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Thiamine Is Synthesized by a Salvage Pathway in *Rhizobium leguminosarum* bv. viciae Strain 3841

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In the absence of added thiamine, *Rhizobium leguminosarum* bv. viciae strain 3841 does not grow in liquid medium and forms only "pin" colonies on agar plates, which contrasts with the good growth of *Sinorhizobium meliloti* 1021, *Mesorhizobium loti* 303099, and *Rhizobium etli* CFN42. These last three organisms have *thiCOGE* genes, which are essential for de novo thiamine synthesis. While *R. leguminosarum* bv. viciae 3841 lacks *thiCOGE*, it does have *thiMED*. Mutation of *thiM* prevented formation of pin colonies on agar plates lacking added thiamine, suggesting thiamine intermediates are normally present. The putative functions of ThiM, ThiE, and ThiD are 4-methyl-5-(β-hydroxyethyl) thiazole (THZ) kinase, thiamine phosphate pyrophosphorylase, and 4-amino-5-hydroxymethyl-2-methyl pyrimidine (HMP) kinase, respectively. This suggests that a salvage pathway operates in *R. leguminosarum*, and addition of HMP and THZ enabled growth at the same rate as that enabled by thiamine in strain 3841 but elicited no growth in the *thiM* mutant (RU2459). There is a putative *thi* box sequence immediately upstream of the *thiM*, and a *gfp-mut3.1* fusion to it revealed the presence of a promoter that is strongly repressed by thiamine. Using fluorescent microscopy and quantitative reverse transcription-PCR, it was shown that *thiM* is expressed in the rhizosphere of vetch and pea plants, indicating limitation for thiamine. Pea plants infected by RU2459 were not impaired in nodulation or nitrogen fixation. However, colonization of the pea rhizosphere by the *thiM* mutant was impaired relative to that of the wild type. Overall, the results show that a thiamine salvage pathway operates to enable growth of *Rhizobium leguminosarum* in the rhizosphere, allowing its survival when thiamine is limiting.

Rhizobia form a species-specific symbiotic relationship with leguminous plants in which atmospheric N_2 is reduced to NH_3. To establish this symbiosis, bacteria must survive in the soil environment, competing with many organisms for nutrients. The availability of vitamins such as biotin, thiamine, and riboflavin in the rhizosphere limits the growth of *Sinorhizobium meliloti* (48). Auxotrophy for vitamins has also been described as the cause of ineffective nodule formation by some rhizobial strains, but effective module formation can be restored by adding the vitamins externally (46). Related to this, *Mesorhizobium loti* strains found in soil are often auxotrophic for the vitamins biotin, thiamine, and nicotinate and are converted to prototrophy by the acquisition of a symbiotic island (51).

Several metabolic processes, such as the synthesis of polyhydroxybutyrate and the excretion of amino acids and organic acids, are strongly affected by the absence of the vitamins biotin and thiamine (14). Soluble vitamins such as niacin, thiamine, riboflavin, pantothenic acid, and biotin are liberated from legume roots and are also produced at biologically active levels by many bacteria and fungi isolated from rhizosphere soil or the plant root surface (50). The production of these water-soluble vitamins by rhizospheric microorganisms such as *Pseudomonas* and *Azospirillum* spp. has been found to be related to the ability of these bacteria to enhance nitrogen fixation and the growth of legumes nodulated by *Rhizobium* (11, 42). External addition of biotin greatly enhances the bacterial growth and colonization of alfalfa roots by *Sinorhizobium meliloti* (15, 49). Overall, this suggests that vitamin supply may limit the growth of rhizobia in the rhizosphere and soil.

Thiamine (vitamin B1) is an essential cofactor required for carbohydrate and branched-chain amino acid metabolism. It is derived from thiamine monophosphate (TMP), the synthesis of which involves a complex multistep pathway (5). In the case of *R. etli*, the *thiCOGE* genes, present on the plasmid pRetCFN42b, have been shown to be essential for the de novo synthesis of thiamine (31). Several bacteria, including *Escherichia coli* and *Bacillus subtilis*, have a set of salvage kinases in addition to this method of de novo synthesis. These utilize dephosphorylated intermediates of the thiamine biosynthetic pathway present in the environment for TMP synthesis (30) (Fig. 1). The formation of TMP by one of these salvage pathways involves the condensation of two intermediates: 4-methyl-5-(β-hydroxyethyl) thiazole monophosphate (THZ-P) and 4-amino-5-hydroxymethyl-2-methyl pyrimidine pyrophosphate (HMP-PP) (34, 56). This condensation step is mediated by the ThiE protein (thiamine phosphate synthase). HMP-PP is derived from 4-amino-5-hydroxymethyl-2-methyl pyrimidine (HMP) by phosphorylation by the bifunctional HMP kinase/HMP monophosphate kinase, ThiD (43). THZ-P is derived from 4-methyl-5-(β-hydroxyethyl) thiazole (THZ) kinase by phosphorylation by the 4-methyl-5-(β-hydroxyethyl) thiazole kinase, ThiM (43).

