MgtE From *Rhizobium leguminosarum* Is a Mg^{2+} Channel Essential for Growth at Low pH and N_2 Fixation on Specific Plants

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MgtE is predicted to be a *Rhizobium leguminosarum* channel and is essential for growth when both Mg^{2+} is limiting and the pH is low. N_2 was only fixed at 8% of the rate of wild type when the crop legume *Pisum sativum* was inoculated with an *mgtE* mutant of *R. leguminosarum* and, although bacteroids were present, they were few in number and not fully developed. *R. leguminosarum* MgtE was also essential for N_2 fixation on the native legume *Vicia hirsuta* but not when in symbiosis with *Vicia faba*. The importance of MgtE and the relevance of the contrasting phenotypes is discussed.

Rhizobia are α -proteobacteria that form symbioses principally with plants of the legume family. They induce the formation of root nodules on plants, providing appropriate conditions, such as the correct nutrient environment and low oxygen concentration, to drive the differentiation of rhizobia into N_2 -fixing specialists (bacteroids). To establish a root nodule, a highly specific exchange of signaling compounds is required, i.e., rhizobia attach to root hairs and grow down plant-derived infection threads into the root cortex (Oldroyd and Downie 2004; Terpolilli et al. 2012; Udvardi and Poole 2013). During their release from infection threads, rhizobia become surrounded by a plant-derived membrane (peribacteroid membrane), creating a controlled and optimal environment for N_2 fixation to occur (Day et al. 2001). The enzyme responsible for N_2 fixation is nitrogenase, which requires at least sixteen molecules of magnesium-ATP ($MgATP$) [$N_2 + 8H^+ + 16 MgATP + 8e^- \rightarrow 2NH_3 + H_2 + 16 MgADP + 16Pi$] (Seefeldt et al. 2009). Despite this essential role of Mg^{2+} , very little is known about how this cation is transported into the bacteroid during symbiosis.

Random mutagenesis of *Rhizobium leguminosarum* bv. *viciae* 3841 (Rlv3841), followed by mass-screening on its legume-host *Pisum sativum* (pea), identified several mutants defective for symbiosis (Karunakaran et al. 2009). One such mutant was found to carry a mutation in a gene encoding a putative MgtE channel (*mgtE*, locus tag RL1461). MgtE channels are highly selective for Mg^{2+} and contain cystathionine β -synthase domains that are proposed to regulate an ion-conducting pore in response to intracellular Mg^{2+} levels (Hattori et al. 2009; Ishitani et al. 2008).

Knowing the importance of Mg^{2+} to N_2 fixation and our lack of understanding of how it is transported during symbiosis, the primary aims of this investigation were to ascertain whether

Rlv3841 MgtE is a Mg^{2+} channel and explore its role in free-living cells. We then sought to examine the symbiotic phenotype of the *mgtE* mutant more closely and understand the role of Mg^{2+} during bacteroid development. Finally, the *mgtE* mutant was screened on other legumes within the host range of Rlv3841, to determine whether differences in the nodule environment can influence the requirement for MgtE.

RESULTS

The *mgtE* gene encodes a Mg^{2+} channel.

To determine if MgtE is permeable to Mg^{2+} , *mgtE* from Rlv3841 was expressed in an *E. coli* triple knockout mutant ($\Delta mgtA\Delta corA\Delta yhiD$) that can only grow on Luria Bertani (LB) when supplemented with 100 mM $MgSO_4$ (Hattori et al. 2009). Once amplified from the Rlv3841 genome (Young et al. 2006), *mgtE* was cloned downstream of and in the same orientation as a *lac* promoter in a broad-host-range vector. When introduced into the *E. coli* mutant, *mgtE* fully restored growth on non-supplemented LB (Fig. 1). In contrast, growth was not restored by the introduction of the empty vector or *mgtE* cloned in the reverse orientation of the *lac* promoter (Fig. 1). This confirms that the putative MgtE channel from Rlv3841 promotes Mg^{2+} accumulation.

