Manganese transport is essential for N₂-fixation by *Rhizobium leguminosarum* in bacteroids from galegoid but not phaseoloid nodules

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Summary

*Rhizobium leguminosarum* has two high-affinity Mn²⁺ transport systems encoded by *sitABCD* and *mntH*. In symbiosis, *sitABCD* and *mntH* were expressed throughout nodules and also strongly induced in Mn²⁺-limited cultures of free-living cells. Growth of a *sitA mntH* double mutant was severely reduced under Mn²⁺ limitation and *sitA* and *mntH* single mutants were more sensitive to oxidative stress. The double *sitA mntH* mutant of *R. leguminosarum* was unable to fix nitrogen (Fix−) with legumes belonging to the galegoid clade (*Pisum sativum*, *Vicia faba* and *Vicia hirsuta*). The presence of infection thread-like structures and sparsely-packed plant cells in nodules suggest that bacteroid development was blocked, either at a late stage of infection thread progression or during bacteroid-release. In contrast, a double *sitA mntH* mutant was Fix⁺ on common bean (*Phaseolus vulgaris*), a member of the phaseoloid clade of legumes, indicating a host-specific symbiotic requirement for Mn²⁺ transport.

Introduction

The nitrogen (N₂)-fixing symbioses between rhizobia and legumes are a subject of intense study. To achieve effective symbioses, rhizobia must first initiate the formation of specialist organs, known as nodules, on the roots of legumes. Rhizobia then descend through the plant-made infection threads and colonise nodules. The bacteria are engulfed by plant cells and are surrounded by a plant-derived membrane, where they enlarge and differentiate into N₂-fixing bacteroids. These bacteroids and surrounding plant membrane are analogous to organelles and are called symbiosomes. It is the bacteroids that reduce N₂ to ammonia (NH₃) by a process known as N₂ fixation (Terpolilli *et al.*, 2012; Udvardi and Poole, 2013).

Some of the best-characterised and agriculturally important legumes e.g. pea (*Pisum sativum*), alfalfa (*Medicago sativa*), broad bean (*Vicia faba*) and clovers (*Trifolium* species) belong to the galegoid clade. Two characteristics of galegoid legumes are their indeterminate nodules (Ferguson *et al.*, 2010) and the presence in their genomes of genes encoding nodule-specific cysteine-rich (NCR) peptides (Mer-gaard *et al.*, 2006; Van de Velde *et al.*, 2010; Kondorosi *et al.*, 2013). These NCR peptides, which show similarities to antimicrobial peptides, are responsible for the enlarged pleomorphic shapes of bacteroids, their chromosomal endoreduplication, altered membrane integrity and terminal differentiation (Oono *et al.*, 2009; Karunakaran *et al.*, 2010; Haag *et al.*, 2011; 2013). In contrast to galegoid legumes, legumes belonging to the phaseoloid clade e.g. soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*), form determinate nodules and lack NCR peptides. As a result, the bacteroids of these legumes are non-swollen, do not endore-duplicate, do not have altered membrane permeability and are able to regrow outside the nodule (Mergaert *et al.*, 2006; Kondorosi *et al.*, 2013).

Microarray analyses of *Rhizobium leguminosarum* bv. viciae 3841 (Rlv3841) isolated from the nodules of the galegoid legume *P. sativum*, furthered our understanding of the fundamental differences between free-living bacteria and swollen bacteroids (Karunakaran *et al.*, 2009). Furthermore, the clustering of expression profiles at different time points revealed that rhizobia isolated from young nodules [harvested 7 days post inoculation (dpi)] had a significant number of up-regulated-genes that were not up-regulated in mature bacteroids (15, 21 and 28 dpi) or in bacteria isolated from the rhizosphere surrounding the roots (Ramachandran *et al.*, 2011). These highly
up-regulated genes are likely to be relevant to understanding processes integral to nodule colonisation and bacteroid development, and include genes encoding two putative Mn$^{2+}$ transport systems, SitABCD (ABC transport system) and MntH (proton coupled system).

The requirement for Mn$^{2+}$ uptake during symbiosis is not clear due to reported conflicting phenotypes when either sitABCD or mntH are mutated in rhizobia. For example, Sinorhizobium meliloti sitABCD mutants are compromised for growth under oxidative stress and have reduced rates of N$_2$ fixation in symbiosis with the galegoid legume M. sativa (Chao et al., 2004; Davies and Walker, 2007a,b). Bradyrhizobium japonicum lacks SitABCD and relies on MntH for Mn$^{2+}$ uptake; however, deletion of mntH had no obvious effect on symbiosis with the phaseoloid legume G. max (Hohle and O’Brian, 2009). One explanation for this apparent difference in requirements for manganese may be that the requirement of Mn$^{2+}$ uptake is dependent on whether the plant-host is a galegoid or phaseoloid-legume. To explore this further, SitABCD and MntH were studied in R. leguminosarum and their requirement for symbiosis examined in both galegoid and phaseoloid legumes.