We report here that the genome of *Rhizobium leguminosarum* genome lacks *thiCOGE* but has *thiMED* genes on plasmid pRL11JI (pRL110441-110443) (55). ThiMED catalyze a salvage pathway that operates under thiamine limitation in the rhizosphere. Some rhizobia exclusively use the de novo ThiCOGE pathway; others, such as strain 3841, use only the ThiMED salvage pathway, while *R. etli* strain CFN42 uses both.

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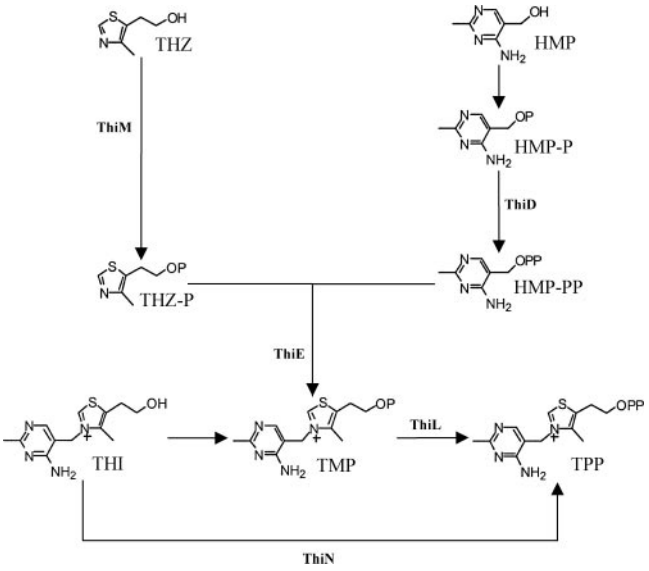

FIG. 1. Proposed thiamine salvage pathway in *R. leguminosarum* bv. viciae 3841. THZ and HMP are intermediates in a salvage pathway. The conversion of thiamine (THI) to TPP may be catalyzed by ThiN (RL4610), which has 25% amino acid identity and 41% similarity to ThiN from *Bacillus subtilis*. The step from TMP to TPP is catalyzed by ThiL in *E. coli* but is undefined in *R. leguminosarum*. HMP-P, HMP monophosphate.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are detailed in Table 1. *Rhizobium* strains were grown at 28°C on either tryptone-yeast extract (TY) (6), standard acid minimal salts medium (AMS), or acid minimal salts agar (37) with 10 mM D-glucose and 10 mM ammonium chloride. The only exception was *S. meliloti*, for which the EDTA level in AMS was reduced to 1 μM. Antibiotics were used at the following concentrations (μg ml^{-1}): streptomycin, 500; kanamycin, 20; tetracycline (Tet), 2 (in AMS) and 5 (in TY); gentamicin (Gm), 20; nyastatin, 50; and spectinomycin (Sp), 100. The concentrations of thiamine and its intermediate HMP (kindly provided by T. P. Begley, Cornell University, Ithaca NY) and 4-methyl-5-(β-hydroxyethyl) thiazole (Sigma-Aldrich) were 1 μg ml^{-1}.

Genetic modification of bacterial strains. All DNA cloning and analysis were performed as previously described (45). The DNA fragment containing the *thiM* promoter was PCR amplified using primers p639 and p640. PCRs were conducted in 50 μl, using 2.5 units of *Pfu* Turbo (Stratagene), 10 to 30 ng of genomic DNA, 1× PCR buffer (Stratagene), 0.2 mM deoxynucleoside triphosphates, and 1 μM primers. The cycling conditions were as follows: 1 cycle of 95°C for 2 min; 30 cycles of 95°C for 45 s, 57°C for 45 s, and 72°C for 2 min; and a final extension of 72°C for 10 min. The PCR product was cloned into the broad-host-range vector pRU1097 D-TOPO, resulting in plasmid pRU1619. Plasmid pRU1097 was custom adapted with topoisomerase by Invitrogen and was used according to their standard protocols for TOPO-adapted vectors.

Primers used in this study. Primers used in this study were as follows: p639, CACCAACGACGAGTTCGGGGCGAT; p640, GAACCTTCCGCCTGTTCGAC; p641, AAGCACGTCAGGGCTCTCGT; p753, TCTAGAATGTCGCGGTGGTCAGTCAGCGC; p754, AAGCTTTTCGCCAAAGAGATCAAGCCGGG; p755, TCTAGAAGGTCTGGCACCTGTCACTCCTCC; p789, GTTCTCTTCGACATCGCGGACGGC; p790, GACCTTGAGGTTGATGCCGAGAAG; p827, CGCCTGCATGCCGTCGATCC; and p828, CGCCGCAAATGTCCTGCTCG.

Construction of a *thiM* mutant in strain 3841. Primers p639 and p641 were used to PCR amplify the *thiM* region from *R. leguminosarum* 3841 genomic DNA, and the 2.4-kb PCR product was cloned into the vector pCR8-GW-TOPO (Invitrogen), producing plasmid pRU1734. An ΩTet cassette from pHP45 (38) was cloned into the FspI site of *thiM* in pRU1734 to produce pRU1755. The insert in pRU1755 containing the ΩTet cassette in *thiM* was Gateway cloned using Gateway LR clonase enzyme mix (Invitrogen) into pGW1, resulting in plasmid pRU1774. Plasmid pGW1 was made by inserting the Gateway cassette reading frame A into the SmaI site of pJQ200SK. To enable compatibility with subsequent plasmids used in complementation experiments, the ΩTet cassette in pRU1774 was replaced by an ΩSp cassette by SmaI digestion and religation to produce plasmid pRU1776. Plasmid pRU1776 was conjugated into strain 3841, and a *thiM* mutant was isolated by selecting for recombination using the *sac* mutagenesis strategy as previously described (24).

