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ShmR Is Essential for Utilization of Heme as a Nutritional Iron Source in *Sinorhizobium meliloti*[▽]

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The bacterium *Sinorhizobium meliloti* is able to use heme as a nutritional iron source. Here, we show that the iron-regulated *shmR* gene encodes an outer membrane protein required for growth on heme. Furthermore, an *shmR* mutant is resistant to the toxic heme analog gallium protoporphyrin. Thus, the receptor protein of the heme transport system has been identified in *S. meliloti*.

Heme is an iron protoporphyrin, which serves as the prosthetic group of heme proteins. Many bacteria, including *Sinorhizobium meliloti* and other rhizobia, can use heme as a nutritional iron source (8). In gram-negative bacteria, a heme-binding outer membrane receptor is a component of a heme transport system. Heme uptake systems in *Rhizobium leguminosarum* (14) and *Bradyrhizobium japonicum* (7) have been described, but a heme receptor was identified only in the latter species. Bioinformatic analysis of the *S. meliloti* 1021 genome identified two putative heme receptor genes, smc02726 and smc04205. We previously characterized the Smc02726 homolog in *S. meliloti* 242. This protein was shown to bind heme; thus, it was named ShmR (Sinorhizobium heme receptor) (2). In the *S. meliloti* 1021 genome, the *shmR* gene is on the chromosome and is not clustered within an operon. In this study, we investigate the role of ShmR in heme utilization in *S. meliloti* 1021.

In order to assess differential expression of outer membrane proteins in response to iron, cells were grown in M3 (2) iron-replete media or in media where iron was chelated with ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDHA). Outer membrane fractions were prepared as previously described by Battistoni et al. (2), which includes a step to solubilize inner membrane proteins and thus separate them from the insoluble outer membrane proteins. Analysis of the outer membrane protein profile of the *S. meliloti* 1021 cells revealed the presence of two proteins, 82 and 91 kDa in size, that were absent in iron-replete cultures. These two proteins were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry as RhtA and ShmR, respectively (data not shown). ShmR migrated more slowly than its predicted size of ca. 80 kDa, which is not unusual for membrane proteins. RhtA is an outer membrane receptor for the siderophore rhizobactin, which is specifically expressed under iron limitation (6). We did not attempt to identify other outer membrane proteins.

To further address the function of *shmR*, we constructed a mutant strain disrupted in that gene as follows. A 2.7-kb DNA fragment containing the *shmR* gene was amplified from the *S. meliloti* 1021 genome by PCR using primers 5′-ATTCGTCTC GCTCCGTAAAA and 5′-CAAATTGTGCTGAAACT GAGG as the forward and reverse primers, respectively, and cloned in the EcoRV site of pBluescript II SK (Stratagene). The *shmR* gene was disrupted by introducing the *lacZ*-Gm^r cassette from plasmid pAB2001 (3) into the SphI site of *shmR*, which creates a transcriptional *shmR*::*lacZ* fusion (Fig. 1). An EcoRI fragment containing the disrupted *shmR* gene was subcloned in the EcoRI site of pK18mobsacB (11) and mobilized into *S. meliloti* strain 1021 by triparental mating using DH5α(pRK2013) as a helper strain (5). Double recombinants were initially identified based on streptomycin, gentamicin, and sucrose resistance and confirmed by Southern blotting using the 2.7-kb PCR fragment as a probe.

We compared the outer membrane profiles of the wild type with the *shmR* mutant strain by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Battistoni et al. (2). A protein band of about 91 kDa, corresponding to ShmR according to mass spectrometry data, was present in the wild-type strain but not in the *shmR* mutant strain grown in iron-limited cultures (Fig. 2).

