SIGNALS AFFECTING THE UREASE STATUS OF PLANT-ASSOCIATED BACTERIA, *METHYLOBACTERIUM* SPP.

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The Faculty of the Graduate School
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In Partial Fulfillment
of the Requirements for the Degree

Master of Science

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by
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AUGUST 2007

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled:

SIGNALS AFFECTING THE UREASE STATUS OF PLANT-ASSOCIATED BACTERIA, *METHYLOBACTERIUM* SPP.

Presented by Stephen B. Witzig,

A candidate for the degree of Master of Science,

And hereby certify that, in their opinion, it is worthy of acceptance.

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Professor Joseph Polacco

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Professor David Emerich

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ABSTRACT

This research focused on furthering our understanding of the interactions between Pink-Pigmented Facultative Methylotrophic bacteria (PPFMs) and plants. PPFMs (*Methylobacterium* spp.) have been found to be the most abundant microorganisms among phylloplane microflora, and have been recovered from all plants examined. I focused on the plant influence on production of active PPFM urease. *Arabidopsis thaliana* and *Glycine max* (soybean) are two dicots which provide valuable urease-negative mutants. However, while genetic and genomic analysis of each plant are advancing, little is known about the identity of the PPFMs with which they associate. I established phylogenetic relationships of various PPFM isolates recovered from plants and elsewhere. I examined the ability of resident PPFMs to mimic the urease-negative phenotype of two mutant classes of urease-negative soybean hosts. The working model is that there is a signal from the plant that either inhibits the production of the urease gene products in the associated bacteria or inhibits the function or transport of Ni^{2+} from the plant to the bacteria. This signal could be a nitrogenous signal (ureides, urea, ammonia) or simply a block in Ni^{2+} transport from the plant or plant cell to the associated bacteria. Examination of urease expression *in planta* or in culture requires knowledge of the urease genes and the regulation of urease in the PPFMs. Urease expression is directly related to

its role in nitrogen assimilation. My studies led to the overall conclusion that urease is essential for assimilation of urea and of ureides, that urease has a constitutive basal level of expression and is "induced" by the ureide allantoin and "repressed" by the preferential nitrogen source, ammonium. However, these nitrogenous signals are not responsible for the urease-negative status of the plant-associated PPFMs. Our working model has shifted to a block in nickel uptake necessitating examination of nickel content in these bacteria, as well as interactions between PPFM and host variously mutated in urease structural and Ni-insertion (*ureG*) genes. To examine the interactions of mutated partners I attempted the recolonization of plants with PPFMs. In the course of these studies it became obvious that the interactions between PPFMs and the host plant is an intimate one because seed-reintroduced strains, though colonizing the host plant, were not seed-transmitted. In addition, PPFM interactions with *Arabidopsis* and with soybean were distinguishable, in that only in the latter were endogenous PPFMs urease-negative on mutant hosts defective in urease accessory genes.

1. INTRODUCTION

1.1 Pink-Pigmented Facultative Methylotrophs (PPFMs)

Plant interactions with microorganisms are well-documented phenomena. The plant pathogen *Agrobacterium tumefaciens* is known for its ability to cause crown gall disease [84]. Symbiotic bacteria that inhabit the rhizosphere and form nodules on the root of legumes are able to assimilate atmospheric nitrogen and provide it to the host plant. The association is initiated via signals that are still not completely understood [85]. Rhizosphere bacteria, including members of the genera *Rhizobium* and *Bradyrhizobium*, however, are not the only players involved in plant-microbe symbiosis. Many bacteria are present on the plant leaf surface (in the phylloplane) and there is evidence that these inhabitants have a significant impact on plant growth and development. One such inhabitant is PPFMs.

Pink-Pigmented Facultative Methylotrophic bacteria, or PPFMs, are members of the genus *Methylobacterium* and are gram-negative alpha-proteobacteria. These plant-associated bacteria are easily detected by their pink color and ability to utilize one carbon compounds, such as methanol, as sole carbon and energy sources. They are phylogenetically related to both plant-associated bacteria *Agrobacterium* and *Rhizobium* (Figure 1.1-1) [86] and have more recently been placed in a clade including a *Methylobacterium* strain that is able to nodulate and fix nitrogen in symbiosis with legumes (Figure 1.1-2) [87]. PPFMs have been isolated from virtually all land plants examined [1]. Although they do not grow as rapidly as other phylloplane bacteria on multicarbon sources, they compete well for leaf surface colonization. Hirano and Upper