Primers p753/p754 and p754/p755 were used to amplify the *thiME* and *thiMED* genes from strain 3841. The PCR products were cloned into pCR2.1TOPO (pRU1738 and pRU1752, respectively), digested with HindIII/XbaI, and then cloned into pRK415-1, resulting in plasmids pRU1783 and pRU1784, respectively. All plasmids were conjugated into rhizobial strains by using pRK2013 as a helper plasmid to provide the transfer genes as previously described (35).

Measurement of reporter fusion activity. Green fluorescent protein fluorescence was measured using a Tecan GENios fluorometer equipped with an excitation filter (485 nm) and an emission filter (510 nm). Cells of strain 3841 containing the plasmids pRU1515 (self-ligated vector) and pRU1619 (p*thiM*::*egfp*) were grown overnight in AMS supplemented with 10 mM glucose and 10 mM ammonium chloride and thiamine (1 μg/ml). The cells were harvested and washed in AMS three times to eliminate most entrained thiamine. Cells were then reinoculated into AMS (glucose, 10 mM; ammonium, 10 mM) with the following additions: none (control); thiamine; HMP; THZ; HMP and THZ; and HMP, THZ, and thiamine. Cells were taken at different time points, and the specific fluorescence was measured by dividing the fluorescence of the sample by the optical density at 590 nm of the culture.

Microscopy. Plasmid pRU504 carrying the *thiMED* operon in pOT2 was previously isolated by optical trapping (3). Strain RU1302 (3841 pRU504) was used to study the expression of *thiM* in the rhizosphere. Microscopy was performed with a Carl Zeiss Axioskop 2.0 epifluorescence microscope with appropriate fluorescence sets. Images were captured using an Axiocam digital camera (22).

Plant growth and inoculation. Vetch (*Vicia sativa*) seeds were surface sterilized in 95% ethanol for 30 seconds and then immersed in a solution of 2% sodium hypochlorite for 10 min. The seeds were washed extensively with sterile water and then allowed to germinate on sterile filter paper for 3 days in the dark. Plants were then placed on microscope slides overlaid with 0.75% agarose containing nitrogen-free rooting solution (35). Strain RU1302 was inoculated into the agarose at 10^7 CFU per plant, while control plants were inoculated with strain 3841 containing the vector (pOT2). Coverslips were placed over the agarose, and the microscope slides were inserted into 50-ml Falcon tubes with a few ml of nitrogen-free rooting solution at the bottom. The Falcon tubes were placed in a growth chamber (23°C, 16-h/8-h light/dark period). At 3 to 7 days postinoculation, the plant roots were observed for bacterial *gfp* expression.

Quantitative RT-PCR (Q-RT-PCR) of *thiM*. For measurement of *thiM* expression in free-living *R. leguminosarum* 3841, bacteria were grown in 50 ml of AMS with glucose, ammonium, and thiamine (35) prior to being transferred into thiamine-free medium. For measurement of *thiM* expression in the rhizosphere, 7-day-old pea (*Pisum sativum*) seedlings grown in 25 ml of vermiculite in 50-ml sterile Falcon tubes were inoculated with 10^8 CFU of strain 3841 and grown at 23°C with a 16-h/8-h light/dark period. After 7 days of growth, the bacteria were harvested by adding sterile water (6 ml) plus RNAprotect (12 ml) to the roots, and this was mixed by vortexing for 30 s. The supernatant was filtered through four layers of sterile muslin cloth and spun down at 1,000 rpm in a Microfuge for 1 min at 4°C. The supernatant was further spun down at 8,000 rpm for 5 min to collect the bacteria. To isolate RNA, cells were resuspended in RNAprotect (RNA stabilization reagent) as described by the manufacturer (QIAGEN). RNA was isolated with an RNeasy Mini kit (QIAGEN), and contaminating DNA was removed by on-column treatment with RNase-free DNase (QIAGEN). RNA concentrations were determined with an Experion microfluidic RNA analyzer (Bio-Rad Laboratories). Reverse transcription-PCR (RT-PCR) was performed with a OneStep RT-PCR kit (QIAGEN) as recommended by the manufacturer, with 300 ng of the appropriate RNA sample and with *mdh* (malate dehydrogenase) serving as a reference gene. The data were analyzed by the relative quantification method (comparative cycle threshold method) to calculate the expression (*n*-fold) (9, 10).

Acetylene reduction and dry weight determination. Acetylene reduction was determined for plants incubated in 95% air and 5% acetylene for 1 h in 250-ml Schott bottles (2). For determination of plant dry weight, the shoot was removed from the root and dried in at 70°C in a dry-heat incubator for 3 days before being weighed.

