 2005) as well as the activation mediated by the HmuP protein (Amarelle et al. 2010). RirA is the primary iron-responsive transcriptional regulator in *E. meliloti*, while the Fur homolog, renamed as Mur, is mainly involved in the control of manganese homeostasis (Chao et al. 2005; Diaz-Mireles et al. 2004; Platero et al. 2004). The fate of exogenous heme once inside *E. meliloti* cytosol is unknown.

Different enzymes and mechanisms allowing iron release from heme have been described in bacteria (Benson and Rivera 2013; Wilks and Ikeda-Saito 2014). In *Corynebacterium diphtheria*, the pioneering work of Schmitt (1997) and Wilks and Schmitt (1998) demonstrated that once heme is taken up by bacterial cell, the tetrapyrrole ring is cleaved by heme oxygenases (HOs) in order to release iron. HOs are ubiquitous enzymes that degrade heme to biliverdin IX-α, iron and carbon monoxide, using O_2 and a reductant such as NADPH cytochrome P450 reductase for oxidation (Benson and Rivera 2013; Montellano 2000). Bacterial heme oxygenases have limited sequence similarity to each other but are structurally and mechanistically similar to those of the eukaryotic HO (Hirotsu et al. 2004; Schuller et al. 1999, 2001; Wilks and Ikeda-Saito 2014).

A novel class of heme-degrading enzyme, IsdG (Iron-regulated surface determinant) structurally unrelated to canonical HOs, was discovered in *Staphylococcus aureus* (Reniere et al. 2010; Skaar et al. 2004; Wu et al. 2005). Homologous to this heme-degrading enzyme, which belongs to the group of the antibiotic biosynthesis monooxygenase family (ABM), were also found in a limited number of gram-positive and gram-negative bacteria (Ojeda et al. 2012; Park et al. 2014; Puri and O'Brian 2006; Skaar et al. 2006). Interestingly, some members of this group of enzymes such as IsdG and IsdI of *S. aureus* and HmoB of *Bacillus subtilis,* catalize the degradation of heme to staphylobilins (15-oxo bilirubin IX-β and 5-oxo-bilirubin IX-δ) and formaldehyde rather than to biliverdin and CO (Matsui et al. 2013; Park et al. 2014; Reniere et al. 2010). In *Mycobacterium tuberculosis*, the IsdG homolog named MhuD, catalyzes heme degradation producing a novel heme catabolite, mycobilin, without the release of CO or formaldehyde, and retaining the *meso*-carbon of the porphyrin ring as an aldehyde (Nambu et al. 2013). In *B. japonicum*, two heme-degrading enzymes belonging to the ABM group of enzymes, HmuQ and HmuD, have been described. HmuQ has weak homology with *S. aureus* IsdG, being biliverdin the heme degradation product (Puri and O'Brian 2006).

The *hemS* (or *hmuS*) gene is annotated as a "heme degrading enzyme" in the genome databases although this activity has been demonstrated only for few bacteria. Particularly, this activity has been reported for ChuS in *Escherichia coli* and PhuS in *Pseudomonas aeruginosa* (Lee et al. 2014; Suits et al. 2005). The crystal structure of ChuS from *E. coli* O157:H7 has been characterized and presents a different folding structure than that of known mammalian and bacterial HOs (Suits et al. 2005). Additionally, iron release from heme without tetrapyrrole ring disruption was found in *E. coli* (Letoffe et al. 2009; Turlin et al. 2014).

HO function has been associated with cellular signaling, antioxidant defense and biosynthesis of light-sensing bilins. HO also participates in the use of

Author's personal copy

heme as nutritional iron source in numerous bacteria (Benson and Rivera 2013; Frankenberg-Dinkel 2004).

Here we report the identification and characterization of two enzymes from *E. meliloti* 1021 with heme degrading activity in vitro: HmuQ and HmuS. We demonstrate that both proteins bind heme, are capable of degrading this compound and produce biliverdin. Moreover we demonstrate that *humQ* and *hmuS* gene expression is regulated by iron and heme, and that both proteins are involved in heme metabolism in *E. meliloti* in vivo.

Materials and methods

Bacteria, plasmids and growth conditions

Bacteria and plasmids used in this study are listed in Table 1. *E. coli* strains were grown aerobically at 37 °C in Luria–Bertani (LB) medium or 2xYT medium (Sambrook et al. 1989). *E. meliloti* strains were grown at 30 °C in tryptone-yeast extract medium (TY) (Beringer 1974), defined minimal medium M9 (Sambrook et al. 1989) supplemented with 6 mM glutamate, 200 μM methionine and 1 μM biotin (M9S) or LB medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LBMC). Low iron conditions were obtained by supplementation with ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDHA) and high iron conditions were obtained by adding $FeCl_3$ to the media. The micromolar concentrations of $FeCl_3$, EDDHA (abbreviated as E) and hemin (Hm) used for each assay are indicated with subscripts When required, 50 μg/ml kanamycin (Km), 100 μg/ml neomycin (Nm), 100 μg/ml streptomycin (Sm), 10 μg/ml gentamycin (Gm) (for *E. coli*), 5 μg/ml gentamycin (for *E. meliloti*), 50 μg/ml ampicillin (Ap) or 25 μg/ml cloramphenicol (Cm) were added to the media.

Construction of Sm1021-VAQ and Sm1021-VAS mutants

To generate an *hmuQ* mutant (Sm1021-VAQ), a 2350 bp DNA fragment containing the *hmuQ* gene and flanking regions was amplified from *E. meliloti* 1021 genome using primers 5́-CGTAACCCCAAAACGAACCTGTCC-3́ and 5́-CACCGTGGAAGTGTCGCTGCT-3́. The fragment obtained was digested with PstI/XhoI and cloned in pBlueScriptSK (pSK) (Stratagene) obtaining plasmid pSK*hmuQ*. To interrupt the *hmuQ* gene, the *lacZ*-Gm^r cassette of pAB2001 (Becker et al. 1995) was cloned in the EcoRI site of pSK*hmuQ* generating pSK*hmuQ::lacZ*-Gm^r. Cloning in the correct orientation was confirmed by restriction mapping. The *hmuQ::lacZ*-Gm^r fragment was sub-cloned in the BamHI site of pK18mobSacB (pK18) (Schafer et al. 1994) obtaining plasmid pK18*hmuQ::lacZ*-Gm^r which was then mobilized into *E. meliloti* 1021 by triparental mating using DH5α-pRK2013 (Figurski and Helinski 1979) as a helper strain. Colonies Sm^r, Gm^r and able to grow in 15 % (w/v) sucrose (Suc^r) were selected and the mutation was confirmed by Southern blot hybridization.

To obtain an *hmuS* mutant (Sm1021-VAS), a 2054 bp DNA fragment containing the *hmuS* gene and flanking regions was amplified from *E. meliloti* 1021 genome using primers 5́-ATAGGATCCACGAAGGTCAACTTGCA-3́ and 5́-TCTGGATCCAAGGGAATGTTCGCCTC-3́. BamHI restrictions sites are underlined. The amplicon was cloned at the EcoRV site of pSK obtaining plasmid pSK*hmuS*. An in-frame deletion of *hmuS* gene was done by inverse PCR using primers 5́-ATAGATATCGGTGGGGCGAATGCT-3́ and 5́-ATAGATATCGTCGCGGCCTGATGGTG-3́ and pSK*hmuS* as template. EcoRV restriction sites are underlined. PCR product was digested with EcoRV and autoligated in order to obtain plasmid pSK*hmuS*if. The *hmuS* deleted gene was cloned in the BamHI site of pK18 in order to generate plasmid pK18*hmuS*if which was then mobilized into *E. meliloti* 1021 and Sm1021-VAQ strains by triparental mating as previously described, in order to generate Sm1021-VAS mutant and Sm1021-VAQS double mutant, respectively. Sm^r and Suc^r colonies were selected and the mutations were confirmed by PCR.