Results

In Rlv3814 sitA and mntH are expressed throughout nodules and are regulated by Mur

To verify the microarray data reported by Karunakaran et al., 2009, promoters of sitA (sitAp) and mntH (mntHp) were fused to a gusA-reporter in the broad-host-range vector pJP2 (Prell et al., 2002). P. sativum was inoculated with Rlv3841 carrying either sitAp-gusA or mnt Hp-gusA and after three weeks, nodules were stained for $\beta$-glucuronidase (GUS) activity. In both cases, GUS activity was detected throughout the nodule (Fig. 1).

It was already known that in R. leguminosarum the sitABCD operon is regulated in response to Mn$^{2+}$ by a Fur-like repressor called Mur (manganese uptake regulator) (Diaz-Mireles et al., 2004; 2005). The DNA-binding site of Mur is well defined (Diaz-Mireles et al., 2004; Rodionov et al., 2006) and can be found upstream of both sitABCD and mntH (Fig 2C). To confirm that mntH is regulated by Mur in response to Mn$^{2+}$, expression of sitAp-gusA and mntHp-gusA constructs were compared in free-living cells. As expected, higher levels of GUS activity were observed when the sitAp-gusA strain was grown under Mn$^{2+}$ limitation relative to when it was grown with excess MnSO$_4$ (Fig. 2). The pattern of GUS activity was very similar for the mntHp-gusA strain (Fig. 2), indicating that mntH is also regulated in response to Mn$^{2+}$-limitation. To see if this response is regulated by Mur, the gusA-fusions were introduced into a mur mutant (Wexler et al., 2001). In the

Fig. 1. Nodules stained for GUS activity showing spatial expression of sitAp-gusA or mnt Hp-gusA. Nodules are colonised with Rlv3841 (A) or strains carrying either sitAp-gusA (B) or mntHp-gusA (C). Blue staining indicates the presence of GUS activity.
Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid | Description | Source or Reference |
|-------------------|-------------|---------------------|
| pJET 1.2/Blunt    | PCR product cloning vector; Ap⁰ | Thermo Scientific |
| pK19mob           | Integration plasmid; mob⁻; Km¹ | (Schafer et al., 1994) |
| pRK2013           | Helper plasmid; mob⁺; Km¹ | (Ditt et al., 1980) |
| pJP2              | Broad-host-range gusA transcriptional promoter probe vector; Tc⁰ | (Prell et al., 2002) |
| pH4503-Spc        | Vector carrying the Tn5 intersposon Spc cassette; Amp⁺ Spc⁻ | (Fellay et al., 1987) |
| pH4503-Km         | Vector carrying the Tn5 intersposon Km⁰ cassette; Amp⁺ Km¹ | (Fellay et al., 1987) |
| pJQ200SK          | Suicide vector sacB gene; Gm¹ | (Quandt and Hynes, 1993) |
| pLMB452           | Internal fragment of sitA cloned into pK19mob; Km¹ | This study |
| pLMB543           | mtIH cloned into pJET; Amp⁺ | This study |
| pLMB544           | mtIHSpc in pJET; Amp⁺ | This study |
| pLMB546           | mtH02Spc cloned into pJQ200SK; Gm¹ Spc⁻ | This study |
| pLMB592           | Internal fragment of oxyR cloned into pK19mob; Km¹ | This study |
| pLMB597           | sitA promoter cloned into pJP2; Tc⁰ | This study |
| pLMB600           | mtH promoter cloned into pJP2; Tc⁰ | This study |
| pLMB679           | sitA amplified from Rip4292 and cloned into pJET; Amp⁺ | This study |
| pLMB691           | sitA::Km in pJET; Amp⁺ Km¹ | This study |
| pLMB694           | sitA::Km cloned into pJQ200SK; Gm¹ Km¹ | This study |
| pLMB766           | mtH cloned into pJP2; Tc⁰ | This study |
| pOPS0393          | sitA along with its native promoter cloned into pJP2; Tc⁰ | This study |
| pOPS0394          | sitABCD operon along with its native promoter cloned into pJP2; Tc⁰ | This study |