Plant assays. For nodulation competition experiments, pea plants were grown in sterile vermiculite (250-ml flasks) and watered with sterile nitrogen-free rooting solution as described previously (35). An inoculum of 10^6 CFU, confirmed by plate count, was applied to each plant. Plants were harvested 4 weeks postinoculation. To determine nodule occupancy, 120 nodules from 12 plants were surface sterilized as previously described (37), crushed, and plated on TY medium and then on TY medium containing either streptomycin by itself or streptomycin plus spectinomycin.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*)ϕ80d*lacZ*Δ*M15* Δ*lacX74 recA1 ara* Δ*139*Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
E. coli DH5α	F$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1*	Invitrogen
R. leguminosarum bv. viciae		
R. leguminosarum 3841	Str derivative of *R. leguminosarum* bv. viciae strain 300	19
R. leguminosarum VF39	*R. leguminosarum* bv. viciae wild type, Sm^r	39
R. leguminosarum JI336	*R. leguminosarum* bv. viciae	13
R. leguminosarum 248	*R. leguminosarum* bv. viciae	20
R. leguminosarum RBL1309	*R. leguminosarum* bv. viciae	53
R. leguminosarum WU235	*R. leguminosarum* bv. viciae	36
R. leguminosarum strain 3855	*R. leguminosarum* bv. viciae	7
R. leguminosarum 128C53	*R. leguminosarum* bv. viciae	44
R. leguminosarum A34	*R. leguminosarum* bv. viciae (formerly known as 8401/pRL1JI)	12
R. leguminosarum bv. trifolii		
R. leguminosarum bv. W14-2	*R. leguminosarum* bv. trifolii wild type, Sm^r	4
R. leguminosarum bv. RCR5	*R. leguminosarum* bv. trifolii wild type	18
Rhizobium sp. strain NGR234	Rif^r derivative of NGR234	47
M. loti MAFF303099	Wild type	21
R. tropici CIAT899	Wild-type *R. tropici*	29
S. meliloti 1021	Wild type	26
S. meliloti RP254	Bean isolate from Morocco	Mouhsine et al., unpublished
R.. etli CFN42	Wild type contains 6 plasmids (p42a–p42f)	41
R.. etli CE3	Str derivative of CFN42	33
R. etli CFNX183	*R. etli* CFN42 cured of pRetCFN42b	8
R. leguminosarum RU1302	3841 pRU504; Sm^r Gm^r	3
R. leguminosarum RU2292	3841 pRU1515; Sm^r Gm^r	This work
R. leguminosarum RU2295	3841 pRU1619 p*thiM*::*egfp*; Sm^r Gm^r	This work
R. leguminosarum RU2459	3841 *thiM*::Ω; Sm^r Sp^r	This work
Plasmids		
pOT2	Promoter probe vector with promoterless *gfp-UV*; Gm^r	3
pCR2.1TOPO	PCR product cloning vector; Ap^r Km^r	Invitrogen
pCR8/GW/TOPO	PCR product cloning vector; GW1 priming site, *att*L1 and *att*L2 sites for Gateway cloning; Sp^r	Invitrogen
pRU1097-D-TOPO	PCR product cloning vector*; gfp-mut3.1* reporter gene; Gm^r	This work
pGW1	*att*R1 and *att*R2 entry vector for gateway cloning (pJQ200SK); Gm^r	This work
pJQ200SK	pACYC derivative, P15A origin of replication; Gm^r	40
pRK2013	ColEI replicon with RK2 *tra* genes, helper plasmid used for mobilizing plasmids; Km^r	16
pHP45Ω	pBR322 derivative carrying ΩpHP45 replicon; Ap^r Sp^r	38
pHP45Ω	pBR322 derivative carrying ΩpHP45 replicon; Ap^r Tet^r	38
pRK415-1	Broad-host-range P-group cloning vector; Tet^r	23
pRU504	*thiMED* operon in pOT1identified by optical trapping	3
pRU1515	Self-ligated pRU1097-D-TOPO; Gm^r	This work
pRU1619	p639/p640 PCR product in pRU1097-D-TOPO; Gm^r	This work
pRU1734	p639/p641 PCR product in pCR8/GW/TOPO; Sp^r	This work
pRU1738	p753/p754 PCR product (*thiME*) in pCR2.1TOPO; Km^r	This work
pRU1752	p754/p755 PCR product (*thiMED*) in pCR2.1TOPO; Km^r	This work
pRU1755	pRU1734 with ligated ΩTet cassette into *thiM* gene via FspI digestion to generate *thiM* mutant; Sp^r Tet^r	This work
pRU1774	pGW1 carrying *thiM* gene with inserted Tet cassette; Gm^r Tet^r	This work
pRU1776	pRU1774 with ΩTet cassette replaced with ΩSp cassette; Gm^r Sp^r	This work
pRU1781	*thiME* gene was cloned from pRU1738 as HindIII/XbaI into pRK415; Tet^r	This work
pRU1782	*thiMED* gene was cloned from pRU1752 as HindIII/XbaI into pRK415; Tet^r	This work

To determine rhizosphere colonization levels, bacteria were inoculated onto pea seedlings as described above for Q-RT-PCR. Strains 3841 and RU2459 were inoculated at the following CFU ratios: 1,000:0, 0:1,000, 1,000:1,000, 10,000:1,000, and 1,000:10,000. After 7 days of growth, sterile phosphate-buffered saline (20 ml) was added to roots in the vermiculite and vortexed for 30 s. Bacteria were serially diluted and plate counted on TY medium containing either streptomycin and nyastatin or streptomycin, nyastatin, and spectinomycin, giving the total number of viable rhizosphere- and root-associated bacteria.