In order to have the *hmuQ* and *hmuS* genes mutated in a background where no endogenous siderophore is produced, mutation in the *rhrA* gene of strain Sm1021-H38 (Platero et al. 2003) was transduced with phage ΦM12 (Finan et al. 1984) to the Sm1021-VAQ, Sm1021-VAS and Sm1021-VAQS mutant strains. Transductions were done as previously described (Finan et al. 1984). Briefly, ΦM12 lysates of Sm1021-H38 mutant were generated by growing Sm1021-H38 in LBMC, culture diluted to an OD_{600nm} of 0.3–0.4 in LBMC was incubated with ΦM12 at 30 °C until complete lysis occur. Lysates were filtered and diluted in order to infect Sm1021-VAQ, Sm1021-VAS and Sm1021-VAQS cells previously grown in

Author's personal copy

Table 1 Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	References
E. meliloti		
Sm1021	Streptomycin derivative of wild-type strain SU47 (Sm^r)	(Meade et al. 1982)
Sm1021-H38	Sm1021 *rhrA*::Tn5-1063a. Siderophore deficient strain (Nm^r, Sm^r)	(Platero et al. 2003)
Sm1021-VAQ	Sm1021 *hmuQ::lacZ*-Gm^r (Sm^r, Gm^r)	This work
Sm1021-VAS	Sm1021 *ΔhmuS* (Sm^r)	This work
Sm1021-VAQS	Sm1021 *hmuQ::lacZ*-Gm^r; *ΔhmuS* (Sm^r, Gm^r)	This work
Sm1021-H38VAQ	Sm1021-H38 *hmuQ::lacZ*-Gm^r (Nm^r, Sm^r, Gm^r)	This work
Sm1021-H38VAS	Sm1021-H38 *ΔhmuS* (Nm^r, Sm^r)	This work
Sm1021-H38VAQS	Sm1021-H38 *hmuQ::lacZ*-Gm^r; *ΔhmuS* (Nm^r, Sm^r, Gm^r)	This work
Sm1021-TR2	Sm1021, Δ*rirA* (Sm^r)	(Chao et al. 2005)
Sm1021-TR2VAQ	Sm1021-TR2, *hmuQ::lacZ*-Gm^r (Sm^r, Gm^r)	This work
Sm1021 (pOT)	Sm1021 containing plasmid pOT1 (Sm^r, Gm^r)	(Platero et al. 2003)
Sm1021-TR2 (pOT)	Sm1021-TR2 containing plasmid pOT1 (Sm^r, Gm^r)	This work
Sm1021 (pOT-pr*hmuS*)	Sm1021 containing plasmid pOT-pr*hmuS* (Sm^r, Gm^r)	This work
Sm1021-TR2 (pOT-pr*hmuS*)	Sm1021-TR2 containing plasmid pOT-pr*hmuS* (Sm^r, Gm^r)	This work
E. coli		
DH5α	*sup*E44Δ*lac*U169(ϕ80*lacZ*ΔM15)*hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	(Hanahan 1983)
BL21DE3(pLysS)	F^- *omp*T *gal dcm hsd*S_B(r_B^- m_B^-) λ(DE3) pLysS (Cm^r)	Novagen
Plasmids		
pBlueScript SK	Cloning vector (Ap^r)	Startagene
pAB2001	Carrying *lacZ*-Gm^r promoter-probe cassette (Gm^r, Ap^r)	(Becker et al. 1995)
pRK2013	ColE1 replicon with RK2 *tra* genes. Used for mobilizing incP and incQ plasmids (Km^r)	(Ditta et al. 1980)
pK18mobSacB	Broad host range mobilizable vector. Non replicative in *E. meliloti*, *sac*B, *ori*V (Km^r)	(Schafer et al. 1994)
pSK*hmuQ*	pBlueScript SK containing *hmuQ* coding sequence (Ap^r)	This work
pSK*hmuS*	pBlueScript SK containing *hmuS* coding sequence (Ap^r)	This work
pSK*hmuQ::lacZ*-Gm^r	pBlueScript SK containing *hmuQ* coding sequence interrupted with *lacZ*-Gm^r cassette of pAB2001 (Ap^r, Gm^r)	This work
pK18*hmuQ::lacZ*-Gm^r	pK18mobSacB containing *hmuQ* coding sequence interrupted with *lacZ*-Gm^r cassette of pAB2001 (Km^r, Gm^r)	This work
pSK*hmuS*if	pBlueScript SK containing *hmuS* coding sequence with an in-frame deletion (Ap^r)	This work
pK18*hmuS*if	pK18mobSacB containing *hmuS* coding sequence with an in-frame deletion (Km^r)	This work
pSK-pr*hmuS*	pBlueScript SK containing the promoter region of *hmuS* (Ap^r)	This work
pET14b	IPTG-inducible expression vector with a 6 × his tag at 5′ end (Ap^r)	Novagen
pET14b-HmuQ	pET14b containing *hmuQ* coding sequence (Ap^r)	This work
pET14b-HmuS	pET14b containing *hmuS* coding sequence (Ap^r)	This work
pOT1	Wide host range *gfp*-UV promoter-probe plasmid, derivative of pBBR1 (Gm^r)	(Allaway et al. 2001)
pOT-pr*hmuS*	pOT1 containing the promoter region of *hmuS* (Gm^r)	This work

Author's personal copy

LBMC to an OD_{600nm} of 0.8. Infection was carried on for 20 min, cells were immediately washed twice with TY and plated on TY supplemented with Sm and Nm. Transductants were re-streaked twice in order to avoid phage contamination.

With the aim to obtain the *hmuQ* gene mutated in a background where the global iron regulator RirA is not present, the *hmuQ* mutation of Sm1021-VAQ strain was transduced with phage ΦM12 to the *rirA* mutant strain. Transduction was performed as above-mentioned and Sm^r and Gm^r colonies were selected.

Growth assays

For colony size experiments, appropriate dilutions of mid-exponential (OD_{620nm} of 0.8–1) cultures grown in TY media were made. Dilutions were spotted with a replica plater in TY solid medium, TY supplemented with 150 μM EDDHA (TYE_{150}) or TY supplemented with 150 μM EDDHA and either 1 μM hemin ($TYE_{150}Hm_1$), 10 μM hemin ($TYE_{150}Hm_{10}$) or 50 μM hemin ($TYE_{150}Hm_{50}$). Cells were grown 5 days at 30 °C and colony sizes were recorded. For growth experiments in liquid media, cells were grown in M9S until mid-exponential phase (OD_{620nm} of 0.8–1). 50-fold dilutions were done in M9S liquid medium supplemented with 300 μM EDDHA, 300 μM EDDHA and different concentrations of hemin, or 37 μM $FeCl_3$. Cultures were incubated with shaking at 30 °C and growth was determined as optical density at 600 nm using a Varioskan Flash® (Thermo).

Expression of *hmuQ* and *hmuS* genes

Three different approaches were performed:

i. RNA purification and real-time qPCR. Expression of *hmuQ* and *hmuS* genes was assessed by quantitative real-time PCR (qPCR). For RNA isolation, mid-exponential phase cultures of *E. meliloti* 1021 grown in M9S supplemented with 300 μM EDDHA, 300 μM EDDHA and 1 μM Hm, 300 μM EDDHA and 20 μM Hm or 37 μM $FeCl_3$ were used. RNA purification was performed as described elsewhere (Amarelle et al. 2010). Reverse transcription was carried out with total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). Conditions used for real-time PCR were as mentioned elsewhere (Amarelle et al. 2010). Primers used for *hmuQ* quantification were 5́-TACTTCGCCATGAACCGTTTCCGT-3́ and 5́-CGCAGCAGATGGAATTCGATGAAG-3́ and for *hmuS* quantification were 5́-AATGGCGGCGTCATCCAGATTCAT-3́ and 5́-GCAGATGCAGATGGAAGGTTTCGT-3́. The generation of specific PCR products was confirmed by melting curve analysis. Gene *smc03224,* a 16S rDNA, was used as a control using primers 5́-GTGTCTCAGTCCCAATGT-3́ and 5́-TCTACGGAATAACGCAGG-3́ previously described (Dominguez-Ferreras et al. 2006). Reactions without reverse transcriptase addition were used as negative controls. For relative quantification, the standard curve method was performed using *E. meliloti* 1021 genomic DNA as PCR template. The data are expressed as the relative starting quantities (SQ) of mRNA normalized to *smc03224* and presented as the average from three independent determinations.