To see whether MgtE is permeable to other cations, disk assays were used to measure the sensitivity of the Rlv3841 *mgtE* mutant (Karunakaran et al. 2009) to a range of metals at toxic concentrations. These assays revealed that the *mgtE* mutant is more resistant than Rlv3841 to toxic concentrations of $CoCl_2$ (Fig. 2), suggesting that MgtE also has some permeability to Co^{2+} . However, the *mgtE* mutant was not changed relative to Rlv3841 in resistance to $NiSO_4$ or $ZnCl_2$ (Fig. 2). These findings agree with the in vitro patch-clamp experiments conducted on MgtE from *Thermus thermophilus*, in which the channel was only found to be permeable to Mg^{2+} and to a lesser extent to Co^{2+} (Hattori et al. 2007). A study on *Bradyrhizobium japonicum* found that certain gain-of-function mutations affecting the gating mechanism of MgtE can also induce Mn^{2+} -permeability (Hohle and O'Brian 2014).

The *mgtE* mutant grows poorly at low pH when Mg^{2+} is limited.

Routine growth in nutrient-rich tryptone yeast (TY) medium did not show the *mgtE* mutant to have any obvious growth phenotype. To determine if there is any requirement for MgtE in free-living cells, therefore, growth of the *mgtE* mutant was studied in a defined minimal medium. Acid minimal salts (AMS) were modified for this purpose by altering the level of

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MgSO₄ to a limiting (0.01 mM) or nonlimiting (2 mM) concentration. As expected, there were no strong differences between the growth of Rlv3841 and the *mgtE* mutant in AMS glucose containing nonlimiting levels of MgSO₄ (Fig. 3). However, there were also no strong differences between the two strains when levels of MgSO₄ were limited (Fig. 3).

Analysis of the Rlv3841 genome (Young et al. 2006) offered a likely explanation for why no obvious growth phenotype was observed in this initial experiment. Rlv3841 encodes several other putative importers for Mg²⁺, including a second MgtE homolog (RL2551) and three copies of a gene encoding another type of putative Mg²⁺ channel, CorA (RL0921, RL0964, and pRL120701). Indeed, studies on other prokaryotes have also reported that more than one Mg²⁺ importer has to be eliminated in order to cause a growth defect (Hattori et al. 2009; Hmiel et al. 1989; Snively et al. 1989). What is unusual about Rlv3841, however, is that the mutation of *mgtE* alone has a severe impact on its ability to fix N₂ on *P. sativum* (Karunakaran et al. 2009).

Knowing the importance of MgtE during symbiosis, we hypothesized that conditions within the nodule may heighten the requirement for MgtE. The strongest candidate was pH, because it is known to affect the functionality of other transporters (Hicks et al. 2003; Hirano et al. 2011; Lü et al. 2011; Mahmood et al. 2009), and rhizobia are exposed to low pH in the peribacteroid space during symbiosis (Pierre et al. 2013). Therefore, instead of adjusting the pH to the routine 7.0, Rlv3841 and the *mgtE* mutant was tested in modified AMS medium at pH 5.75. At pH 5.75, growth of the *mgtE* mutant was greatly reduced in limiting MgSO₄, as compared with Rlv3841 (Fig. 3). However, when MgSO₄ was not limiting, the *mgtE* mutant grew at rates similar to Rlv3841 (Fig. 3).

To be sure that the change in pH was the cause of the growth defect, the experiment was repeated with the addition of MES buffer (pKa 6.1), which has an effective buffering range of pH 5.5 to 6.7. Once again, at low pH, the *mgtE* mutant showed poor growth in limiting MgSO₄ (Fig. 3). Measurements of pH before and after growth tests confirmed no significant changes were occurring. To confirm that MgtE is essential for growth at low

pH in limiting Mg²⁺, the plasmid-expressed copy of *mgtE* (pJP2*mgtE*) was shown to fully complement the low pH growth phenotype of the *mgtE* mutant (Fig. 3).

MgtE is required for bacteroid development.

The *mgtE* mutant was originally identified because it was symbiotically defective on *P. sativum* (Karunakaran et al. 2009). However, due to the large number of mutants being tested in the same screen, it was not possible to investigate its

Fig. 2. Sensitivity of Rlv3841 and the *mgtE* mutant to toxic concentrations of CoCl₂, NiSO₄, and ZnCl₂. Disc assays were used to measure the sensitivity of Rlv3841 and the *mgtE* mutant (*mgtE*) to 50 mM CoCl₂, 50 mM NiSO₄, and 100 mM ZnCl₂. Each assay was done in triplicate and average measurements are shown.