Bioinformatic analysis of the *thi* box. The presence of *thi* box riboswitches and RNA secondary structure in the *thiM* gene was analyzed using the RibEx website (1).

RESULTS

Growth of rhizobia on minimal medium with and without thiamine. *R. leguminosarum* bv. viciae strain 3841 did not grow

TABLE 2. Growth of *Rhizobium* strains in the presence and absence of thiamine and salvage intermediates

Strain[a]	Growth on minimal medium with[b]:				
	Thiamine	No addition	HMP	THZ	HMP and THZ
R. leguminosarum 3841	++	+	+	+	++
R. leguminosarum VF39	++	+	+	+	++
R. leguminosarum RBL1309	++	+	+	+	++
R. leguminosarum 3855	++	++	ND	ND	ND
R. leguminosarum WU235	++	++	ND	ND	ND
R. leguminosarum A34	++	++	ND	ND	ND
R. leguminosarum 248	++	++	ND	ND	ND
R. leguminosarum JI336	++	++	ND	ND	ND
R. leguminosarum 128C53	++	++	ND	ND	ND
R. leguminosarum bv. trifolii RCR5	++	++	ND	ND	ND
R. leguminosarum bv. trifolii W14-2	++	++	ND	ND	ND
RU2459 (*thiM* mutant)	++	−	−	−	−
RU2459 (pRU1781)	++	+	+	+	++
RU2459 (pRU1782)	++	+	+	+	++
R. etli CFN42	++	++	ND	ND	ND
R. etli CFNX183	++	+	+	+	++
R. etli CE3	++	++	ND	ND	ND
R. tropici CIAT899	++	++	ND	ND	ND
M. loti MAFF 303099	++	++	ND	ND	ND
S. meliloti 1021	++	++	ND	ND	ND
S. meliloti RP254	++	++	ND	ND	ND
Rhizobium sp. strain NGR234	++	++	ND	ND	ND

[a] Plasmids pRU1781 and pRU1782 contain *thiME* and *thiMED*, respectively.
[b] Growth on minimal medium was scored as follows: ++, good growth; +, pin colony formation; −, no growth; ND, not determined.

without thiamine either in liquid minimal medium or on medium solidified with agarose. However, it formed "pin" colonies on agar-solidified minimal medium in the absence of added thiamine (Table 2). These data are consistent with the absence of a complete *thiCOGE* biosynthetic operon in the genome sequence of strain 3841 (55). A *gfp-UV* fusion to the *thiE* gene from strain 3841 had previously been isolated from the rhizosphere of peas (3), and therefore we could use bioinformatic analysis of the region surrounding *thiE* to identify three genes, *thiMED*, which are present on plasmid pRL11JI.

BLAST analysis was used to determine the distribution of the *thiMED* and *thiCOGE* genes in different sequenced *Rhizobium* strains (*Mesorhizobium loti*, *S. meliloti*, *Bradyrhizobium japonicum*, *R. leguminosarum*, *R. etli*, and *Agrobacterium tumefaciens*). Only *R. leguminosarum* 3841 and its close relative *R. etli* CFN42 have the *thiMED* genes. The ThiMED proteins from *R. etli* and *R. leguminosarum* 3841 have 95%, 92%, and 91% identity, respectively (17). Excepting *R. leguminosarum* 3841, all species have *thiCOGE*. *R. etli* CFN42 is unusual in that the genes for both the de novo thiamine synthesis pathway (*thiCOGE*) and the putative salvage pathway (*thiMED*) are present on plasmids pRetCFN42b and pRetCFN42e, respectively (17). To test whether the presence of *thiCOGE* enables rhizobial strains to grow in the absence of added thiamine, the sequenced strains *R. etli* CFN42, *M. loti* MAFF 303099, and *S. meliloti* 1021, as well as several other common laboratory strains, were grown in the presence and the absence of thiamine (Table 2). Three out of 11 strains of *R. leguminosarum* (3841, VF39, and RBL1309) did not grow well in the absence of added thiamine. All of the other rhizobia tested grew well in the absence of added thiamine, implying they have a *thiCOGE* operon or the equivalent.

Since strain 3841 formed only pin colonies on agar medium lacking thiamine, one possibility is that *thiMED* constitutes a salvage pathway that uses intermediates in thiamine biosynthesis normally present in agar and presumably in the soil environment. The putative reactions catalyzed by ThiM, the 4-methyl-5-(β-hydroxyethyl) thiazole kinase, by ThiE, the thiamine phosphate synthase, and by ThiD, the HMP kinase, suggest that HMP and THZ might be the intermediates used (Fig. 1). When added alone, neither HMP nor THZ rescued growth of strain 3841; however, when these were added together, the wild-type strain grew as well as it did when thiamine was added (Table 2). Strains VF39 and RBL1309, which do not grow in the absence of thiamine, also grew well on HMP and THZ (Table 2). To determine if the pin colony growth seen on solid medium results from the operation of the salvage pathway, a *thiM* mutant (RU2459) was made by the insertion of an omega interposon into *thiM* of strain 3841. Strain RU2459 no longer formed pin colonies on agar plates and, as expected, was unable to grow in the presence of HMP and THZ but could use thiamine (Table 2). Strain RU2459 was complemented for growth on HMP and THZ with plasmids containing *thiME* (pRU1781) and *thiMED* (pRU1782) (Table 2). These results indicate that a salvage pathway for condensation of HMP and THZ to thiamine, using ThiM, ThiE, and ThiD, operates in *R. leguminosarum* 3841. In addition, the *thiM* gene could be amplified by PCR from genomic DNA of VF39 and RBL1309 (data not shown), indicating they also have this pathway.