Figure 1.1-1. Unrooted phylogenetic relationship based on 16S rRNA analysis among methylotrophic bacteria and other representatives within the class *Proteobacteria*. The graph is taken from Bratina et al. [87]. *Methylobacterium* is grouped in the lower left, within the α-2 subdivision. The abbreviations on the tree represent the following organisms: α-subclass reference organisms *Agrobacterium tumefaciens* (A. tume.) and *Rhodospirillum rubrum* (R. rubrum); α-subclass methylotrophs *Methylobacterium* sp. strain DM4 (M. sp. DM4), *Methylobacterium* sp. strain M27 (M. sp. 27), *Methylobacterium extorqens* (M. ext.), *Methylobacterium exorquens* AM1 (M. ext. AM1), *Methylobacterium organophilum* XX (M. org. XX), strain PK-1 (PK-1), and strain PR-6 (PR-6); α-subclass methanotrophs *Methylocystis parvus* OBBP (M. par. OBBP), "*Methylosinus*" sp. strain B (M. sp. B), "*Methylosinus*" sp. strain LAC (M. sp. LAC), "*Methylosinus methanica*" 81Z (M. meth. 81Z), "*Methylosinus sporium*" (M. spor.), and "*Methylosinus trichosporium*" OB3b (M. t. OB3b); β-subclass reference organism *Pseudomonas testosteroni* (P. test.); β-subclass methylotrophs "*Methylobacillus flagellatus*" KT1 (M. flag.), *Methylobacillus glycogenes* (M. gly.), "*Methylomonas methanolica"* (M. lica.), "*Methylomonas methylovora*" (M. vora), and *Methylophilus methylotrophus* AS1 (M. meth. AS1); γ-subclass reference organism *Escherichia coli* (E. coli.); γ-subclass methanotrophs *Methylococcus capsulatus* (M. cap.), *Methylomonas* sp. strain A4 (M. sp. A4), *Methylomonas alba* BG8 (M. alba BG8), *Methylomonas lutea* (formerly *Methylococcus luteus*) M. luteus), *Methylomonas methanica* (M. meth.), and *Methylomonas rubra* (M. rubra).