Fig. 1. Complementation of *Escherichia coli* triple knockout strain with Rlv3841 *mgtE*. The *E. coli* triple knockout strain (MgKO) grew on Luria Bertani (LB) medium supplemented with 100 mM MgSO₄ but did not grow without the supplement. Rlv3841 *mgtE* under the control of the *lac* promoter (*plac-mgtE*) restored growth of the MgKO mutant on nonsupplemented LB. Neither the empty vector nor the plasmid containing *mgtE* in the reverse orientation restored growth.

phenotype in further detail. A more comprehensive analysis of the symbiotic phenotype was, therefore, required in order to fully understand the role of MgtE in N₂ fixation and bacteroid development. The *mgtE* mutant was confirmed to induce small and white nodules on *P. sativum*, in contrast to the elongated,

Fig. 3. Growth of Rlv3841 and the *mgtE* mutant with different levels of MgSO₄ and pH. Rlv3841 (diamonds), *mgtE* mutant (triangles), and *mgtE* mutant complemented with pJP2*mgtE* (squares) were grown in modified acid minimal salts glucose containing 0.01 mM or 2 mM MgSO₄ adjusted to pH 7.0 or pH 5.75. Experiments were conducted both in the absence (triplicate) and presence (duplicate) of MES buffer. Average values are shown $\pm$ standard error of the mean.

pink nodules induced by Rlv3841. Dry weight measurements showed that nodules induced by the *mgtE* mutant weighed $<50\%$ that of nodules induced by Rlv3841 (4.69 ± 0.1 compared with 10.51 ± 0.2 mg per plant, $n = 48$ plants).

Only low levels of acetylene reduction could be detected for nodules induced by the *mgtE* mutant, 8% the rate of Rlv3841, suggesting that the mutant is severely defective for N₂ fixation (Fig. 4). Acetylene reduction rates returned to wild-type levels when the *mgtE* mutant was complemented with pJP2*mgtE* (Fig. 4). The presence of some N₂ fixation, however, did suggest that

Fig. 4. Acetylene reduction rates for plants inoculated with Rlv3841 or the *mgtE* mutant. Rates of acetylene reduction were measured for *Pisum sativum*, *Vicia hirsuta*, and *V. faba* inoculated with Rlv3841, *mgtE* mutant, or *mgtE* complemented with plasmid pJP2*mgtE*. Average values shown $\pm$ standard error of the mean (SEM). The 100% rates of acetylene reduction for *P. sativum*, *V. hirsuta*, and *V. faba* were, respectively, 6.82 ± 0.2 , 0.67 ± 0.05 , and 5.78 ± 1.0 μ moles per hour per plant $\pm$ SEM.

Fig. 5. Sections of *Pisum sativum* nodules induced by Rlv3841 or the *mgtE* mutant. Nodules colonized by either Rlv3841 or *mgtE* mutant were sectioned and stained with toluidine blue and were visualized under a light microscope.

a proportion of the *mgtE* mutant population is released from the infection threads and goes on to infect plant cells. Examination of nodule sections confirmed this (Fig. 5), but there were also a large number of uninfected cells in contrast to the few that were seen in nodules containing Rlv3841 (Fig. 5). The low number of infected cells was confirmed by measuring the bacteroid dry weight per milligram of nodule dry weight; the collective weight of the *mgtE* mutant was approximately 30% less than the collective weight of Rlv3841 (162 ± 26 compared with 247 ± 3 $\mu\text{g}/\text{mg}$, $n = 48$). Examination of ultrathin nodule sections using transmission electron microscopy revealed that the *mgtE* mutant does produce the classical branch-shape bacteroid morphology, but it also revealed (Fig. 6) that many of these bacteroids contain numerous granules of poly- β -hydroxybutyrate, which usually signifies excess carbon that cannot be used for N_2 fixation.