In order to check whether the *thiMED* genes in *R. etli* CFN42 confer on it a putative salvage pathway, strain CFNX183, a derivative of CFN42 lacking plasmid pRet CFN42b, which contains the *thiCOGE* genes, was grown on various media. It formed pin colonies when grown on media lacking thiamine but grew well on the intermediates HMP and THZ, which are used by the ThiMED salvage pathway (Table 2). This suggests that the ThiMED salvage pathway operates in *R. etli*.

Regulation of the *thiM* promoter. It has been shown that a *gfp-UV* fusion to *thiE* is expressed under thiamine limitation and in the rhizosphere of peas (3). However, this fusion contains 1.8 kb of DNA upstream of *thiE*, which contains *thiM* and a gene coding for a hypothetical protein. To see if the *thiMED* genes are regulated from a promoter immediately upstream of *thiM*, an 889-bp fragment of this region was fused to *gfp-mut3.1* in plasmid pRU1097, producing pRU1619. Strains RU2292 (strain 3841 containing pRU1515, which is self-ligated pRU1097) and RU2295 (strain 3841 containing pRU1619, i.e., p*thiM*::*gfp-mut3.1*) were grown in the presence of thiamine overnight and then transferred into AMS containing various combinations of thiamine, HMP, and THZ (Fig. 2). The expression of *thiM*::*gfp-mut3.1* was very low when thiamine was present but high in growth medium from which thiamine was absent. Together, but not alone, the thiamine intermediates HMP and THZ also repressed the *thiM* promoter. These data confirm that there is a thiamine-repressible promoter immediately upstream of *thiM*. Furthermore, immediately upstream of *thiM* in both *R. leguminosarum* 3841 and *R. etli* CFN42 there is a *thi* box consisting of three thiamine pyrophosphate [TPP]

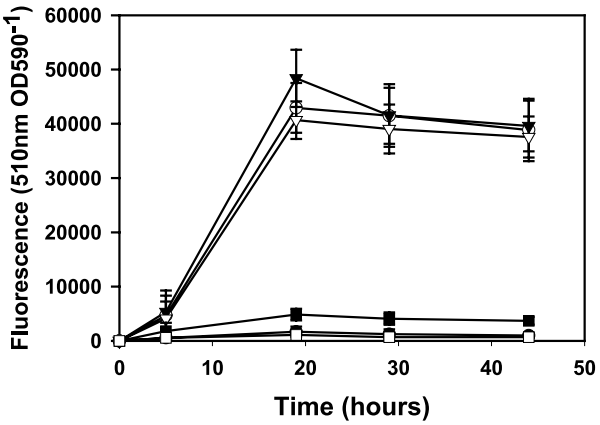

FIG. 2. Expression of p*thiM*::*gfp-mut3.1* on different thiamine intermediates. Symbols: ●, thiamine; ○, no addition; ▼, HMP; ▽, THZ; ■, HMP and THZ; □, HMP, THZ, and thiamine. OD590, optical density at 590 nm.

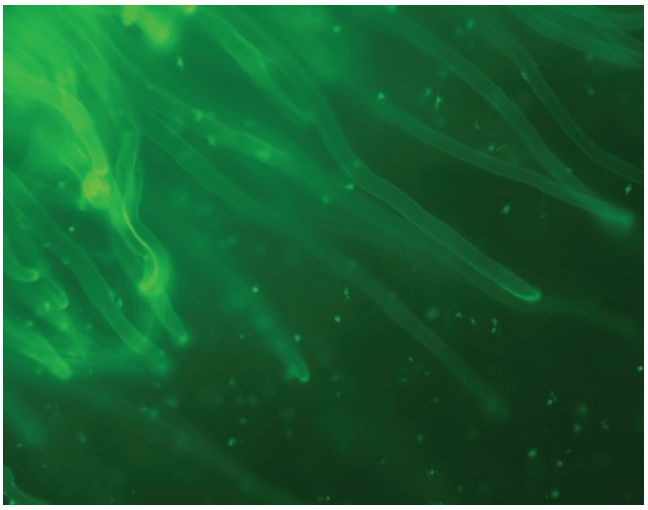

FIG. 3. Expression of the *thiME* fusion (pRU504) in the rhizosphere of vetch. Fluorescent bacteria can be seen throughout the rhizosphere. No fluorescent bacteria were seen in the absence of the *thiME* promoter in the parent plasmid pOT1.

riboswitches named 1, 2, and 3, which have 65, 63, and 61% identity, respectively, to the characterized *R. etli thiC* TPP riboswitch subregions.