ii. B-galactosidase transcriptional fusion. The transcriptional fusion with the promoterless cassette *lacZ*-Gm^r, generated as a result of Sm1021-VAQ mutant construction, was used also in the case of *hmuQ* gene expression. β-galactosidase activity was assessed in the Sm1021-VAQ mutant and in *rirA/hmuQ* double mutant. *E. meliloti* 1021 parental strain and *rirA* mutant strain were used as controls. Mid-exponential phase cultures grown in M9S medium were diluted 100-fold in M9S medium supplemented with either 37 μM $FeCl_3$, 300 μM EDDHA, 300 μM EDDHA plus 1 μM Hm or 300 μM EDDHA plus 20 μM Hm. Mid-exponential phase cultures were used for β-galactosidase assay, performed according to the kinetic protocol described by Thibodeau et al. (Thibodeau et al. 2004). Briefly, cells were lysed with 1 % (v/v) triton X-100, 1 % (w/v) Sarkosyl and 1 mg/ml of lysozyme for 15 min at room temperature. An aliquot of 40 μl was transfer to a Nunc F-bottom 96 well plate and 130 μl of Z buffer (Miller 1972) with 0.27 % (v/v) β-mercaptoethanol was added, and the plate was loaded in a Varioskan Flash® (Thermo) for substrate dispensing. Plate was incubated 10 min at 30 °C, 30 μl of 4 mg/ml *o*-Nitrophenyl β-D-galactopyranoside (ONPG) in Z buffer was added to each well, and the absorbance at 420 nm was recorded every 2 min over a period of one hour. The maximum

Author's personal copy

rate (ΔAbs/Δmin) was measured and the enzymatic activity, expressed as nmoles of o-Nitrophenol produced per min per optical density at 620 nm per ml, was determined. Each experiment was performed independently three times.

iii. GFP-UV promoter fusion. In order to evaluate the activity of the promoter region corresponding to *hmuS* gene, a plasmid-borne transcriptional fusion with *gfp*-UV was constructed. For this purpose, a 464 bp DNA fragment containing *hmuPSTUV* promoter region was amplified from *E. meliloti* 1021 genome by PCR using primers 5́-GGCCTTTCTGATCGAAGAGCACG-3́ and 5́-CAGCGGCGCCACGGTTTG-3́. The amplicon was cloned at the EcoRV site of pBSK generating plasmid pSK-pr*hmuS*. Correct orientation was confirmed by restriction mapping. A HindIII-XbaI fragment was subcloned in plasmid pOT1 (Allaway et al. 2001) generating plasmid pOT-pr*hmuS*. Plasmid pOT-pr*hmuS* and the pOT1 vector were mobilized into *E. meliloti* 1021 parental and *rirA* mutant strains by triparental mating in order to generate Sm1021 (pOT-pr*hmuS*), Sm1021-TR2 (pOT-pr*hmuS*) and Sm1021-TR2 (pOT1) strains. Plasmid presence in the Sm^r and Gm^r obtained colonies was confirmed by PCR. Previously constructed Sm1021 (pOT1) (Platero et al. 2004) was used as negative control. Cells were grown until mid-exponential phase in M9S medium and diluted 100-fold in M9S medium supplemented with either 300 μM EDDHA, 300 μM EDDHA plus 1 μM Hm, 300 μM EDDHA plus 20 μM Hm or 37 μM $FeCl_3$. Expression of the green fluorescent protein (GFP-UV) was evaluated in a Varioskan Flash® (Thermo) using a 405 nm excitation filter and 505 nm emission filter. Quantitative relative fluorescence (RF) was determined according the method of Allaway et al. (Allaway et al. 2001) expressed as fluorescence emission at 505 nm/OD_{620nm}. RF values calculated for *hmuS* promoter fusions in *E. meliloti* 1021 parental and *rirA* mutant strains were normalized to the RF values obtained for control strains Sm1021 (pOT1) and Sm1021-TR2 (pOT1) respectively. Results are the mean of three replicates in the same experiment. Each experiment was repeated independently three times.

Cloning, overexpression and purification of HmuQ and HmuS

Using pSK*hmuQ* or pSK*hmuS* plasmids as template, *hmuQ* or *hmuS* open reading frames were amplified by PCR. For *hmuQ* amplification, primers 5́-TCGGATCCATCTCAACAGGCCTGAG-3́ and 5́-GCAATTCATATGTACTTCGCCATG-3́ were used. For *hmuS* amplification, primers 5′-GGGAATTCCATATGACGATGACTGAG-3′ and 5′-CGCGGATCCGCCGTTCATCATCACTT-3′ were used. Restriction sites included in the primers are underlined. Products obtained were cloned in the NdeI and BamHI sites of pET14b (Novagen) generating plasmids pET14b-HmuQ and pET14b-HmuS. *E. coli* BL21DE3(pLysS) (Novagen) strain was transformed with each of these plasmids. Overexpression and purification of HmuQ and HmuS recombinant proteins was performed as described by Friedman et al. (Friedman and O'Brian 2004). Correct overexpression and purification were confirmed by SDS-PAGE. Fractions containing HmuQ protein were equilibrated with 50 mM phosphate buffer, pH 8.0, containing 300 mM NaCl and 10 % (v/v) glycerol. Fractions containing HmuS protein were equilibrated with 100 mM phosphate buffer, pH 8.0, and 10 % (v/v) glycerol. Aliquots of 10 μM solution of proteins were stored at −80 °C for further use.

Heme binding and heme degradation assays

Heme binding and heme degradation assays were performed as detailed elsewhere (Puri and O'Brian 2006). To determine the stoichiometry of heme binding to HmuQ or HmuS, spectra were recorded after each heme addition. Absorption at 413 nm was plotted versus the heme concentration.

Heme degradation was determined spectrophotometrically as the decrease in the heme absorbance in the presence of the protein and either ascorbate or human NADPH-cytochrome P450 reductase (Calbiochem) as reductant. A heme degradation control without enzyme in similar conditions was also performed.

Author's personal copy

Detection of biliverdin as HmuQ or HmuS reaction products

Heme degradation reactions were made as mentioned above. A mixture without HmuQ/HmuS protein was used as a negative control to monitor heme degradation without enzymes (Hm control). Product extraction and HPLC–MS analysis were performed as described by Puri et al. (Puri and O'Brian 2006).

Detection of dimethyl-esterified biliverdin isomers as HmuQ or HmuS reaction products

Product extraction and esterification was performed as described elsewhere (Wang et al. 2004). Dimethyl-esterified HmuQ and HmuS products were dissolved in CH_2Cl_2 and subsequently diluted in 0.1 % (v/v) formic acid in acetonitrile, 1/2 and 1/20 respectively. Samples were loaded in a C_{18} phenomenex luna analytical HPLC column and a 1 ml/min flow of 0.1 % (v/v) formic acid in acetonitrile was applied. The gradient of acetonitrile (v/v) used was: 30 % acetonitrile 5 min, from 30 to 80 % 35 min, from 80 to 95 % 5 min, 95 % 5 min, from 95 to 30 % 5 min and maintained at 30 % during 5 min.

Dimethyl-esterified biliverdin isomers of the IX series, obtained as previously described (Bonnett and McDonagh 1973), were suspended in 0.1 % (v/v) formic acid in acetonitrile and used as a standards.

Results

In silico studies

We have previously shown that heme is used as an iron source by *E. meliloti* 1021 and that the ShmR outer membrane protein is required for heme transport (Amarelle et al. 2008; Battistoni et al. 2002; Noya et al. 1997). Here, we wanted to identify HO-encoding genes in *E. meliloti* in order to evaluate their roles in iron release from the tetrapyrrole ring. The analysis of *E. meliloti* 1021 genome revealed no canonical HOs in its genome, although two putative genes encoding heme degrading enzymes, *smc01518* and *hmuS*, were identified. SMc01518 showed sequence similarity to the ABM group of enzymes to which IsdG-family of monooxygenases belong. The SMc01518 predicted protein has 50 and 59 % sequence identity (SI) with *B. japonicum* HmuD and HmuQ, respectively, and 35 % SI with *S. aureus* IsdG with only 42 % of coverage. Based on this homology and on data described below, we renamed *smc01518* as *hmuQ*.