| Strain            | Description | Source or Reference |
|-------------------|-------------|---------------------|
| Rlv3841           | Wild type R. leguminosarum bv. viciae; Str⁻ derivative of strain Rlv300; Str¹ | (Johnston and Beringer, 1975) |
| J325              | R. leguminosarum bv. viciae J251; mtthSpc; Spc¹ | (Wexler et al., 2001) |
| RlvA34            | R. leguminosarum bv. viciae formerly known as 8401/pRL1JI | (Downie et al., 1983) |
| Rip4292           | Derivative of field bean isolate 8002 with sym plasmid pRP2J1; Rif² | (Lamb et al., 1982) |
| LMB364            | pLMB452 integrated into Rlv3841; sitApK19mob; Neo¹ | This study |
| LMB460            | pLMB546 conjugated into Rlv3841; mtIH::Spc; Spc¹ | This study |
| LMB466            | mtIH::Spc transduced from LMB460 into LMB364; sitApK19mob mtIH::Spc; Neo¹ Spc¹ | This study |
| LMB497            | pLMB596 integrated into Rlv3841; oxyR::Km; Spc²; Tc² | This study |
| LMB498            | pLMB597 conjugated into Rlv3841; sitAp-gusA; Tc² | This study |
| LMB505            | pLMB600 conjugated into Rlv3841; mtth-gusA; Tc² | This study |
| LMB511            | pLMB597 conjugated into LMB497; sitAp-gusA; Tc² | This study |
| LMB512            | pLMB600 conjugated into LMB497; mtth-gusA; Tc² | This study |
| LMB525            | sitApK19mob transduced from LMB364 into RlvA34; Neo¹ | This study |
| LMB526            | mtIH::Spc transduced from LMB460 into RlvA34; Spc⁰ | This study |
| LMB539            | sitApK19mob transduced from LMB364 into LMB526; Neo¹ Spc² | This study |
| LMB541            | pLMB546 integrated into Rip4292; mtIH::Spc; Spc² | This study |
| LMB550            | pLMB597 conjugated into J325; sitAp-gusA; Tc² | This study |
| LMB551            | pLMB600 conjugated into J325; mtth-gusA; Tc² | This study |
| LMB624            | pLMB694 conjugated into 4292; sitA::Km; Neo¹ | This study |
| LMB630            | pLMB694 conjugated into LMB541; sitA::Km mtIH::Spc; Neo¹ Spc² | This study |
| LMB683            | pLMB786 (pJP2::mtth) conjugated into LMB466 | This study |
|                        | (sitApK19mob mtIH::Spc); Neo¹ Spc² Tc² | |
| OPS0925            | pOPS0393 (sitA along with its native promoter cloned into pJP2) conjugated into LMB466 (sitApK19mob mtIH::Spc); Neo¹ Spc² Tc² | This study |
| OPS0926            | pOPS0394 (sitABCD operon along with its native promoter cloned into pJP2) conjugated into LMB466 (sitApK19mob mtIH::Spc); Neo¹ Spc² Tc² | This study |

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mur mutant, GUS activity with both sitAp-gusA and mtth-p-gusA remained high regardless of the MnSO₄ levels (Fig. 2). Therefore, both the sitABCD operon and mtth are regulated by the Mur-repressor in response to Mn²⁺ levels in Rlv3841.

Requirement of high affinity Mn²⁺ transport systems in response to Mn²⁺ limitation and oxidative stress

To confirm SitABCD and MntH as the main Mn²⁺ transport systems in Rlv3841, growth of the single sitA and mtth mutants and the sitA mtth double mutant (Table 1) were 2717–2726

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tested under Mn$^{2+}$ limitation. As expected, the single mutations by themselves did not cause a strong growth phenotype, although the sitA mutant did have a longer generation time relative to Rlv3841 under Mn$^{2+}$ limitation (5.5 h c.f. 4.5 h) (Fig. 3). Growth of the double mutant however, was severely defective under Mn$^{2+}$ limitation (Fig. 3), confirming SitABCD and MntH are the main transporters for Mn$^{2+}$ in Rlv3841. Growth of the double mutant was restored by introducing a stable plasmid (pJP2) carrying sitA alone (along with its native promoter), indicating that the sitA-pK19mob mutation is non-polar (Supporting Information Fig. S1).

Due to the presence of reactive oxygen species (ROS) in the infection threads of nodules (Santos et al., 2001; Rubio et al., 2004; Cardenas et al., 2008), the requirement for SitABCD and MntH was also examined under oxidative stress. Mn$^{2+}$ is known for its critical role when cells are exposed to oxidative stress, acting both as a cofactor for protective enzymes (Santos et al., 2000; Kehres and Maguire, 2003; McEwan, 2009) and by suppressing Fenton's reaction by replacing Fe$^{2+}$ in mononuclear enzymes (Anjem and Imlay, 2012; Imlay, 2013). To measure the sensitivity of the wild type and mutants to oxidative stress, cultures were exposed to hydrogen peroxide (H$_2$O$_2$), viable cells counted and it was found that the single mutants were hypersensitive to H$_2$O$_2$ (Fig. 4). It was not possible to test the double mutant in the same way as it requires Mn$^{2+}$-rich medium for growth and high Mn$^{2+}$ protects against oxidative stress (Fig. 4). However, the phenotypes of the single mutants show that SitABCD and MntH help protect cells against oxidative stress. However, expression of sitABCD and mntH are not regulated in response to H$_2$O$_2$ and their expression is independent of the

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**Fig. 3.** Growth curves of single and double sitA mntH mutants under Mn$^{2+}$ limitation. Rlv3841 (diamonds), single mutants sitA (squares) and mntH (circles), and the double mutant sitA mntH (triangles) were grown in media limited (0.05 μM) or not limited (25 μM) for MnSO$_4$. Averaged from three independent experiments. For clarity only plus SEM bars are shown at 4 h intervals.