***shmR* expression responds to exogenous hemin.** High-affinity heme transport systems are induced under iron limitation as a strategy to acquire iron from heme compounds (13). Previously we reported the iron responsiveness of the *shmR* promoter using a plasmid-borne transcriptional fusion with *gfpUV* (9), and similar results were obtained with a chromosomal *lacZ* fusion in this work (data not shown). Here, we examined the responsiveness of the *shmR* promoter to heme as an iron source using the plasmid-borne transcriptional fusion with *gfpUV* (Fig. 3). Under low-iron conditions with no exogenous heme, *shmR* promoter activity was induced, but activity decreased with increasing heme concentrations. Thus, expression of *shmR* is responsive to exogenous hemin and qualitatively similar to the response to iron (9).

The *S. meliloti shmR* mutant is resistant to the toxic heme analog Ga-PPIX. Bacteria that can transport heme into cells are sensitive to killing by the heme analogue gallium protoporphyrin (Ga-PPIX) (12). To test whether ShmR is required for internalization of heme, we examined the effects of Ga-

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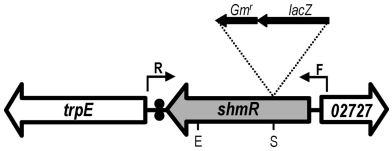

FIG. 1. Physical map of the *S. meliloti* 1021 DNA region containing the *shmR* gene. The *trpE* gene encodes an anthranilate synthase homolog; 02727 encodes a hypothetical protein. Black dots indicate the factor rho-independent transcriptional terminator. F and R indicate forward and reverse primers, respectively. S, SphI restriction site where the *lacZ*-Gm^r reporter was inserted; E, EcoRI restriction site used for subcloning the *shmR*::*lacZ*-Gm^r transcriptional fusion into the suicide plasmid.

PPIX on growth inhibition of the wild-type and *shmR* mutant strains. Ga-PPIX was spotted into wells of iron-chelated solid-medium plates containing cells, and the Ga-PPIX effect was scored as an inhibition halo around the wells. Growth of wild-type strain *S. meliloti* 1021 was affected by Ga-PPIX, as shown by an inhibition halo around the well indicating that Ga-PPIX enters cells. However, growth of the *shmR* mutant was not inhibited, showing that ShmR is required for internalization of the heme analogue. When the strains were grown in iron-sufficient solid media, the presence of Ga-PPIX did not inhibit bacterial growth (data not shown), which is consistent with iron-dependent regulation of *shmR*.

The *shmR* gene is required for utilization of heme as a nutritional iron source. To investigate the effect of a mutation in the *S. meliloti shmR* gene on heme-mediated iron nutrition, we tested the ability of the *shmR* mutant to use different iron sources for growth. Under iron limitation, *S. meliloti* synthesizes and transports the siderophore rhizobactin 1021 as an iron scavenging system (6). The expression of this system may interfere with the ability to establish whether the *shmR* strain can use heme as an iron source. To circumvent this problem, we carried out the studies using the rhizobactin-deficient strain H38 (10). This strain is disrupted in the *rhrA* gene, which encodes an activator of the rhizobactin 1021 system (6). This mutant does not produce the endogenous siderophore but retains the ability to utilize heme compounds and heterologous siderophores (10). As a consequence, the *rhrA* mutant is unable to grow in iron-restricted medium unless a nutritional iron source other than ferric-rhizobactin 1021 is added to the medium. The *shmR* gene was disrupted in strain H38 as described above to construct an *rhrA shmR* double mutant. When cultured in iron-replete M3 medium, the *rhrA* mutant and *rhrA*

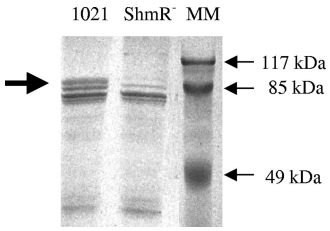

FIG. 2. Outer membrane protein profile. Wild-type *S. meliloti* 1021 and *shmR* mutant (ShmR$^-$) strains were grown in M3 minimal medium containing 500 μM EDDHA. The arrow indicates the protein band corresponding to ShmR. MM, molecular mass markers.