The *hmuS* gene is annotated as a putative gene encoding a hemin transport protein in the *E. meliloti* genome. It is clustered together with genes involved in heme, Ferrichrome and Ferrioxamine B transport (*hmuTUV*) and in the regulation of *shmR* expression (*hmuP*) (Fig. S1) (Amarelle et al. 2010; Cuiv et al. 2008). The HmuS amino acid predicted sequence has a 45 % SI with PhuS of *P. aeruginosa* and 37 % SI with both ChuS of *E. coli* and HemS of *Yersinia enterocolitica*. These orthologs of *E. meliloti* HmuS have been reported to be involved in heme metabolism, either acting as a heme-degrading enzyme or as a heme chaperone and heme oxygenase titratable regulator of heme uptake (Lee et al. 2014; O'Neill and Wilks 2013; Stojiljkovic and Hantke 1994; Suits et al. 2005). Thus, we decided to characterize *hmuQ* and *hmuS* of *E. meliloti* in order to assess their roles in heme degradation.

HmuQ and HmuS bind one heme per molecule and catalyze heme degradation

Recombinant *E. meliloti* HmuQ and HmuS were overexpressed in *E. coli* as N-terminal His_6-tag fusion proteins and purified by affinity chromatography. Both proteins migrated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to their calculated molecular masses as his_6-tag fusions, 13,924 Da for HmuQ and 41,441 Da for HmuS (Fig. S2). Heme binding capability of 10 μM HmuQ and 10 μM HmuS was assessed spectrophotometrically (Fig. 1a, b respectively). Both proteins displayed a Soret peak at 413 nm. When proteins were titrated with increasing amounts of heme, the Soret peak absorbance increased, showing an inflection point at 10 μM hemin (Fig. 1a, b, insets). The data indicate a single site of heme binding for each one of the proteins. Spectroscopic analyses indicate that HmuQ has a K_d of 5.1 ± 1.2 μM and HmuS a K_d of 4.4 ± 0.7 μM.

With the aim at determining heme degradation activity of HmuQ and HmuS, a single turnover assay was performed in the presence of catalase. Catalase is required to avoid the possible molecular oxygen-mediated couple oxidation of heme in the presence of

Author's personal copy

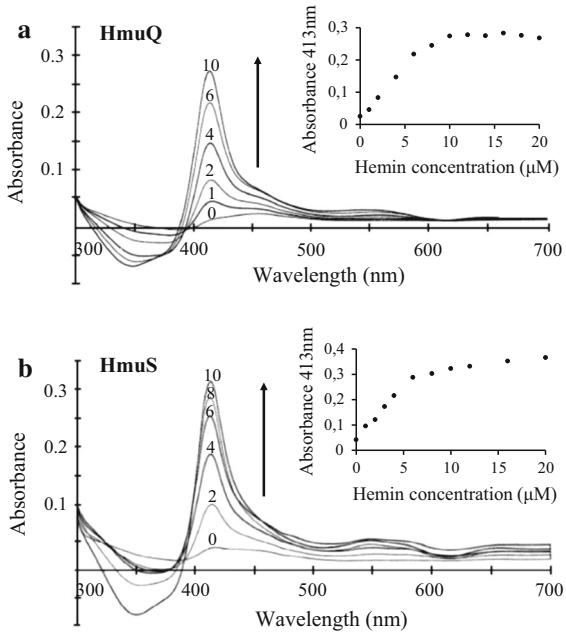

Fig. 1 Hemin binding assays. Absorption spectra of recombinant HmuQ (**a**) and HmuS (**b**) proteins in the presence of increasing concentrations of hemin are shown. The sample and reference cuvettes were titrated with increasing hemin concentrations as described in the text, and the spectrum was recorded after each addition. The sample cuvette also contained 10 μM of either recombinant protein HmuQ or HmuS. Numbers over the spectra indicate increasing amounts of hemin (μM). The *arrow* indicates an increase in absorption with increasing amounts of added hemin. In the insets, the absorption at 413 nm displayed by each protein in the binding assays was plot against hemin concentrations. Data shown in this figure are from a single experiment representative of at least three independent assays

free H_2O_2 (Sigman et al. 2001). As the in vivo electron donor of bacterial heme oxygenases is not known and, taking into account previous works on bacterial heme oxygenases (Frankenberg-Dinkel 2004; Puri and O'Brian 2006; Unno et al. 2007; Zhu et al. 2000), ascorbate or NADPH-cytochrome P450 reductase were used as reductants in the in vitro assays. Analysis of HmuQ-heme and HmuS-heme complexes in the presence of ascorbic acid showed a decrease in the Soret peak at 409 nm as a function of time as expected for a heme-degrading enzyme. As shown in Fig. 2a, b, HmuS-heme degradation was faster than HmuQ-heme degradation while heme in the absence of enzymes showed no significant spectral change during the time of the assay (Fig. S3). HmuQ-heme and HmuS-heme complexes were also incubated with NADPH-cytochrome P450 reductase, and in these assays, degradation of heme was monitored spectrophotometrically with the addition of 10 μM increments of NADPH. A decrease in the Soret peak at 409 nm was also observed; nonetheless, in this condition, no significant differences between HmuQ and HmuS activities were detected (Fig. 2c, d). An increase in a peak at 340 nm was observed corresponding to absorption of NADPH, presumably because the substrate became depleted and the NADPH was not completely oxidized. Taken together, results presented here clearly show that HmuQ and HmuS proteins catalyze heme degradation in vitro using either ascorbate or NADPH-cytochrome P450 reductase as reductants.

Biliverdin is produced from HmuQ- and HmuS-catalyzed heme degradation

The HmuQ and HmuS products were analyzed by HPLC–MS. Commercialized biliverdin IX-α was used as a standard (Fig. 3a), and a main peak with a retention time (RT) of 6.48–7.85 was identified. A reaction without enzymes was used as a control and biliverdin was not detected in this reaction (Fig. 3b). Products obtained after HmuQ-catalyzed reaction gave one peak with the same atomic mass as biliverdin ($m/z = 583.2$) (Fig. 3c) with a RT of 5.96–6.16. Degradation products of HmuS displayed two peaks with the same atomic mass to biliverdin (Fig. 3d). These two peaks showed retention times of 6.44–6.64 and 6.05–6.27, demonstrating that HmuS-dependent heme degradation yields two biliverdin isomers. These results indicate that heme degradation products catalyzed by HmuQ and HmuS are biliverdins and suggest that the isomers produced are not biliverdin IX-α. From the relative abundance (RA) of biliverdin isomers obtained, we can conclude that heme degradation catalyzed by HmuS produces 3.3 times more biliverdin than HmuQ when ascorbate was used as reductant.

In order to identify the biliverdin isomers produced by heme degradation by HmuQ and HmuS, the dimethyl esters of biliverdin IX-α, IX-β, IX-γ and IX-δ, obtained by chemical oxidation of Fe(III)-protoporphyrin IX dimethyl ester, were used as standards for the HPLC analysis (Fig. S4a). The dimethyl-esterified derivatives of the products obtained from HmuQ and HmuS catalysis were co-injected with each one of the four isomers. The HmuQ