**Fig. 4.** Sensitivity to H$_2$O$_2$. Rlv3841 (diamonds), single mutants sitA (squares), mntH (circles) and double mutant (crosses) were exposed to 0 mM (solid line) or 0.5 mM (broken line) H$_2$O$_2$ after growth in AMS or modified AMS glucose supplemented with 25 μM MnSO$_4$. Survival (%) corresponds to number of colony forming units (CFU) relative to number of CFUs at time 0 h. Data from average of three independent experiments for ‘AMS’ or one experiment for ‘AMS supplemented with 25 μM MnSO$_4$. It was not possible to test the double mutant in the same way as it requires Mn$^{2+}$-rich medium for growth and high Mn$^{2+}$ protects against oxidative stress.
H$_2$O$_2$-responsive regulator OxyR (Supporting Information Fig. S2).

Requirement of Mn$^{2+}$ transporters for symbiosis with *P. sativum*, *V. faba* and *V. hirsuta* (galegoid legumes)

To test the requirement of SitABCD and MntH for nodule colonisation on galegoid legumes, the single and double mutants were inoculated onto *P. sativum*. Three weeks post inoculation, *P. sativum* nodules colonised by the single mutants were elongated, pink in colour and indistinguishable from nodules colonised by wild type Rlv3841 (Fig. 5a), whereas nodules induced by the double mutant were small, spherical, white in colour and typical of an ineffective symbiosis (Fig. 5e). Acetylene reduction assays indicated a lack of N$_2$ fixation (Fig. 6) and after six weeks’ growth, *P.

![Images and graphs showing symbiotic phenotypes and acetylene reduction rates.]

Fig. 5. Symbiotic phenotypes of double mutant on *P. sativum*. Nodules colonised by Rlv3841 (A–D) or the *sitA mntH* double mutant (E–H) were harvested after three weeks. Images of whole nodules (A and E), nodule sections stained with toluidine blue (B, C, F and G) and electron micrographs (D and H) are shown. Red arrows (C and G) indicate infection thread-like structures. Electron micrograph of nodule colonised by double mutant shows presence of starch.

Fig. 6. Rates of acetylene reduction for Rlv3841, single and double *sitA mntH* mutants on galegoid legumes *P. sativum*, *V. faba* and *V. hirsuta*. Measurements taken three weeks post inoculation. Averaged from four to ten plants or twenty-four plants for *V. hirsuta* ± SEM.

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*P. sativum* inoculated with the double mutant were indistinguishable from the uninoculated control (Fig 7.). N₂ fixation was restored to the double mutant by complementing with *mntH* cloned in the stable plasmid pJP2 (Fig. 6), confirming the phenotype is due to Mn²⁺ transport.

Even though infection-thread-like structures could be seen (Figs. 5c and 5g) only a few of the nodule cells were infected by the double mutant and these cells were sparsely packed with bacteria (Figs. 5f and 5h), relative to the many densely-packed plant cells containing Rlv3841 (Figs. 5b and 5d). Large starch granules were also present in nodules inoculated with the double mutant (Fig. 5h), indicative of a failed symbiosis (Udvardi and Poole, 2013).

To see if this Fix⁻ phenotype extends to other galegoid legumes that fall within the host-range of Rlv3841, the double mutant was tested on *V. faba* and *V. hirsuta*. As with *P. sativum*, a Fix⁻ phenotype was observed for the double mutant on *V. faba* while no symbiotic defect was observed with the single mutants (Fig. 6). *V. hirsuta* inoculated with the double mutant was also Fix⁻ (Fig. 6). Thus on three separate galegoid legumes, Mn²⁺ uptake by Rlv3841 is essential for an effective N₂-fixing symbiosis.