To test whether the absence of MgtE just delays or indefinitely precludes the onset of N_2 fixation, plants were grown for a longer period of six weeks. After six weeks, *P. sativum* inoculated with *mgtE* mutant looked similar to the uninoculated plants and the shoots weighed a third of *P. sativum* inoculated with Rlv3841 (Fig. 7A). This confirms that the absence of MgtE prevents nitrogen fixation.

The *mgtE* mutant is symbiotically defective on *V. hirsuta* but not *V. faba*.

After characterizing the defective symbiosis between the *mgtE* mutant and *P. sativum*, the requirement for MgtE on other plant hosts was investigated. The wild legume *V. hirsuta* and the crop legume *V. faba* were used to achieve this, as they both lie within the host range of Rlv3841. As with *P. sativum*, on *V. hirsuta*, the *mgtE* mutant exhibited severely reduced rates of acetylene reduction (Fig. 4) and, consequently, the plants showed signs of chlorosis.

However, when *V. faba* was inoculated with the *mgtE* mutant, healthy pink, elongated nodules were formed and rates of

acetylene reduction equaled those of Rlv3841 (Fig. 4). After 3 weeks, the dry weights of nodules occupied by either the *mgtE* mutant or Rlv3841 were similar (8.29 ± 2.3 compared with 9.35 ± 2.6 mg per plant, $n = 48$ plants) as were the dry weights of the bacteroids (277.5 ± 12.4 compared with 277.4 ± 6.2 μg of nodule dry weight per milligram). Even after a growth period of 6 weeks, *V. faba* inoculated with the *mgtE* mutant could not be distinguished from *V. faba* inoculated with Rlv3841, either in appearance or dry weight (Fig. 7B).

DISCUSSION

MgtE is essential for N_2 fixation on *P. sativum* and *V. hirsuta* but is nonessential for N_2 fixation on *V. faba*. It is unlikely that there is a difference in the requirement for Mg^{2+} , as it is essential for N_2 fixation and many other cellular processes (Moomaw and Maguire 2008; Seefeldt et al. 2009; Smith and Maguire 1998). One possible explanation is that there is a difference in the bioavailability of Mg^{2+} . Bacteroids are enclosed within the peribacteroid membrane, so if bioavailability is the reason, it would be caused by differing levels of Mg^{2+} being transported across this membrane and into the peribacteroid space. To this date, no plant transporter for Mg^{2+} has been located in the peribacteroid membrane, but the number of plant transporters important to symbiosis is growing (Hakoyama et al. 2012; Kaiser et al. 2003; Krusell et al. 2005; Moreau et al. 2002; Takanashi et al. 2013).

Differences in nodule environment could also influence the requirement of MgtE. It was demonstrated that free-living cells require MgtE at low but not at neutral pH (Fig. 3), and so, a difference in pH between the peribacteroid space of *P. sativum*

Fig. 6. Electron micrographs of nodules harvested from *Pisum sativum*. As compared with **A** and **C**, Rlv3841, **B** and **D**, poly- β -hydroxybutyrate is much more abundant in *mgtE* mutant, indicating excess carbon in the mutant bacteroids.

Fig. 7. *Pisum sativum* and *Vicia faba* inoculated with Rlv3841 or the *mgtE* mutant. **A**, *P. sativum* and **B**, *V. faba* were inoculated with Rlv3841 or the *mgtE* mutant alongside an uninoculated control. Photos were taken after six weeks. Shoot dry weights were averaged from 10 plants ($\text{g}^{-1} \pm$ standard error of the mean).

and *V. faba* nodules would explain the contrasting phenotypes. It is not known how pH affects the requirement of MgtE, but it is possible that, at low pH, MgtE is the only Mg^{2+} channel in an open conformation. Although pH-dependent gating has never been seen for MgtE, changes in pH have been shown to influence gating of the potassium channel KcsA through dictating the charge of amino acids located between both cytoplasmic and transmembrane domains (Hirano et al. 2011). To this date, pH has only been measured in the peribacteroid space of *Medicago truncatula* nodules (Pierre et al. 2013). Acidification of the peribacteroid space is an important part of symbiosis and is driven by plant-encoded H^+ ATPases located in the peribacteroid membrane (Catalano et al. 2004; Fedorova et al. 1999; Saalbach et al. 2002; Wienkoop and Saalbach 2003). It is necessary to both energize the peribacteroid membrane and protonate NH_3 to NH_4^+ , preventing its reuptake once it has left the bacteroid and allowing it to be exported into the plant cytosol through a NH_4^+ channel (Day et al. 2001; Tyerman et al. 1995). This acidification would clearly put pressure on bacteroids to adapt to a low pH environment and make components like MgtE critical for N_2 fixation.