Author's personal copy

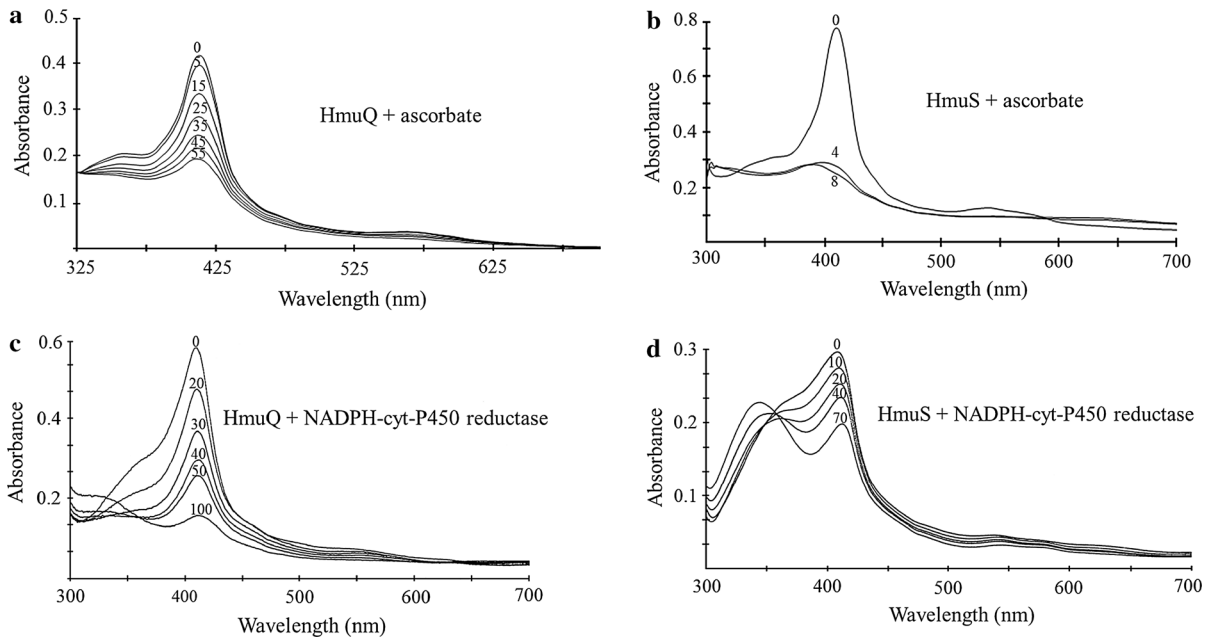

Fig. 2 Spectroscopic analysis of heme degradation by HmuQ and HmuS. Degradation of 8 μM hemin by 10 μM HmuQ (**a** and **c**) and 10 μM HmuS (**b** and **d**) in the presence of catalase was initiated by the addition of 10 mM ascorbic acid (**a** and **b**) or 10 μM NADPH (**c** and **d**). Spectra were recorded at different time intervals. Numbers over the spectra indicate either time (**a** and **b**) or NADPH micromolar concentration (**c** and **d**). Data shown in this figure are from a single experiment representative of at least three independent assays

heme degradation product co-eluted with biliverdin IX-δ dimethyl ester (Fig. S4b). The dimethyl-esterified derivatives of the products obtained from the oxidation of heme by HmuS showed two peaks, one of them eluted together with biliverdin IX-β dimethyl ester and the other with biliverdin IX-δ dimethyl ester (Fig. S4c).Thus, these observations demonstrate that heme is oxidized by HmuQ to produce biliverdin IX-δ while HmuS catalyzes heme oxidation with the production of two different biliverdin isomers (biliverdin IX-β and biliverdin IX-δ).

The expression of *hmuQ* and *hmuS* genes respond to iron and hemin

In a previous report, Chao et al. (2004) described *hmuPSTUV* to be iron regulated in a RirA-dependent manner, which is consistent with the presence of a putative RirA box in the promoter region reported by Rodionov et al. (Rodionov et al. 2006). Also, the ORFs *smc*01514, *smc*01516, *smc*01517 and *hmuQ* were reported to have a similar RirA-dependent regulation pattern (Chao et al. 2005). In order to evaluate the role of hemin in the regulation of *hmuQ* and *hmuS,* we compared by qPCR the expression of both genes in cells grown in different conditions of iron and hemin availability. The *smc*03224 gene encoding 16S rDNA was used as a constitutive control gene. As shown in Fig. 4, *hmuQ* and *hmuS* genes were expressed in iron restricted medium and repressed in iron sufficient conditions. Expression of both genes was inversely correlated with the hemin concentration of growth medium in presence of the free-iron chelator EDDHA. In addition, *hmuS* gene expression was almost two times higher than *hmuQ* gene expression in medium with 300 μM EDDHA and 1 μM hemin.

To determine if *hmuS* regulation depends on RirA, a plasmid-borne *hmuS*-GFP-UV promoter fusion was introduced in the parental and in the *rirA* mutant strains, and GFP activity was measured (Fig. S5a). Repression of *hmuS* by iron or hemin was lost in a *rirA* mutant background, demonstrating that this repression is mediated by the RirA protein. A similar regulatory pattern was observed for *hmuQ* (Fig. S5b).

Author's personal copy

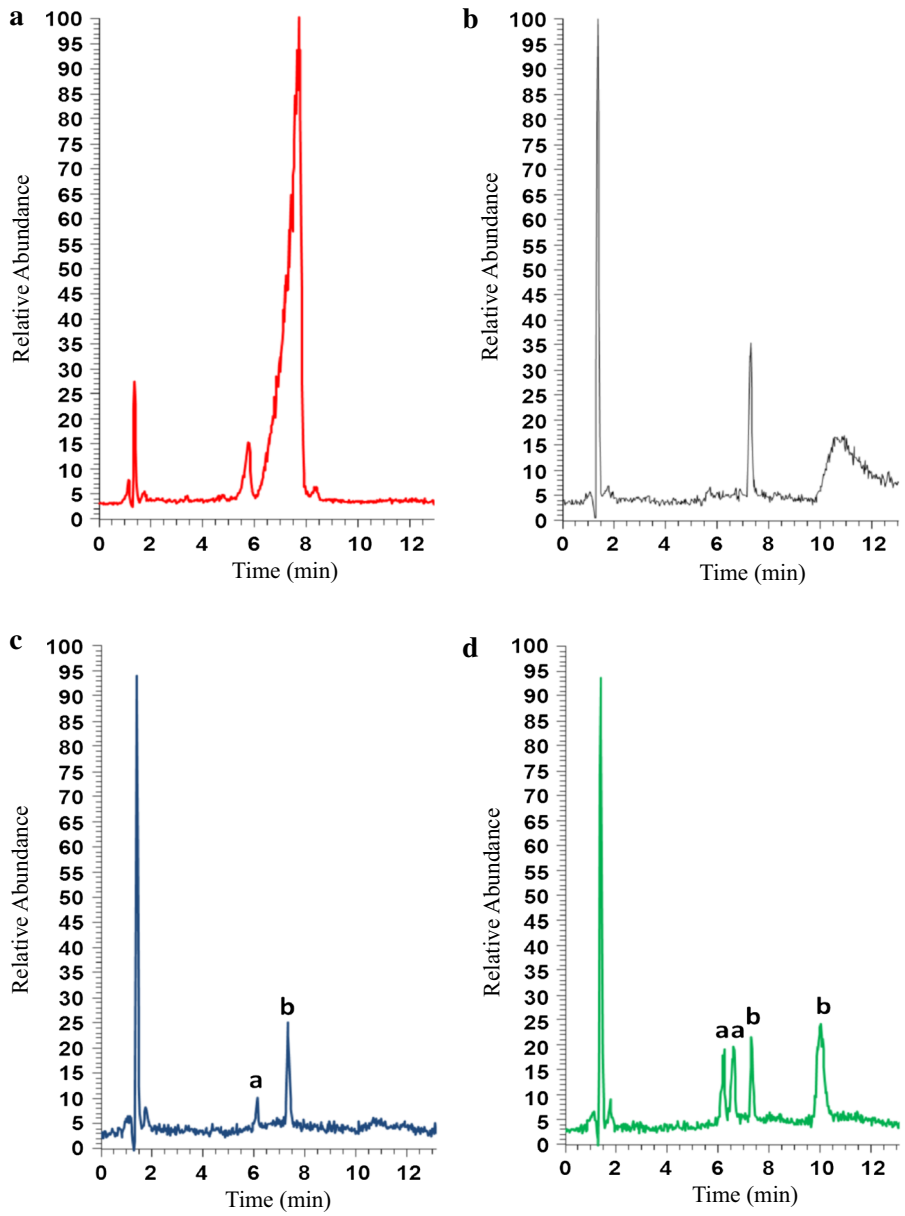

Fig. 3 HPLC–MS analysis of the heme degradation products of HmuQ and HmuS. Purified recombinant HmuQ and HmuS were assayed for heme oxygenase activity. The reaction products were extracted and analyzed by HPLC-MS using biliverdin IX-α as standard. Chromatograms of biliverdin IX-α (**a**), products from the control reaction without HmuS or HmuQ proteins (**b**) and, heme degradation products of HmuQ (**c**) or HmuS (**d**) are shown. Letter "a" over the peaks indicates those peaks displaying the same atomic mass than biliverdin IX (m/z = 583.2) and letter "b" indicates those peaks with the same atomic mass than peaks present in the control reaction shown in **b**