**Requirement of Mn²⁺ transporters for symbiosis with the phaseoloid legume *P. vulgaris***

In light of the apparent lack of a requirement for Mn²⁺ transport for symbiotic N₂ fixation with the phaseoloid legume *G. max* (Hohle and O'Brian, 2009), we decided to test the requirement for Mn²⁺ uptake in *R. leguminosarum* with the phaseoloid legume, *P. vulgaris*. The legume *P. vulgaris* is not a host of Rlv3841 but it is symbiotically compatible with *R. leguminosarum* bv. phaseoli 4292 (Rlp4292) (Lamb et al., 1982; Downie et al., 1983). Therefore, to check the requirement of Mn²⁺ uptake on *P. vulgaris*, sitA and *mntH* were mutated in Rlp4292. As a control, the two mutations were also introduced into *R. leguminosarum* bv. viciea A34 (RlvA34), a strain derived from Rlp4292 but possessing a different Sym plasmid, allowing it to nodulate and fix N₂ on *P. sativum* (Lamb et al., 1982; Downie et al., 1983).

Similar to the Rlv3841 double mutant, growth of the Rlp4292 and RlvA34 double mutants were severely impaired under Mn²⁺ limitation (Fig. 8) confirming that in this different genetic background, SitABCD and MntH are the main transporters for Mn²⁺. When *P. sativum* was inoculated with the RlvA34 double mutant a Fix⁻ phenotype was observed (Fig. 9), in agreement with the symbiotic phenotype of the Rlv3841 double mutant (Fig. 6). In contrast, when the phaseoloid legume *P. vulgaris* was inoculated with the Rlp4292 double mutant, the appearance of the nodules and the N₂ fixation rates were indistinguishable from plants inoculated with Rlp4292 wild type (Fig. 9).

**Discussion**

We show here that the Mn²⁺ transporters SitABCD and MntH are required by *R. leguminosarum* for symbiosis with legumes belonging to the galegoid clade i.e. *P. sativum, V. faba* and *V. hirsuta*. The presence of infection thread-like structures and the sparsely-packed plant cells suggest that bacteroid development of the double mutant is either blocked at a late stage of infection thread progression or during bacteroid-release. A similar developmental phenotype was seen when bacA was mutated in *R. leguminosarum*, where BacA confers resistance against NCR peptides (Karunakaran et al., 2010; Haag et al., 2013). However, the *sitA mntH* double mutant showed no
symbiotic phenotype on the phaseoloid legume *P. vulgaris*. Similarly, no symbiotic phenotype was observed when a *B. japonicum* mutant defective for Mn$^{2+}$ uptake was used to inoculate the phaseoloid legume *G. max* (Hohle and O’Brian, 2009).

One simple explanation as to why no symbiotic phenotypes have been seen with phaseoloid legumes would be if there is much more Mn$^{2+}$ available in the nodules of these legumes; consequently, high-affinity Mn$^{2+}$ transporters like SitABCD and MntH would become non-essential. Consistent with this it has been reported that *P. vulgaris* grown symbiotically with rhizobia require high levels of Mn$^{2+}$ for optimum growth (Pelaez *et al*., 2010). A second explanation could be that there is a lower concentration of ROS in the infection threads of phaseoloid relative to galegoid legumes. The presence of ROS in infection threads has been widely reported although we are not aware of direct comparison between galegoid and phaseoloid legumes (Santos *et al*., 2001; Rubio *et al*., 2004; Cardenas *et al*., 2008). The superoxide dismutase (SodA) in *S. meliloti* can use Mn$^{2+}$ as a cofactor and its absence results in abnormal infection and senescent bacteroids (Santos *et al*., 1999; 2000). Although this seems similar to the phenotype of the *sitA mntH* double mutant, disruption of *sodA* in Rlv3841 was shown not to affect nodulation or N$_2$ fixation on *P. sativum* (personal communication, Allan Downie). The symbiotic requirement of Mn$^{2+}$ cannot therefore be completely attributed to SodA and instead may also reflect its ability to replace Fe$^{2+}$ as a cofactor under oxidative stress (Anjem *et al*., 2009). Alternatively, the requirement of Mn$^{2+}$ may not be restricted to oxidative stress resistance as some bacteria utilise the metal as a cofactor for enzymes central to metabolism (Eyzaguir *et al*., 1973; Hohle and O’Brian, 2012).

Mn$^{2+}$ transporters, in particular SitABCD, have also been shown to be important for *S. meliloti* 1021 when colonising the nodules formed on the galegoid legume *M. sativa* (Chao *et al*., 2004; Davies and Walker, 2007a). Interestingly, the symbiotic phenotype of the *R. leguminosarum* double mutant is more severe than that of the *S. meliloti sitA* mutant. For example, when *M. sativa* was...
inoculated with the sitA mutant a mixture of small-white and intermediate-sized nodules were observed (Davies and Walker, 2007a) in contrast to the homogenous small-white nodules initiated by the R. leguminosarum double mutant on all three galegoid legumes tested. In addition to this, mutation of sitA in S. meliloti only caused a ~50–75% decrease in acetylene reduction whereas no acetylene reduction could be detected for the R. leguminosarum double mutant. A possible explanation for this discrepancy is that S. meliloti 1021 encodes another Mn\(^{2+}\) transporter. Indeed, it has been reported that the S. meliloti genome contains an uncharacterised gene (locus tag SMa115) that encodes a putative Nramp transporter that shares 26% amino acid identity with MntH from E. coli (Patzer and Hantke, 2001; Platero et al., 2007). Furthermore, this might also explain why mutation of sitB and sitD in S. meliloti strain 242 caused no symbiotic phenotype (Platero et al., 2003). In contrast to R. leguminosarum however, disruption of sitABCD alone in strain 242 caused a strong growth phenotype under Mn\(^{2+}\) limitation (Platero et al., 2003; Diaz-Mireles et al., 2004), suggesting that SitABCD is the major Mn\(^{2+}\) transporter in S. meliloti under free-living conditions.