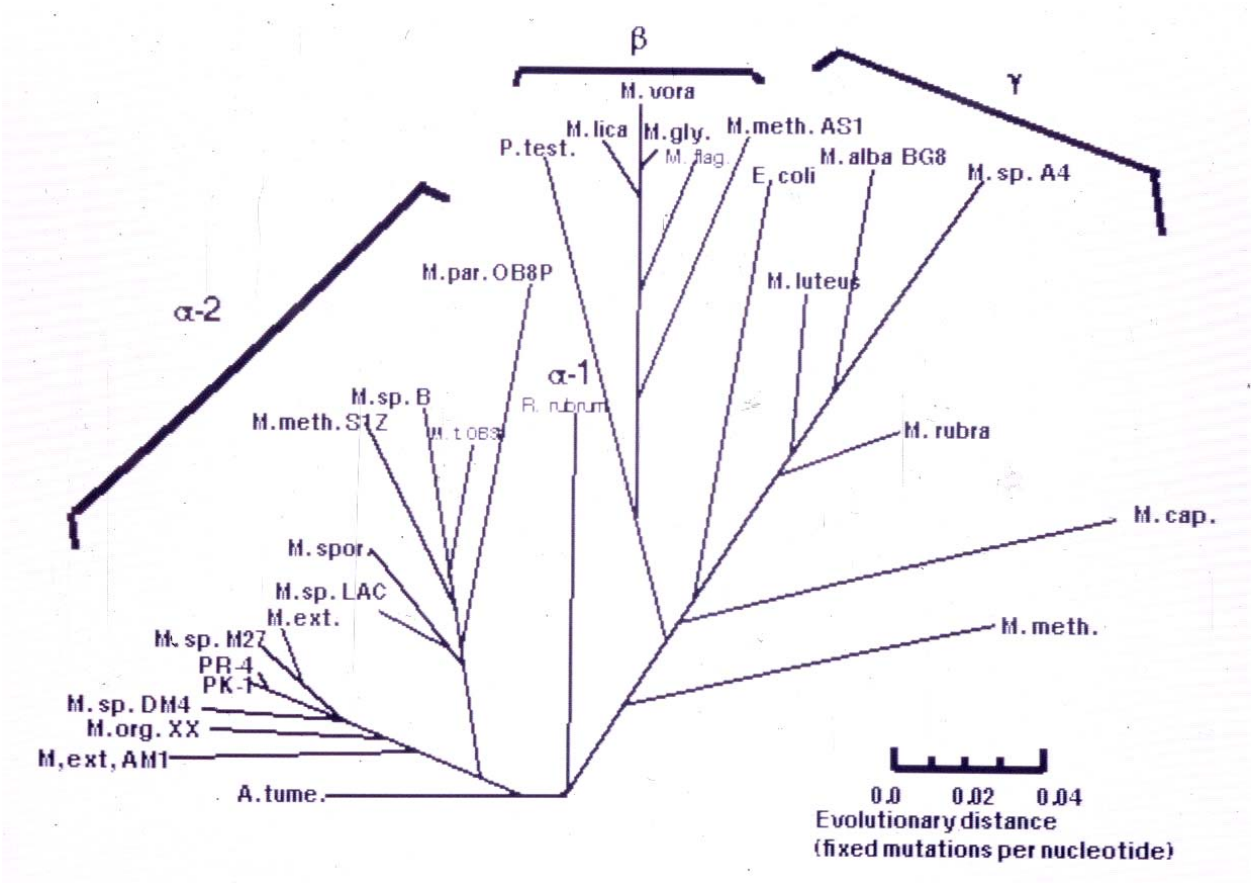
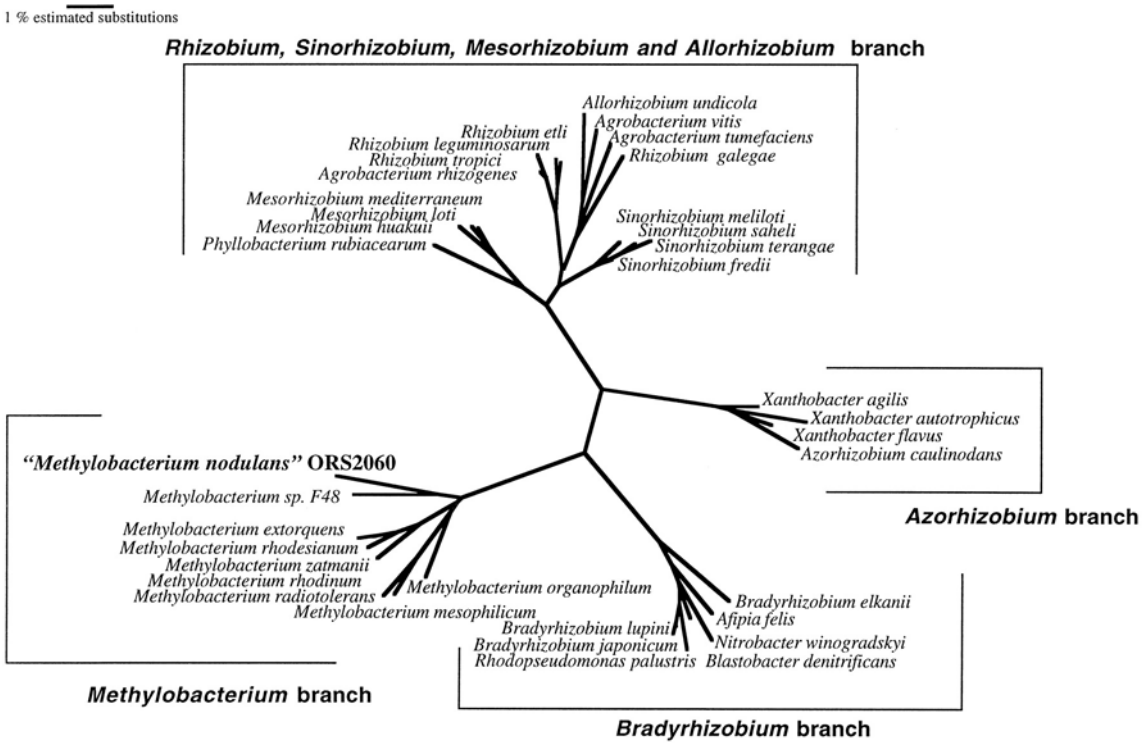

β
γ
α-2
α-1
M. vora
M. lica
M.gly.
M. flag.
M.meth. AS1
P.test.
M. alba BG8
E. coli
M. sp. A4
M.par. OB8P
M. luteus
M.sp. B
M.meth. S1Z
M. t OB3
R. rubrum
M. rubra
M. cap.
M. spor.
M.sp. LAC
M.ext.
M. meth.
M. sp. M2Z
PR-4
PK-1
M. sp. DM4
M.org. XX
M,ext, AM1
A. tume.
0.0
0.02
0.04
Evolutionary distance
(fixed mutations per nucleotide)