This is the first report of a Mg^{2+} channel in rhizobia having a role in symbiosis. It also adds to a growing list of mutants that exhibit defective symbiosis with some but not all compatible legumes (Miller et al. 2007; Karunakaran et al. 2010; Koch et al. 2010; Lindemann et al. 2010; Osterås et al. 1991; Zhang et al. 2012) highlighting how the nodule environment can differ between legumes and how these differences can influence the host range of bacteroids.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions.

Strains and plasmids used in this study are detailed in Table 1. *Escherichia coli* strains were grown at 37°C in LB broth or on LB agar. LB was supplemented with 100 mM $MgSO_4$ when growing the *E. coli* triple knockout strain (Hattori et al. 2009). *R. leguminosarum* strains were grown at 28°C in either TY extract (Beringer 1974) or AMS supplemented with 10 mM glucose and 10 mM NH_4Cl (Poole et al. 1994a). Antibiotics were used at the following concentrations ($\mu g/ml$): neomycin, 80; spectinomycin, 100 (50 for *E. coli*); streptomycin, 500; and tetracycline, 2.

Cloning and conjugations.

To study *mgtE* in the *E. coli* triple knockout strain, a 1.6-kb fragment containing *mgtE* was amplified from Rlv3841 genomic DNA and was cloned into pJET1.2/blunt (pLMB553). A *Bgl*II-digested fragment from pLMB553 (containing *mgtE*) was cloned into the broad-host-range vector pRK415 (Keen et al. 1988), in the same orientation and downstream of the *lac* promoter, resulting in the plasmid pLMB562 (*plac-mgtE*). The same *Bgl*II-digested fragment was also cloned into pRK415 in the reverse orientation, resulting in pLMB565 (*mgtE*). The *E. coli* triple knockout strain was transformed with empty pRK415 (LMB469), pLMB562 (LMB470), or pLMB565 (LMB471).

For in planta experiments and growth complementation assays, the 1.6-kb fragment containing Rlv3841 *mgtE* was also cloned into the environmentally stable plasmid pJP2 (Prell et al. 2002). First, this fragment was cloned into pJET1.2/blunt (pLMB569). An *Xba*I/*Kpn*I-digested fragment from pLMB569 (containing *mgtE*) was then cloned into pJP2, yielding the plasmid pLMB576 (pJP2*mgtE*). This plasmid was conjugated from *E. coli* into *R. leguminosarum* strains, using the helper plasmid pRK2013, as previously described (Poole et al. 1994b).

Disk assays.

Strains were grown on TY agar slopes and after 2 days of growth, the slopes were washed with TY broth to obtain a bacterial suspension at an optical density at 600 nm (OD_{600}) = 0.2. A volume of 200 μl was taken from this suspension and was used to inoculate 3 ml of soft TY agar (0.9% [wt/vol]) incubated at 42°C. Immediately after inoculation, the soft agar was poured over solid TY agar (1.75% [wt/vol]) and was left to solidify at room temperature. After 30 min, sterile filter disks were placed on top of the soft TY agar and 15 μl of $CoCl_2$ (50 mM), $NiSO_4$ (50 mM), and $ZnCl_2$ (100 mM) were added to individual disks. After 2 days of growth, the zone of growth inhibition was measured for each metal.

Growth experiments.