An intriguing possibility is the presence of NCR peptides in galegoid legumes and their absence in phaseoloid legumes alter the susceptibility of rhizobia to oxidative stress. Antimicrobial peptides like NCR peptides have been shown to stimulate HO Formation via Fenton's reaction by inflicting damage on Fe-S clusters (Kohanski et al., 2007). If this is the case, there would be a demand for Mn\(^{3+}\) to supress Fenton's reaction by replacing Fe\(^{2+}\) as a cofactor wherever possible (Anjem and Imlay, 2012). NCR peptides are also known to increase membrane permeability of bacteroids, which may lead to the leakage of cellular content (Galvez et al., 1991; Okereke and Montville, 1992; Matifah et al., 1993; Matsuzaki et al., 1997; Xu et al., 1999; Brogden, 2005; Bolintineanu et al., 2010). It is feasible therefore that transporters like SitABCD and MntH would become essential for retrieving the metal ions that are lost on exposure to NCR peptide. Membrane damage would also disrupt the proton motive force (pmf) of the membrane, conservation of which is critical to a range of divergent metal transporters (Karlinsey et al., 2010). In galegoid legumes therefore, the presence of active divergent metal transporters with a low affinity for Mn\(^{2+}\) may be able to compensate for the loss of SitABCD and MntH. In galegoid legumes however, where the functionality of these low affinity transporters may be compromised by the disruption of pmf and the rate of Mn\(^{2+}\) uptake may be insufficient to compensate for the loss of SitABCD and MntH.

For plant-infecting bacteria this demonstrates how a bacterium's requirement for metal transporters depends on the host plant. The requirement of an Mg\(^{2+}\) channel by R. leguminosarum for \(\text{N}_2\) fixation on specific legumes is another example of how the plant-host dictates the requirement for the transporters used by rhizobia (Hood et al., 2015). However, in that case the difference in fixation phenotype was observed between plants that all belong to the galegoid tribe (e.g. P. sativum and V. faba). This raises an important caveat about our observation that Mn\(^{2+}\) transport by SitABCD and MntH is important for \(\text{N}_2\) fixation in galegoid legumes versus the phaseoloid P. vulgaris. The absence of a fixation phenotype in P. vulgaris may be specific to this plant and independent of nodule development. However, the absence of an effect on \(\text{N}_2\) fixation when Mn\(^{2+}\) transport is prevented in both soybean and P. vulgaris suggests that nodule development may be important. In future it would be interesting to extend this by testing other host of R. leguminosarum including lentil (Lens culinaris). Fundamentally, these studies highlight the stark differences between nodules of different legumes and consequently, alter what is required by rhizobia for successful colonisation.

**Experimental procedures**

**Bacterial strains, plasmids and culture conditions**

The strains and plasmids used in this study are detailed in Table 1. R. leguminosarum strains were grown at 28°C in either tryptone-yeast (TY) extract (Beringer, 1974) or Acid Minimal Salts (AMS) supplemented with 10 mM glucose and 10 mM NH\(_4\)Cl (Poole et al., 1994). When growing the sitA mntH double mutant, solid TY medium was supplemented with 50 \(\mu\)M MnSO\(_4\). Escherichia coli strains were grown at 37°C in Luria Bertani (LB) broth or on LB-agar. Antibiotics were used at the following concentrations (\(\mu\)g/ml): neomycin, 80; spectinomycin, 100 (for E. coli); streptomycin, 500; tetracycline, 2.

**Mutagenesis**

Mutagenesis of sitA (RL3884) was achieved by amplifying the internal gene fragment with primers pr0970 and pr0971 and cloning the resulting product into Xbal-digested pK19mob (pLMB452). Plasmid pLMB452 was then conjugated into Rlv38541 and site-directed integration of the recombinant suicide vector was selected for with Neomycin as previously described (Karunakaran et al., 2009). The position of insertion was mapped using the primer pr0416 and the pK19mob mapping primer pK19/18B. Insertion in sitA was chosen as it is first gene in the operon and we expected it to completely disrupt transport by the Sit complex. All primers are shown in Supporting Information Table S1. Cloning utilised the BD Infusion\textsuperscript{TM} cloning kit (Clontech) and was performed according to the manufacturer's instructions.