HmuQ and HmuS are important for heme iron utilization by *E. meliloti*

To test the role of HmuQ and HmuS in *E. meliloti* physiology, we constructed isogenic *hmuQ* and *hmuS* mutants (Sm1021-VAQ and Sm1021-VAS, respectively) and an *hmuQ, hmuS* double mutant (Sm1021-VAQS). Sm1021-VAS and Sm1021-VAQS mutant strains displayed smaller colony sizes than the parental strain and the Sm1021-VAQ mutant strain, when

Author's personal copy

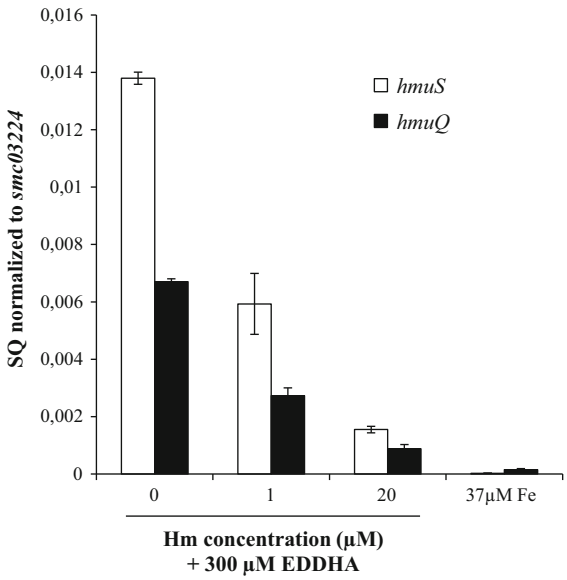

Fig. 4 Effect of iron availability on the expression of *hmuQ* and *hmuS* genes. mRNA from wild type cells grown in M9S minimal media supplemented with either 300 µM EDDHA, 300 µM EDDHA and 1 µM hemin, 300 µM EDDHA and 20 µM hemin or 37 µM $FeCl_3$ were analyzed by qPCR. *hmuQ* and *hmuS* genes were assessed. The data are expressed as the relative starting quantity (SQ) of mRNA normalized to the housekeeping gene *smc*03224. The data are expressed as the mean of three replicates; *error bars*, 1 SD

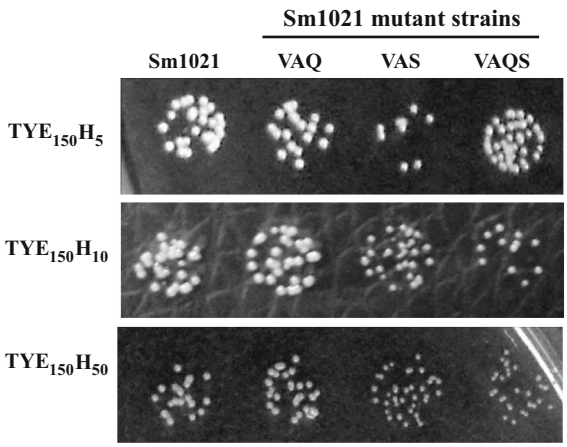

Fig. 5 Growth assays. Cells were grown in TY broth until early stationary phase. Appropriate dilutions were made in TY broth and then plated in TY solid media supplemented with 150 µM EDDHA and either 5, 10 or 50 µM hemin and incubated for 5 days. The experiments were done in triplicate with similar results

10 µM hemin was added to the medium (Fig. 5). In the presence of 50 µM hemin all the strains exhibited smaller colony sizes, indicative of heme toxicity, but the effect was more pronounced in the Sm1021-VAS and Sm1021-VAQS mutant strains, suggesting a role of *hmuS* in heme detoxification.

Taking into account that iron scavenging by the siderophore produced by *E. meliloti* may interfere with heme-dependent phenotypes, growth experiments were also performed using the Rhizobactin 1021-deficient mutant strain, Sm1021-H38, as a genetic background. The *rhrA* mutation present in the Sm1021-H38 mutant strain was transduced to the *hmuQ, hmuS and hmuQS* backgrounds as detailed in materials and methods section, generating mutant strains Sm1021-H38VAQ, Sm1021-H38VAS and Sm1021-H38VAQS. Addition of hemin in the range of 0.5–50 µM clearly improved Sm1021-H38 growth in iron chelated medium (Fig. 6), with maximal growth observed with 5 µM hemin. The Sm1021-H38VAQ mutant strain, displayed a lower growth than the Sm1021-H38 strain in the range of 1 µM to 30 µM hemin. The Sm1021-H38VAS and the Sm1021-H38VAQS mutants presented a similar growth defect being more pronounced at hemin concentrations in the range of 10–50 µM.

Results obtained here suggest that HmuQ and HmuS participate in heme utilization as an iron source at least when heme is present in the range of 0.5–40 µM. Nonetheless, the triple mutant Sm1021-H38VAQS was still able to grow in iron-chelated medium supplemented with hemin suggesting the presence of a third heme degrading enzyme or of another mechanism involved in iron release from heme.

Moreover, results presented in Fig. 5 together with results shown in Fig. 6 indicate that hemin concentrations higher than 40 µM, impaired bacterial growth of the parental strain (colony size on solid medium and OD_{620nm} in liquid medium) and that this effect is more pronounced in the *hmuS* mutant. These observations suggest a role for HmuS in heme detoxification.

Discussion

In this work, we conducted a biochemical and physiological approach to characterize two

Author's personal copy

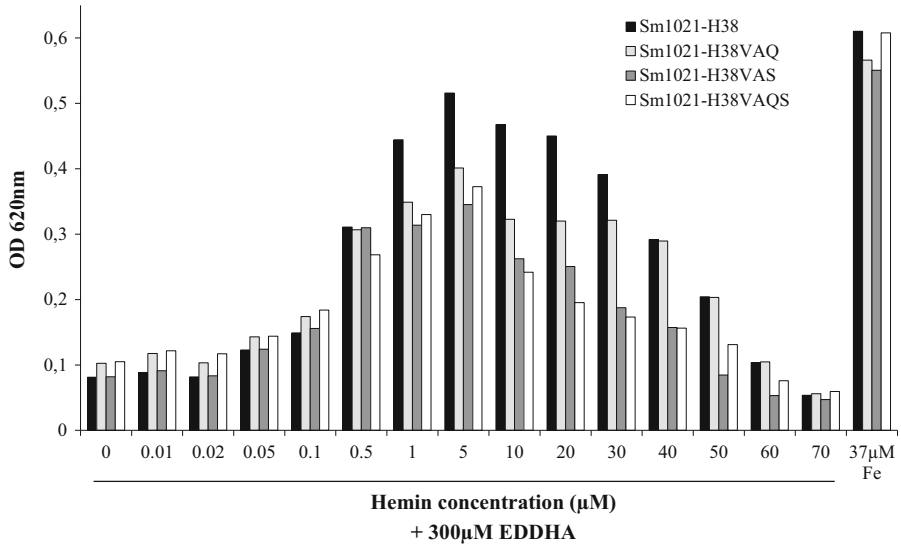

Fig. 6 Growth assays. *E. meliloti* 1021-H38 mutant strain and its derivative mutants *E. meliloti* 1021-H38VAQ, *E. meliloti* 1021-H38VAS and *E. meliloti* 1021-H38VAQS were grown in M9S minimal medium supplemented with 300 μM EDDHA or 300 μM EDDHA with increasing concentrations of hemin. M9S minimal medium supplemented with 37 μM $FeCl_3$ was used as a positive growth control. The experiment was done independently two times with similar results

presumptive heme degrading enzymes in *E. meliloti* 1021. By in vitro assays, we demonstrate that both HmuQ and HmuS recombinant proteins bind heme with a 1:1 stoichiometry (Fig. 1) a common feature for heme oxygenases, IsdG-like monooxygenases and PhuS homologs. Moreover, HmuQ and HmuS affinities for heme (5 and 4 μM, respectively) were similar to those previously reported for other heme oxygenases (Gisk et al. 2012; Skaar et al. 2004). Interestingly, we found that heme is oxidized by *E. meliloti* HmuS to biliverdin IX-β and biliverdin IX-δ a characteristic previously reported for HemO (formerly PigA) a heme oxygenase of *P. aeruginosa* and probably for *Vibrio cholerae* HutZ (Gisk et al. 2012; Ratliff et al. 2001; Uchida et al. 2012; Wegele et al. 2004). However, *E. meliloti* HmuS is not homolg to HemO or to HutZ, but has 45 % SI with the PhuS protein of *P. aeruginosa*. Different functions have been reported for *P. aeruginosa* PhuS: acting as a heme-chaperone for its delivery to HemO, as a HemO titratable regulator of heme uptake (O'Neill and Wilks 2013), or as a heme degrading enzyme (Lee et al. 2014). Lee et al. (Lee et al. 2014) indicate that in vitro, heme degradation by PhuS produces verdoheme as an intermediate in the heme breakdown pathway by canonical heme oxygenases. Those authors suggest that in vivo, PhuS might act not only as a heme trafficking protein, but also as a pre-heme oxygenase in charge of a first step of heme breakdown, subsequently delivering the verdoheme for further processing by HemO. *In vitro* heme-degradation by *E. meliloti* HmuS, in the presence of ascorbate was very efficient being almost all the heme degraded in few minutes (Fig. 2b). However, whether HmuS is a heme oxygenase, and/or a heme trafficking protein in vivo, remains to be determined.