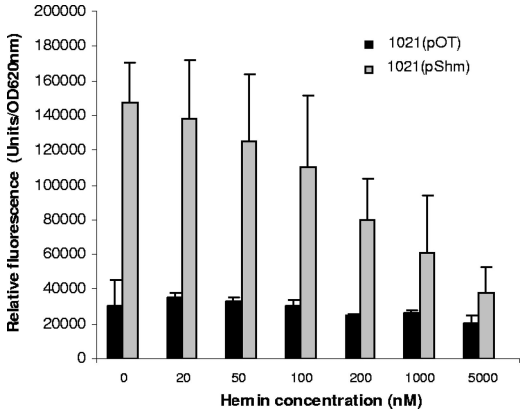

FIG. 3. In vivo effect of hemin concentration on *shmR* promoter activity in *S. meliloti* 1021. Wild-type strains containing either a plasmid with the presumptive *shmR* promoter fused to *gfpUV* [1021(pShm)] (9) or the pOT1 plasmid [1021(pOT)] (1) were grown in M3 minimal medium supplemented with 150 μM EDDHA and different hemin concentrations. 1021(pOT) was used as a control. Relative fluorescence, in arbitrary fluorescence units per unit of optical density at 620 nm (OD620nm), was determined. The data shown are the mean values of four independent experiments done by triplicate. The error bars indicate one standard deviation.

shmR double mutant exhibited similar growth properties. By contrast, media supplemented with the iron chelator EDDHA did not support growth of either strain. However, the addition of hemin to the iron-chelated medium restored growth of the *rhrA* mutant but not of the *rhrA shmR* double mutant (Fig. 4). These data show that *shmR* is essential for the utilization of heme as a sole iron source by *S. meliloti.*

S. meliloti strain 1021 is able to use heme proteins, as well as heme, as sources of iron (8). However, the *shmR* strain was unable to grow on hemoglobin or leghemoglobin but retained the ability to grow on $FeCl_3$, ferrichrome, and ferric-rhizobac-

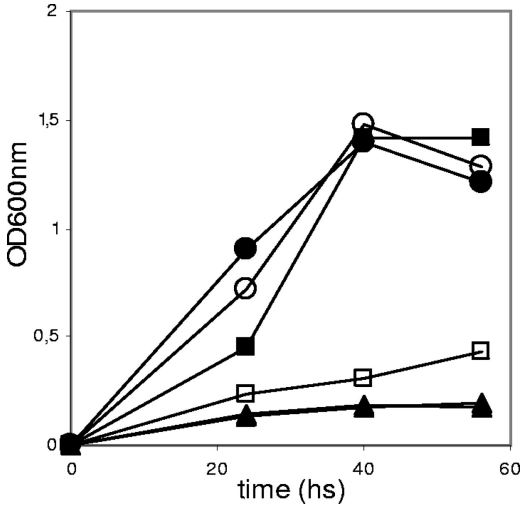

FIG. 4. The *shmR* gene is required to use heme as an iron source. *S. meliloti* H38 strain (black) and its *shmR* derivative mutant (white) were grown in M3 minimal medium supplemented with either 200 μM EDDHA (triangles), 37 μM $FeCl_3$ (circles), or 200 μM EDDHA plus 10 μM hemin (squares). OD600nm, optical density at 600 nm.

TABLE 1. Effect of *shmR* gene mutation on the ability to use different compounds as sole iron sources

S. meliloti strain	Bacterial growth[a] (diam [cm] ± SD) around wells containing[b]:					
	Hm (20 nmol)	Hb (5 nmol)	Lb (5 nmol)	Fc (5 nmol)	Rhb1021 (20 nmol)	$FeCl_3$ (740 nmol)
1021	2.1 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	2.7 ± 0.2	2.9 ± 0.2	3.5 ± 0.2
shmR	0	0	0	3.1 ± 0.2	2.9 ± 0.2	3.9 ± 0.2

[a]Results are averages of three independent experiments.
[b]Hm, hemin; Hb, hemoglobin; Lb, leghemoglobin; Fc, ferrichrome; Rhb1021, ferric-rhizobactin 1021. Solutions to be tested were added to wells in 500 μM EDDHA–tryptone-yeast extract medium (4) containing about 10^6 CFU/ml.

tin 1021 (Table 1). These results demonstrate that the *shmR* gene is necessary for iron acquisition from heme, hemoglobin, and leghemoglobin and that this gene encodes the only functional heme receptor present under the conditions tested.