The gene mntH (RL0940) was disrupted with an omega intersposon cassette carrying Spc\(^{\ast}\) (\(\Omega\)Spc). A 3 kb fragment containing mntH was PCR-amplified from Rlv3841 genomic DNA using primers pr1186 and pr1187 and the resulting product was cloned into pJEt1.2/blunt (pLMB543). A Smal-cut fragment carrying the \(\Omega\)Spc cassette was cloned into pLMB543 at the unique EcoRV site in mntH (mntH\(\Omega\)Spc).
making pLMB544. A 5 kb XbaI/Xhol fragment the carrying mntH\#Spc was then cloned into pJQ200SK to make pLMB546. The plasmid pLMB546 was conjugated into Rlv3841 and the single mntH mutant (LMB460) was isolated using the sacB mutagenesis strategy as described (Kumar et al., 2005). Mutagenesis was confirmed using intersposon primers (pOT forward/pOT forward_far) and mapping primers designed to bind ~1 kb downstream and upstream of mntH (pr1225 and pr1226). To make the double mutant (LMB466), mntH\#Spc was transduced from LMB460 into the single sitA mutant (LMB364) using the bacteriophage RL38 (Buchanan-Wollaston, 1979). Solid TY medium supplemented with 50 μM MnSO₄ was used when selecting for the double mutant.

To construct the single sitA and mntH mutations in RlvA34, sitA\#pK19mob and mntH\#Spc were transduced from LMB364 and LMB460 (respectively) into RlvA34, resulting in LMB525 and LMB526. The double mutant (LMB539) was made by transducing sitA\#pK19mob from LMB364 into LMB526. RL38 is incapable of infecting Rlp4292 so the single and double mutations were created by sac mutagenesis. The plasmid pLMB546 was conjugated into Rlp4292 to make the single mntH mutant (LMB541). To make a single sitA mutant (LMB624), a 3 kb region containing sitA was PCR-amplified from Rlp4292 genomic DNA (using primers pr1378 and pr1394) and cloned into pJET/1.2 blunt (pLM679). An EcoRI-fragment carrying the intersposon cassette ωKm was end-filled using Klenow and then cloned into pLM679 at the unique SmaI site in sitA resulting in pLM691. An XbaI/NotI fragment from pLM691 was then cloned into pJQ200SK making pLMB694, which was used to generate the single sitA mutation in Rlp4292. Mutagenesis was confirmed using intersposon primers (pOT forward/pOT forward_far) and mapping primers designed to bind ~1 kb downstream and upstream of sitA (pr0416 and pr1457). To make the double mutation, pLMB694 was conjugated into LMB541 and the double mutant (LMB630) was isolated by sac mutagenesis on MnSO₄-supplemented TY medium.

**Complementation of the double sitA mntH mutant with pJP2\#mntH**

To complement the double sitA mntH mutant with mntH, a 1.9 kb region containing mntH was amplified from Rlv3841 genomic DNA using primers pr1290 and pr1462. The PCR product was digested with XbaI/HindIII and cloned into XbaI/HindIII-digested pJP2, to make pLMB766. The plasmid pLMB766 was then conjugated into the sitA mntH double mutant to make LMB683. Presence of the plasmid pLMB766 was confirmed with pJP2 mapping primers p611 and pr0096. To complement the sitA deletion in the double sitA mntH mutant, we constructed two plasmids (i) pOPS0393 (sitA along with promoter cloned in pJP2) and (ii) pOPS0394 (sitABCD operon along with promoter cloned in pJP2). Although, the pK19 insertion was only in sitA, we also complemented with sitABCD along with its promoter, to determine whether the pK19 insertion was polar on the rest of the operon. To construct pOPS0393 a 1.44 kb region containing sitA along with its native promoter was amplified from Rlv3841 genomic DNA using primers npxp1221 and npxp1220. The PCR product was digested with XbaI/HindIII and cloned into XbaI/HindIII-digested pJP2. Similarly, to construct pOPS0394 a 4.0 kb region containing sitABCD along with its native promoter was amplified from Rlv3841 genomic DNA using primers npxp1221 and npxp1222. The PCR product was digested with XbaI/HindIII and cloned into XbaI/HindIII-digested pJP2. The plasmids were then conjugated into the sitA mntH double mutant to make OPS0925 and OPS0926 respectively.