Concerning HmuQ, its closest homologs belong to the group of the IsdG family of monooxygenases, displaying 59 % SI with the HmuQ from *B. japonicum*. When ascorbate was used as a reductant, heme degradation by HmuQ was almost ten times slower than HmuS-catalyzed reaction (Fig. 2a, b). Native reductant for HmuQ activity is not known and perhaps this low performance of HmuQ is due to the fact that ascorbate might not be an appropriate reductant. An alternative explanation could be that HmuQ heme-degrading activity requires a chaperone involved in either heme delivery to HmuQ or in facilitating the dissociation of the degradation product from the protein. The product of heme degradation catalyzed by HmuQ is biliverdin IX-δ isomer. This compound has also been reported as the heme-degradation product of HugZ, a novel heme oxygenase from *Helicobacter pylori*, different from the IsdG family of heme-degrading monooxygenase (Guo et al. 2008; Hu et al. 2011). The finding that different biliverdin isomers could be obtained as a result of heme degradation, open the question whether these heme metabolites have unique bacterial functions.

In order to determine the conditions required for *hmuS* and *hmuQ* expression in vivo, we evaluated their expression under iron sufficient, iron deprived and

Author's personal copy

iron deprived medium supplemented with different hemin concentrations. We found that both *hmuQ* and *hmuS* genes are repressed by iron and also by hemin. Previously, Rodionov et al. (Rodionov et al. 2006) described the presence of a putative RirA box in the promoter region of *hmuPSTUV* operon and Chao et al. (Chao et al. 2005) demonstrated that *hmuSTUV* and *hmuQ* are iron regulated in a RirA dependent manner. Results presented here are in accordance with these data, and additionally show that *hmuS* and *hmuQ* expression is also repressed by heme in a RirA dependent way.

It is considered that HOs are able to provide nutritional iron from heme, although this physiological role is difficult to establish because the heme oxygenase mutants usually display a subtle phenotype. Data presented here show that in *E. meliloti,* growth of *hmuS* and *hmuQ* mutants was impaired in iron-limited medium supplemented with hemin as iron source (Fig. 6). These data, together with the fact that *hmuS* and *hmuQ* genes are maximally expressed under iron-deprived conditions (Fig. 4), suggest that both HmuQ and HmuS proteins are important for the use of heme as iron source under iron-limiting conditions. Finally, we consider the possibility that HmuS and/or HmuQ might also be involved in counteract heme toxicity. We found that *hmuS* mutant, but not *hmuQ* mutant, was sensitive to heme concentrations higher than 50 μM (Figs. 5, 6) indicating that HmuS participate in avoiding heme toxicity.

Acknowledgments The authors thank Marcela Martínez from the Analytical Platform of IIBCE for her valuable help with the HPLC analysis. This work was partially supported by ANII (FCE_2009_1_2564 and POS_2011_1_555), PEDECIBA-Uruguay, GB and JMLM are supported by UBACyT 2013-2016/043BA and MO by NIH Grant R01 GM099667.

References

Allaway D, Schofield NA, Leonard ME, Gilardoni L, Finan TM, Poole PS (2001) Use of differential fluorescence induction and optical trapping to isolate environmentally induced genes. Environ Microbiol 3:397–406

Amarelle V, O'Brian MR, Fabiano E (2008) ShmR is essential for utilization of heme as a nutritional iron source in *Sinorhizobium meliloti*. Appl Environ Microbiol 74:6473–6475

Amarelle V, Koziol U, Rosconi F, Noya F, O'Brian MR, Fabiano E (2010) A new small regulatory protein, HmuP, modulates haemin acquisition in *Sinorhizobium meliloti*. Microbiology 156:1873–1882

Battistoni F, Platero R, Duran R, Cervenansky C, Battistoni J, Arias A, Fabiano E (2002) Identification of an iron-regulated, hemin-binding outer membrane protein in *Sinorhizobium meliloti*. Appl Environ Microbiol 68:5877–5881

Becker A, Schmidt M, Jager W, Puhler A (1995) New gentamicin-resistance and lacZ promoter-probe cassettes suitable for insertion mutagenesis and generation of transcriptional fusions. Gene 162:37–39

Benson DR, Rivera M (2013) Heme uptake and metabolism in bacteria Met ions. Life Sci 12:279–332

Beringer JE (1974) R factor transfer in *Rhizobium leguminosarum*. J Gen Microbiol 84:188–198

Bonnett R, McDonagh AF (1973) The meso-reactivity of porphyrins and related compounds. VI. Oxidative cleavage of the haem system. The four isomeric biliverdins of the IX series. J Chem Soc Perkin 1 9:881–888

Chao TC, Buhrmester J, Hansmeier N, Puhler A, Weidner S (2005) Role of the regulatory gene *rirA* in the transcriptional response of *Sinorhizobium meliloti* to iron limitation. Appl Environ Microbiol 71:5969–5982

Cuiv PO, Keogh D, Clarke P, O'Connell M (2008) The *hmuUV* genes of *Sinorhizobium meliloti* 2011 encode the permease and ATPase components of an ABC transport system for the utilization of both haem and the hydroxamate siderophores, ferrichrome and ferrioxamine B. Mol Microbiol 70:1261–1273

Diaz-Mireles E, Wexler M, Sawers G, Bellini D, Todd JD, Johnston AW (2004) The fur-like protein mur of *Rhizobium leguminosarum* is a Mn^{2+}-responsive transcriptional regulator. Microbiology 150:1447–1456

Ditta G, Stanfield S, Corbin D, Helinski DR (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci U S A 77:7347–7351

Dominguez-Ferreras A, Perez-Arnedo R, Becker A, Olivares J, Soto MJ, Sanjuan J (2006) Transcriptome profiling reveals the importance of plasmid pSymB for osmoadaptation of *Sinorhizobium meliloti*. J Bacteriol 188:7617–7625

Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A 76:1648–1652

Finan TM, Hartweig E, LeMieux K, Bergman K, Walker GC, Signer ER (1984) General transduction in *Rhizobium meliloti*. J Bacteriol 159:120–124

Frankenberg-Dinkel N (2004) Bacterial heme oxygenases. Antioxid Redox Signal 6:825–834

Friedman YE, O'Brian MR (2004) The ferric uptake regulator (Fur) protein from *Bradyrhizobium japonicum* is an iron-responsive transcriptional repressor in vitro. J Biol Chem 279:32100–32105

Gisk B, Wiethaus J, Aras M, Frankenberg-Dinkel N (2012) Variable composition of heme oxygenases with different regiospecificities in *Pseudomonas* species. Arch Microbiol 194:597–606

Guo Y et al (2008) Functional identification of HugZ, a heme oxygenase from *Helicobacter pylori*. BMC Microbiol 8:226

Author's personal copy

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580

Hirotsu S et al (2004) The crystal structures of the ferric and ferrous forms of the heme complex of HmuO, a heme oxygenase of *Corynebacterium diphtheriae*. J Biol Chem 279:11937–11947

Hu Y et al (2011) Crystal structure of HugZ, a novel heme oxygenase from *Helicobacter pylori*. J Biol Chem 286:1537–1544