Figure 1.1-2. Unrooted phylogenetic tree showing the different rhizobial branches, including *Methlyobacterium nodulens* in the α subdivision of *Proteobacteria.* This figure is taken from Sy et al. [88]. The tree was constructed by using the neighbor-joining method from almost full-length 16S rDNA sequences. *M. nodulens* is a N-fixing symbiont which forms nodules on *Crotalaria*. The GenBank/EMBL accession numbers are as follows (the first letters of the genus and species are given in parenthesis): D12790 (Pr), D12797 (Mh), X67229 (Ml), L38825 (Mmed), X67224 (Ar), X67234 (Rt), U29386 (Rl), U28916 (Re), Y17047 (Au), X67225 (Av), X67223 (At), X67226 (Rg), X67222 (Sm), X68390 (Ss), X68397 (St), X67231 (Sf), X94198 (Xag), X94201 (Xau), X94199 (Xf), D11342 (Ac), U35000 (Be), M65248 (Af), L11661 (Nw), S46917 (Bd), D25312 (Rp), D12781 (Bj), U69637 (Bl), D32226 (Mo), D32225 (Mmes), D32227 (Mrad), D32229 (Mrhodi), D32230 (Mz), D32228 (Mrhode), D32224 (Me), D32236 (Msp), and AF220763 (Mn).


1 % estimated substitutions
Rhizobium, Sinorhizobium, Mesorhizobium and Allorhizobium branch
Allorhizobium undicola
Agrobacterium vitis
Agrobacterium tumefaciens
Rhizobium galegae
Rhizobium etli
Rhizobium leguminosarum
Rhizobium tropici
Agrobacterium rhizogenes
Mesorhizobium mediterraneum
Mesorhizobium loti
Mesorhizobium huakuii
Phyllobacterium rubiacearum
Sinorhizobium meliloti
Sinorhizobium saheli
Sinorhizobium terangae
Sinorhizobium fredii
Xanthobacter agilis
Xanthobacter autotrophicus
Xanthobacter flavus
Azorhizobium caulinodans
Azorhizobium branch
"Methylobacterium nodulans" ORS2060
Methylobacterium sp. F48
Methylobacterium extorquens
Methylobacterium rhodesianum
Methylobacterium zatmanii
Methylobacterium rhodinum
Methylobacterium radiotolerans
Methylobacterium organophilum
Methylobacterium mesophilicum
Methylobacterium branch
Bradyrhizobium elkanii
Afipia felis
Bradyrhizobium lupini
Nitrobacter winogradskyi
Bradyrhizobium japonicum
Rhodopseudomonas palustris
Blastobacter denitrificans
Bradyrhizobium branch

[2] measured bacterial populations on snap pea throughout a growing season and found PPFMs to be the most abundant organisms in the phylloplane microflora at each sampling date. Utilizing mutants in the pathway for one-carbon metabolism of *Methylobacterium* together with wildtype, Sy et al. [88] demonstrated that methylotrophy is advantageous to the bacterium colonizing *Medicago truncatula* under competitive conditions. Under non-competitive conditions, these methylotrophy mutants were able to colonize the plants as well as wildtype indicating that methanol is not the only carbon-source available to *Methylobacterium* while it is associated with the plant [88]. Populations of *Methylobacterium* on red clover in the field were shown to decrease from the spring towards summer, but then increase again towards the end of the cropping season [89]. PPFMs, however, are not limited to the phylloplane. They are found associated with all parts of the plant, concentrated at the actively growing portions. In a proteomic study of *Methylobacterium extorquens* AM1, Gourion et al. [90] harvested *Methylobacterium* from the roots and the aerial portions of inoculated plants and compared proteins that were up or down regulated during colonization versus those from free-living bacteria grown on minimal medium. Among proteins induced during phyllosphere colonization was PhyR, a two-component response regulator that was shown to play an essential role in plant colonization. They suggested that it is part of a key regulator for adaptation to epiphytic life of *Methylobacterium* [90]. A *phyR* disruption mutant exhibited *in vitro* growth similar to the progenitor isolate. However, during colonization of *Arabadopsis*, *phyR* cell numbers were below the detection limit for 65% of the 3-week old plants. Colonization to wildtype levels was restored when the PhyR gene was expressed in trans [90].

PPFMs and other commensal plant-associated bacteria differ from plant pathogens by not eliciting a hypersensitive response and by not causing disease in associated tissue [reviewed in 3]. Hirano and Upper [131] have studied the various phyllosphere inhabitants and used *Pseudomonas syringae* as a model to explain the complex association of bacteria with plants. Depending on the host plant and the environmental conditions, *P. syringae* can act as an epiphyte, an ice nucleus or as a pathogen in the phyllosphere [131]. Recently, a plant-growth promoting *Methylobacterium* isolate was shown to induce defense responses in groundnut [132]. The induced systemic resistance activity in *Methylobacterium*-associated groundnut provided protection against rot pathogens suggesting that PPFMs could be useful as a means of biological control of pathogens [132]. PPFMs are seed transmitted in soybean [3] and have been detected intra-cellularly in scotch pine buds by *in-situ* hybridization [4]. PPFMs have been studied for their stimulation of seed germination and other aspects of plant growth and development.