Strains were grown on TY agar slopes and, after 2 days of growth, were washed with 5 ml of AMS (omitting $MgSO_4$) to obtain a bacterial suspension. This bacterial suspension was used to inoculate 50 ml of modified AMS containing either 0.01 mM or 2 mM $MgSO_4$ at an initial OD_{600} = 0.01. After 14 h of growth, samples were taken every 3 h and were used for

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Source or Reference
Plasmids		
pJET 1.2/Blunt	Polymerase chain reaction product cloning vector; Ap ^r	Thermo Scientific
pRK2013	mob ⁺ ; Km ^r	Ditta et al. 1980
pRK415	Broad-host-range plasmid; Tc ^r	Keen et al. 1988
pJP2	Stable broad-host-range cloning vector; Tc ^r	Prell et al. 2002
pLMB553	<i>mgtE</i> cloned into pJET; Ap ^r	This work
pLMB562	<i>mgtE</i> cloned into pRK415 in correct orientation of the <i>lac</i> promoter (<i>plac-mgtE</i>); Tc ^r	This work
pLMB565	<i>mgtE</i> cloned into pRK415 in incorrect orientation of the <i>lac</i> promoter; Tc ^r	This work
pLMB569	<i>mgtE</i> cloned into pJET; Ap ^r	This work
pLMB576	<i>mgtE</i> cloned into pJP2; Tc ^r	This work
Rhizobium leguminosarum		
Rlv3841	Str ^r derivative of Rlv300	Johnston and Beringer 1975
RU4107	Rlv3841 <i>mgtE</i> ::mTn5 (<i>mgtE</i> mutant); Nm ^r	Karunakaran et al. 2009
LMB481	pLMB576 (pJP2 <i>mgtE</i>) conjugated into RU4107; Nm ^r Tc ^r	This work
Escherichia coli		
MgKO strain ^b	$\Delta mgtA\Delta corA\Delta yhiD$	Hattori et al. 2009
LMB469	MgKO strain transformed with empty pRK415; Tc ^r	This work
LMB470	MgKO transformed with pLMB562 (<i>plac-mgtE</i>); Tc ^r	This work
LMB471	MgKO transformed with pLMB565 (<i>mgtE</i>); Tc ^r	This work

^a Ap^r, Km^r, Tc^r, Str^r, and Nm^r, = resistance to ampicillin, kanamycin, tetracycline, streptomycin, and neomycin, respectively.

^b An *E. coli* triple knockout strain.

OD₆₀₀ measurements. When measuring growth at low pH, pH of AMS was adjusted to 5.75. Growth experiments were conducted in the presence of MOPS (20 mM) buffer alone and MOPS plus MES (20 mM) buffer. To confirm that pH remained stable, it was measured before and after the experiment.

Plant experiments.

P. sativum cv. Avola and scarified *V. faba* cv. Sutton seeds were surfaced-sterilized (70% EtOH and 2% sodium hypochlorite) and were sown directly into in 1-liter pots containing autoclaved vermiculite and 400 ml of N-free rooting solution (Poole et al. 1994a). *V. hirsuta* seeds were scarified and surface-sterilized with 1% sodium hypochlorite. These seeds were then placed onto 3% wt/vol H₂O agar and were kept in the dark to germinate. After 2 days, germinated seeds were sown into 1-liter pots containing autoclaved vermiculite and N-free rooting solution. Plants were inoculated with 10⁶ CFU of *R. leguminosarum*, grown in a controlled growth room at 22°C with a 16-h light cycle and were then harvested 3 weeks postinoculation (wpi). Acetylene reduction by nodules was determined in 95% air and 5% acetylene for 1 h in 250-ml Schott bottles, as described by Hardy et al. (1973) and Trinick et al. (1976).

To determine shoot dry weights, plants were grown in 2-liter pots and were harvested 6 wpi. Shoots were dried at 60°C for 3 days. To measure bacteroid and nodule dry weights, nodules were collected 3 wpi, were macerated in 10 mM phosphate buffer (pH 7.0), were centrifuged at low speed for 5 min to remove traces of vermiculite and plant debris and, then, at high speed (4,000 rpm) to separate the bacteroids (pellet) from the plant cytosol (supernatant). Both fractions were dried overnight at 100°C and, then, were weighed (nodule dry weight equivalent to combined weights of bacteroid and plant cytosolic fractions).

Root nodules were sectioned and were then stained with toluidine blue. Stained sections were visualized under a Leica DM6000 light microscope. For electron microscopy, ultrathin sections were taken and stained with uranyl acetate and lead citrate (Lodwig et al. 2005).

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