Complementation of a *thiM* mutant (RU2459) with a plasmid containing *thiME* may indicate that there is another promoter downstream of *thiM.* However, while the omega interposons do have strong transcriptional terminators, their insertion may generate outwardly directed promoters which complicate such analysis. RT-PCR products were also obtained for *thiM*, *thiE*, and *thiD* and for primer pairs spanning *thiME* and *thiED*, with RNA obtained from thiamine-limited cultures but not from those with an excess of thiamine (data not shown). This indicates the presence of an operon spanning *thiMED*, with a promoter upstream of *thiM*, although it does not preclude the presence of other promoters.

Quantitation by Q-RT-PCR and rhizosphere visualization. Quantitative RT-PCR was used to measure the mRNA expression levels of strain 3841 *thiM* in the rhizosphere as well as in thiamine-starved cells grown in culture. In cultured bacteria, thiamine starvation resulted in a 16.5 ± 2.1 (mean ± standard error of the mean [SEM])-fold increase in expression ($n = 3$). Likewise, bacteria isolated from the rhizosphere had a 20.8 ± 9.0 (mean ± SEM)-fold increase in *thiM* expression ($n = 3$) relative to laboratory cells grown on excess thiamine. This suggests that thiamine is present at limiting concentrations in the pea rhizosphere.

The previously isolated *thiME*::*gfp-UV* fusion (pRU504) was conjugated into strain 3841 and inoculated in the vetch plants to directly visualize expression in the rhizosphere (Fig. 3). It can be seen that the expression of the fusion was high in the rhizosphere, confirming the Q-RT-PCR results.

Plant properties of the *thiM* mutant and the wild type. The wild type and the *thiM* mutant (RU2459) reduced acetylene at 2.1 ± 0.4 (mean ± SEM) ($n = 6$) and 1.9 ± 0.4 (mean ± SEM) ($n = 6$) μmol ethylene per plant per hour, respectively. The dry weights of plants inoculated with either the wild type or the *thiM* mutant and harvested at 6 weeks were 1.40 g ± 0.24 g (mean ± SEM) ($n = 6$) and 1.61 g ± 0.40 g (mean ± SEM) ($n = 6$), respectively. None of these results are significantly different in *t* tests, indicating that the mutation in *thiM* has no significant effect on nitrogen fixation.

The fact that there is no effect of mutation of *thiM* on the ability of *R. leguminosarum* to nodulate peas and fix nitrogen suggests that the supply of thiamine by the plant to bacteroids and bacteria in infection threads is not limiting. However, the competitive ability of *R. leguminosarum* in the rhizosphere, where thiamine is limiting, may be altered. In order to determine if there is an effect on the nodulation competitiveness of the *thiM* mutant, a large inoculum (10^6 cells of *thiM* and wild-type strains) was placed onto pea seedlings. At 4 weeks postinoculation, 10 nodules from each of 12 plants were picked randomly and checked for occupation by bacteria. The results indicated that the *thiM* mutant was at a modest, but significant, competitive disadvantage relative to the wild type, occupying 35% ± 8% (means ± SEM) of nodules on each plant (*t* test; $P < 0.05$).

Competition between the wild type and the *thiM* mutant for rhizosphere colonization. Competition between the *thiM* mutant and the wild type for growth in the pea rhizosphere was measured by inoculating a low number of bacteria into the pea rhizosphere (10^3 to 10^4 bacteria per seedling) and determining total bacteria after 7 days. When the mutant and the wild type were inoculated alone into a sterile rhizosphere, almost identical numbers of bacteria were recovered after 7 days (Fig. 4). However, when these strains were inoculated together, the *thiM* mutant was at a slight, but significant, disadvantage compared to the wild type (*t* test; $P < 0.05$). Even when strain RU2459 was inoculated at a 10-fold excess over the wild type, it still accounted for only 18% of bacteria recovered (Fig. 4). The ability of the *thiM* mutant to grow in a sterile rhizosphere and to nodulate and fix nitrogen on peas shows that thiamine must be released by pea roots. At higher cell densities, however, the competition for thiamine in the rhizosphere presumably becomes more acute, and the presence of a thiamine salvage pathway becomes increasingly important.

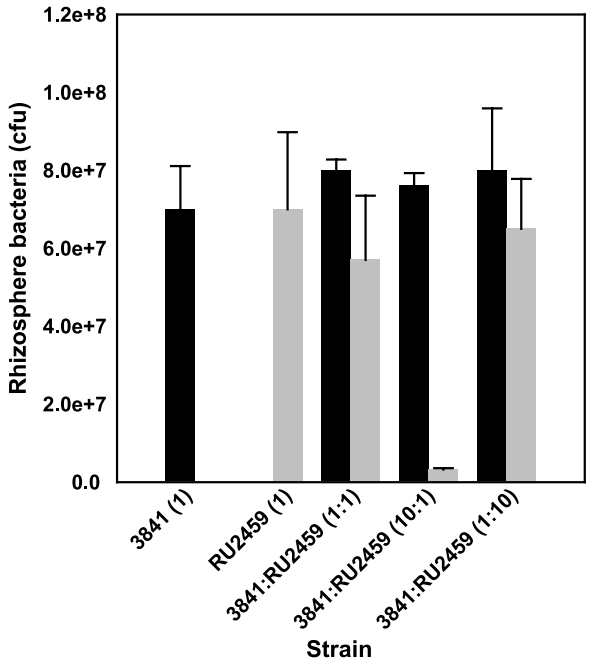

FIG. 4. Competition of the wild type (3841) (black bars) and the *thiM* mutant (RU2459) (gray bars) in sterile rhizospheres. Inoculation ratios are given on the *x* axis, with 1 corresponding to 1,000 CFU. Bacterial numbers recovered from 12 plants (mean ± SEM) are shown.