ShmR is not essential for nitrogen fixation. Two independent plant assays were carried out using the wild-type and *shmR* strains to inoculate 20 germinated seedlings in nitrogen-free Jensen medium as previously described (9). No significant differences could be detected in plant dry weight or visualization of nodules formed between *Medicago sativa* cv. Creola plants inoculated with wild-type or *shmR* mutant strains (data not shown). These results indicate that the ShmR protein is not essential for symbiosis or nitrogen fixation in alfalfa in the condition assayed here.

Conclusions. The results presented herein demonstrate that the outer membrane heme-binding protein ShmR plays an essential role in heme internalization and iron nutrition from heme, hemoglobin, and leghemoglobin in the free-living form of *S. meliloti*. Collectively, our findings show that ShmR is the only functional heme receptor in *S. meliloti* in free-living cells.

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REFERENCES

1. **Allaway, D., N. A. Schofield, M. E. Leonard, L. Gilardoni, T. M. Finan, and P. S. Poole.** 2001. Use of differential fluorescence induction and optical trapping to isolate environmentally induced genes. Environ. Microbiol. **3:**397–406.
2. **Battistoni, F., R. Platero, R. Duran, C. Cervenansky, J. Battistoni, A. Arias, and E. Fabiano.** 2002. Identification of an iron-regulated, hemin-binding outer membrane protein in *Sinorhizobium meliloti.* Appl. Environ. Microbiol. **68:**5877–5881.
3. **Becker, A., M. Schmidt, W. Jager, and A. Puhler.** 1995. New gentamicin-resistance and *lacZ* promoter-probe cassettes suitable for insertion mutagenesis and generation of transcriptional fusions. Gene **162:**37–39.
4. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum.* J. Gen. Microbiol. **84:**188–198.
5. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA **76:**1648–1652.
6. **Lynch, D., J. O'Brien, T. Welch, P. Clarke, P. O. Cuiv, J. H. Crosa, and M. O'Connell.** 2001. Genetic organization of the region encoding regulation, biosynthesis, and transport of rhizobactin 1021, a siderophore produced by *Sinorhizobium meliloti.* J. Bacteriol. **183:**2576–2585.
7. **Nienaber, A., H. Hennecke, and H. M. Fischer.** 2001. Discovery of a haem uptake system in the soil bacterium *Bradyrhizobium japonicum.* Mol. Microbiol. **41:**787–800.
8. **Noya, F., A. Arias, and E. Fabiano.** 1997. Heme compounds as iron sources for nonpathogenic *Rhizobium* bacteria. J. Bacteriol. **179:**3076–3078.
9. **Platero, R., L. Peixoto, M. R. O'Brian, and E. Fabiano.** 2004. Fur is involved in manganese-dependent regulation of *mntA* (*sitA*) expression in *Sinorhizobium meliloti.* Appl. Environ. Microbiol. **70:**4349–4355.
10. **Platero, R. A., M. Jaureguy, F. J. Battistoni, and E. R. Fabiano.** 2003. Mutations in *sitB* and *sitD* genes affect manganese-growth requirements in *Sinorhizobium meliloti.* FEMS Microbiol. Lett. **218:**65–70.
11. **Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Puhler.** 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.
12. **Stojiljkovic, I., V. Kumar, and N. Srinivasan.** 1999. Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. Mol. Microbiol. **31:**429–442.
13. **Wandersman, C., and P. Delepelaire.** 2004. Bacterial iron sources: from siderophores to hemophores. Annu. Rev. Microbiol. **58:**611–647.
14. **Wexler, M., K. H. Yeoman, J. B. Stevens, N. G. de Luca, G. Sawers, and A. W. Johnston.** 2001. The *Rhizobium leguminosarum tonB* gene is required for the uptake of siderophore and haem as sources of iron. Mol. Microbiol. **41:**801–816.