**Construction of gusA-fusions and measurement of β-glucuronidase (GUS) activity**

For the construction of the sitAp-gusA and mntHp-gusA reporter fusions, the promoter-regions were PCR-amplified from Rlv3841 genomic DNA with primers pr1292 and pr1293 for sitA and pr1290 and pr1291 for mntH and cloned into pJP2 at the XbaI/HindIII sites to make plasmids pLB597 (sitAp-gusA) and pLMB600 (mntHp-gusA). Plasmids pLB597 and pLMB600 were then conjugated into Rlv3841 to make LMB498 and LMB505 respectively. The presence of the plasmids was confirmed with pJP2 mapping primers p611 and pr0096.

To detect expression of sitAp-gusA and mntHp-gusA in planta, nodules taken from plants three weeks post inoculation were sectioned with a vibratome and incubated in staining buffer containing 0.025% 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Lodwig et al., 2004). After eighteen minutes, nodule sections were fixed in 1.25% glutaraldehyde and visualised under a Leica DM6000 light microscope.

When detecting expression of sitAp-gusA and mntHp-gusA in free-living cells in response to MnSO₄ levels, strains were grown to an OD₆₀₀ 1–1.2 in modified AMS glucose containing either 0.05 μM or 0.9 μM MnSO₄. Cultures were sampled and β-glucuronidase (GUS) activity measured as described (Lodwig et al., 2004). When detecting expression of sitAp-gusA and mntHp-gusA in free-living cells in response to oxidative stress, cells were cultured in AMS glucose to an OD₆₀₀ 0.2–0.4. The culture was split, where 100 μM H₂O₂ was added to one and 0 μM was added to the other. Samples were taken at 0, 2, 4 and 6 h and used to measure GUS activity.

**Growth assays**

Growth of Rlv3841, single and double mutants was tested in 96-well plates. Strains were pre-cultured in AMS or modified AMS glucose (containing 25 μM MnSO₄) in the case of the double mutant, to an OD₀₀₀ 0.2–0.6. Cultures were then split, spun down and resuspended in modified AMS (omitting MnSO₄). This washing step was repeated twice to remove extracellular traces of MnSO₄. After the final-washing step, strains were suspended in modified AMS glucose containing 0.05 μM or 25 μM MnSO₄ to an OD₀₀₀ 0.1. Samples were then transferred to a 96-well plate and measured at OD₀₀₀ by a BioTek EON™ plate reader. Growth was measured for 24 h at 30 min intervals, with linear shaking.

Growth of Rlv3841, RlvA34, Rlp4292 and their corresponding double mutants were tested in conical flasks. Strains were first grown on TY slopes or in the case of the double mutants, on TY supplemented with 50 μM MnSO₄. After two days, slopes were washed with 5 ml modified AMS (omitting MnSO₄) to obtain a bacterial suspension that was used inoculate modified AMS glucose containing 0.05 μM or 10 μM MnSO₄ to a starting ~OD₀₀₀ 0.005. After 14 h of growth,
samples were taken every 3–4 h and used to measure OD<sub>600</sub>. Note that 25 μM MnSO₄ (used in the 96-well plate assays) was inhibitory to Rlv3841 when cultured in conical flasks, hence 10 μM MnSO₄ was used to restore growth of the double mutant in this assay.

**H₂O₂ sensitivity assay**

To measure H₂O₂-sensitivity, strains were first pre-cultured in AMS glucose to stationary phase (OD<sub>600</sub> 0.9–1.1). Cultures were then pelleted by centrifugation, washed three times in sodium hypochlorite, washed extensively with sterile distilled water and sown into in 1 L pots containing autoclaved vermiculite (only leaving seeds in sterile H₂O for 5 seconds). These seeds were then placed onto 3% w/v H₂O-agar and grown in a controlled growth room at 22°C, with a 16 h light/8 h dark cycle and then harvested three weeks later. Root nodules were sectioned and then stained with toluidine blue. Stained sections were visualised under a Leica DM6000 light microscope. For electron microscopy, ultrathin sections were taken and stained with uranyl acetate and lead citrate as previously described (Lodwig et al., 2003).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Growth curves of the double sitA mntH mutant complemented with pJP2(sitA) or pJP2(sitABCD). Rlv3841 (diamonds), double mutant sitA mntH (triangle), double mutant complemented with pJP2(sitA) and double mutant complemented with pJP2(sitABCD) were grown in media limited (0.05 mM) or not limited (25 mM) for MnSO4. Averaged from three independent experiments. For clarity only plus SEM bars are shown at 4 h intervals.

Fig. S2. GusA activity in strains expressing sitAp-gusA and mntHp-gusA in response to H2O2 and in absence of OxyR. Gus activity in Rlv3841 and the oxyR mutant carrying either sitAp-gusA (a) or mntHp-gusA (b). Measured in the absence or presence of H2O2 (100 μM). Averaged from three independent experiments ± SEM.

Table S1. Primers used in this study.