DISCUSSION

In this paper, we report that the biosynthesis of thiamine in *R. leguminosarum* bv. viciae strain 3841 is mediated by a salvage pathway requiring ThiMED. In medium solidified with agar without thiamine, strain 3841 formed pin colonies, while the presence of the salvage intermediates HMP and THZ allowed normal growth. The *thiM* mutant (RU2459) did not grow without the addition of thiamine. Complementing plasmids containing the *thiM* gene restored growth on HMP and thiazole. This indicates that, as expected, ThiMED are 4-methyl-5-(β-hydroxyethyl) thiazole kinase, thiamine phosphate synthase, and 4-amino-5-hydroxymethyl-2-methyl pyrimidine kinase. In the sequenced rhizobia *S. meliloti* 1021 and *M. loti* MAFF303099, the presence of the ThiCOGE pathway presumably mediates the de novo synthesis of thiamine, while *R. etli* CFN42 has both this and the ThiMED salvage pathway. Three out of 11 tested strains of *R. leguminosarum* will grow only with added thiamine or HMP and THZ, indicating dependence on the ThiMED pathway, but the other strains are capable of growing in the absence of added thiamine. Thus, it is common, but not universal, for strains of *R. leguminosarum* to use the ThiMED pathway for thiamine synthesis. It is noteworthy that the *thiMED* genes are on plasmid pRL11JI in *R. leguminosarum* 3841 and *R. etli* has both the *thiCOGE* and *thiMED* genes on plasmids pRetCFN42b and pRetCFN42e, respectively, while *S. meliloti* has the *thiCOGE* genes on pSymB. Thus, rhizobia often have genes for thiamine biosynthesis on plasmids.

An intriguing example of how vitamins can limit the growth of rhizobia in the environment is found in *Mesorhizobium* sp. strain R7A, in which genes for symbiosis and for biotin, thiamine, and nicotinate biosynthesis are found on a chromosomal (symbiosis) island. Strains found in the soil lack the symbiotic island and are auxotrophic for all three vitamins (or just for thiamine and biotin in some cases), but transfer of the symbiotic island restores prototrophy (51). Overall, it appears to be common for the growth of rhizobia in soil to be limited by the rate at which vitamins such as biotin and thiamine can be synthesized; their growth may be arrested until they are available, perhaps in the rhizosphere.

It is interesting that some rhizobia possess a full pathway for de novo thiamine biosynthesis and others, such as strain 3841, possess only a salvage pathway. The use of a *thiE gfp-UV* biosensor as well as Q-RT-PCR confirmed that the *thiMED* genes are expressed in the rhizosphere, indicating that limiting levels of thiamine are present. Such a limitation suggests that the ability to synthesize thiamine either de novo or via the ThiMED salvage pathway should be important for growth in the rhizosphere. Consistent with this, a *thiM* mutant was shown to be at a competitive disadvantage for growth in the rhizosphere and for nodulation (Fig. 4). We have not attempted to address the experimentally difficult question of whether the *thiMED* genes might be required for long-term survival in soil. However, the example of acquisition of de novo thiamine biosynthesis, via a symbiosis island, by strains of *M. loti* that exist in the soil without this capacity highlights this issue (51). Can such strains exist without the ability to make any thiamine, or do they possess salvage pathways such as ThiMED?

No thiamine-regulatory proteins have been found in bacteria (25, 52). Instead, the corresponding genes have been found to be regulated by riboswitches in different bacteria, including *R. etli* (28, 32). TPP directly regulates the expression of the thiamine biosynthesis genes by a novel mechanism involving the formation of a riboswitch. TPP interacts with the nascent mRNA message at a *cis*-acting region within the 5′ leader, called the *thi* box, to form a secondary structure that allows the formation of a transcription terminator (27, 28, 32, 54). The presence of a *thi* box upstream of *thiM* indicates there is a riboswitch regulating the *thiMED* genes, just as there is one regulating the de novo *thiCOGE* thiamine biosynthetic operon in *R. etli*. This is consistent with the repression of the *thiMED* genes in cultures grown on excess thiamine.

Competition experiments suggest that the *thiMED* salvage pathway will become increasingly important as bacteria increase in number and thereby begin to compete for limiting quantities of thiamine in the rhizosphere. Key questions that we cannot yet answer include what levels of intermediates such as HMP and THZ are in soil and whether they are released at higher levels by plant roots. Overall, *R. leguminosarum* 3841, in common with most rhizobia, appears to adopt a survival strategy in soil, where vitamins will limit its growth and cause it to rely on a plant host for provision of these factors in the rhizosphere.

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