Lee MJ, Schep D, McLaughlin B, Kaufmann M, Jia Z (2014) Structural analysis and identification of PhuS as a heme-degrading enzyme from *Pseudomonas aeruginos*a. J Mol Biol 426:1936–1946

Letoffe S, Heuck G, Delepelaire P, Lange N, Wandersman C (2009) Bacteria capture iron from heme by keeping tetrapyrrol skeleton intact. Proc Natl Acad Sci U S A 106:11719–11724

Matsui T, Nambu S, Ono Y, Goulding CW, Tsumoto K, Ikeda-Saito M (2013) Heme degradation by *Staphylococcus aureus* IsdG and IsdI liberates formaldehyde rather than carbon monoxide. Biochemistry 52:3025–3027

Meade HM, Long SR, Ruvkun GB, Brown SE, Ausubel FM (1982) Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. J Bacteriol 149:114–122

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor

Montellano PR (2000) The mechanism of heme oxygenase. Curr Opin Chem Biol 4:221–227

Nambu S, Matsui T, Goulding CW, Takahashi S, Ikeda-Saito M (2013) A new way to degrade heme: the *Mycobacterium tuberculosis* enzyme MhuD catalyzes heme degradation without generating CO. J Biol Chem 288:10101–10109

Nienaber A, Hennecke H, Fischer HM (2001) Discovery of a haem uptake system in the soil bacterium *Bradyrhizobium japonicum*. Mol Microbiol 41:787–800

Noya F, Arias A, Fabiano E (1997) Heme compounds as iron sources for nonpathogenic *Rhizobium* bacteria. J Bacteriol 179:3076–3078

O'Brian MR, Fabiano E (2010) Mechanisms and regulation of iron homeostasis in the rhizobia. In: Cornelis P, Andrews SC (eds) Iron uptake and homeostasis in microorganisms. Caister Academic Press, Caister

Ojeda JF, Martinson DA, Menscher EA, Roop RM 2nd (2012) The *bhuQ* gene encodes a heme oxygenase that contributes to the ability of *Brucella abortus* 2308 to use heme as an iron source and is regulated by Irr. J Bacteriol 194:4052–4058

O'Neill MJ, Wilks A (2013) The *P. aeruginosa* heme binding protein PhuS is a heme oxygenase titratable regulator of heme uptake. ACS Chem Biol 8:1794–1802

Park S, Kim D, Jang I, Oh HB, Choe J (2014) Structural and biochemical study of *Bacillus subtilis* HmoB in complex with heme. Biochem Biophys Res Commun 446:286–291

Persmark M, Pittman P, Buyer JS, Schwyn B, Gill PR, Neilands JB (1993) Isolation and structure of rhizobactin 1021, a siderophore from the alfalfa symbiont *Rhizobium meliloti*. J Am Chem Soc 115:3950–3956

Platero RA, Jaureguy M, Battistoni FJ, Fabiano ER (2003) Mutations in *sitB* and *sitD* genes affect manganese-growth requirements in *Sinorhizobium melilot*i. FEMS Microbiol Lett 218:65–70

Platero R, Peixoto L, O'Brian MR, Fabiano E (2004) Fur is involved in manganese-dependent regulation of *mntA* (*sitA*) expression in *Sinorhizobium meliloti*. Appl Environ Microbiol 70:4349–4355

Puri S, O'Brian MR (2006) The *hmuQ* and *hmuD* genes from *Bradyrhizobium japonicum* encode heme-degrading enzymes. J Bacteriol 188:6476–6482

Ratliff M, Zhu W, Deshmukh R, Wilks A, Stojiljkovic I (2001) Homologues of neisserial heme oxygenase in gram-negative bacteria: degradation of heme by the product of the *pigA* gene of *Pseudomonas aeruginosa*. J Bacteriol 183:6394–6403

Reniere ML et al (2010) The IsdG-family of haem oxygenases degrades haem to a novel chromophore. Mol Microbiol 75:1529–1538

Rodionov DA, Gelfand MS, Todd JD, Curson AR, Johnston AW (2006) Computational reconstruction of iron- and manganese-responsive transcriptional networks in alpha-proteobacteria. PLoS Comput Biol 2:e163

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor

Schafer A, Tauch A, Jager W, Kalinowski J, Thierbach G, Puhler A (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69–73

Schmitt MP (1997) Utilization of host iron sources by *Corynebacterium diphtheriae*: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. J Bacteriol 179:838–845

Schuller DJ, Wilks A, de Ortiz Montellano PR, Poulos TL (1999) Crystal structure of human heme oxygenase-1. Nat Struct Biol 6:860–867

Schuller DJ, Zhu W, Stojiljkovic I, Wilks A, Poulos TL (2001) Crystal structure of heme oxygenase from the gram-negative pathogen *Neisseria meningitidis* and a comparison with mammalian heme oxygenase-1. Biochemistry 40: 11552–11558

Sigman JA, Wang X, Lu Y (2001) Coupled oxidation of heme by myoglobin is mediated by exogenous peroxide. J Am Chem Soc 123:6945–6946

Skaar EP, Gaspar AH, Schneewind O (2004) IsdG and IsdI, heme-degrading enzymes in the cytoplasm of *Staphylococcus aureus*. J Biol Chem 279:436–443

Skaar EP, Gaspar AH, Schneewind O (2006) *Bacillus anthracis* IsdG, a heme-degrading monooxygenase. J Bacteriol 188:1071–1080

Stojiljkovic I, Hantke K (1994) Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding-protein-dependent transport system in *Yersinia enterocolitica*. Mol Microbiol 13:719–732

Suits MD, Pal GP, Nakatsu K, Matte A, Cygler M, Jia Z (2005) Identification of an *Escherichia coli* O157:H7 heme oxygenase with tandem functional repeats. Proc Natl Acad Sci U S A 102:16955–16960

Thibodeau SA, Fang R, Joung JK (2004) High-throughput beta-galactosidase assay for bacterial cell-based reporter systems. Biotechniques 36:410–415

Turlin E, Heuck G, Simoes Brandao MI, Szili N, Mellin JR, Lange N, Wandersman C (2014) Protoporphyrin (PPIX)

Author's personal copy

efflux by the MacAB-TolC pump in *Escherichia coli*. Microbiol Open 3:849–859

Uchida T, Sekine Y, Matsui T, Ikeda-Saito M, Ishimori K (2012) A heme degradation enzyme, HutZ, from *Vibrio cholerae*. Chem Commun (Camb) 48:6741–6743

Unno M, Matsui T, Ikeda-Saito M (2007) Structure and catalytic mechanism of heme oxygenase. Nat Prod Rep 24:553–570

Viguier C, Cuiv PO, Clarke P, O'Connell M (2011) RirA is the iron response regulator of the rhizobactin 1021 biosynthesis and transport genes in *Sinorhizobium meliloti*. FEMS Microbiol Lett 246:235–242

Wang J et al (2004) Human heme oxygenase oxidation of 5- and 15-phenylhemes. J Biol Chem 279:42593–42604

Wegele R, Tasler R, Zeng Y, Rivera M, Frankenberg-Dinkel N (2004) The heme oxygenase(s)-phytochrome system of *Pseudomonas aeruginosa*. J Biol Chem 279:45791–45802

Wexler M, Yeoman KH, Stevens JB, de Luca NG, Sawers G, Johnston AW (2001) The *Rhizobium leguminosarum tonB* gene is required for the uptake of siderophore and haem as sources of iron. Mol Microbiol 41:801–816

Wilks A, Ikeda-Saito M (2014) Heme utilization by pathogenic bacteria: not all pathways lead to biliverdin. Acc Chem Res 47:2291–2298

Wilks A, Schmitt MP (1998) Expression and characterization of a heme oxygenase (Hmu O) from *Corynebacterium diphtheriae*. Iron acquisition requires oxidative cleavage of the heme macrocycle. J Biol Chem 273:837–841

Wu R, Skaar EP, Zhang R, Joachimiak G, Gornicki P, Schneewind O, Joachimiak A (2005) *Staphylococcus aureus* IsdG and IsdI, heme-degrading enzymes with structural similarity to monooxygenases. J Biol Chem 280:2840–2846

Zhu W, Wilks A, Stojiljkovic I (2000) Degradation of heme in gram-negative bacteria: the product of the *hemO* gene of *Neisseriae* is a heme oxygenase. J Bacteriol 182:6783–6790