1.2 PPFMs and their effect on seed germination and plant growth and development.

It has been demonstrated that seed-associated bacteria affect germination. For example, Klincare et al. [5] showed a correlation between lowered populations of seed microflora and a decline in the germination rates in a variety of species. This observation led to the investigation of a possible role of PPFMs in germination. A procedure to heat cure soybean seeds of their bacteria was developed [6]. Heat-cured seeds had a decrease in

germination frequency, by 30-75% depending on seed lot, that could be corrected by imbibition with PPFMs, their spent medium, or by addition of cytokinins [3].

Since exogenous cytokinins had an effect on germination similar to PPFMs or their spent media, cytokinin production was investigated in PPFMs [3, 7]. Four different leaf isolates and a type culture were shown to produce and secrete *trans*-zeatin by way of tRNA turnover [8]. A cytokinin-null mutant (*miaA*$^-$), however, stimulated germination of soybean seeds as well as wild-type bacteria [8]. The component(s) in PPFM spent medium that is responsible for the germination effect has yet to be characterized. More recently, Ryu et al. [91] demonstrated the production of plant growth regulators, including the auxin indole-3-acetic acid (IAA) as well as the cytokinins *trans*-zeatin riboside (*t*-ZR), dihydrozeatin riboside (DHZR) and isopentenyladenosine (iPA), by two *Methylobacterium* isolates from rice. Inoculation of red pepper and tomato seeds with these two isolates resulted in increased germination percentage as well as increased root length compared to uninoculated controls and plants inoculated with the *miaA*$^-$ mutant described above [91]. Similar results were found in rice. Rice seeds inoculated with these isolates exhibited both an increase in the germination percentage and the germination rate suggested to be a result of phytohormones produced by the PPFMs [92]. There is evidence that bacteria that stimulate plant growth do so by lowering ethylene levels in the plant [93]. *Methylobacterium* spp. that utilizes the direct precursor to ethylene (i.e. have an ACC deaminase) were able to promote root elongation in canola by reducing the level of ethylene in the plant [94]. This suggests that the plant growth

promoting effects of *Methylobacterium* may be due to a combination of substances both produced and utilized by the bacteria.

PPFMs have been shown to produce vitamin B_{12} [9]. In liverworts, Basile et al. showed a correlation between exogenous vitamin B_{12}-enhanced growth and development and PPFM-enhanced growth [10]. *Methylobacterium* spp. isolated from the moss *Funaria hygrometrica* were shown to cause an acceleration of bud formation and growth in the protonemata of *Funaria* [95]. Secretions of other interesting secondary metabolites by PPFMs are being reported in the literature. For example, two character-impact compounds of strawberry flavor, the furanones 2, 5-dimethyl-4-hydroxy-2*H*-furan-3-one (DMHF) and 2,5-dimethyl-4-methoxy-2*H*-furan-3-one (mesifuran) were synthesized by strawberry tissue cultures only after being treated with *Methylobacterium extorquens*. It was demonstrated that the precursor to furanones, 2-hydroxy-propanol (lactaldehyde), was formed by the bacterial oxidation of 1,2-propandediol, which is found in strawberry cells [11].

Sugarcane seeds inoculated with *Methylobacterium* spp. show an accelerated rate of germination and a higher percent germination [96]. When combining seed inoculation with *Methylobacterium* spp. in sugarcane with a soil treatment and a foliar application of the bacteria, researchers demonstrated an increase in specific leaf area, plant height, number of internodes and cane yield [96]. Foliar applications of *Methylobacterium* spp. have resulted in increased growth and yield of cotton as well [97]. *In vitro* regenerated sunflower plantlets from excised hypocotyl segments were inoculated with a

Methylobacterium strain from a field-grown sunflower plant prior to being placed on shoot-induction medium. The plantlets showed an increase in both the number of shoots and roots while having no effect on the length of the shoots [98]. A PPFM strain originally isolated from contaminated rice callus stimulated the growth of recolonized rice callus [99]. This callus isolate and isolates from green leafy plants, were shown to inhibit plantlet generation in two rice cultures resulting in continual embryo-like cell proliferation [99]. However, when rice seeds were inoculated with these isolates and grown in culture, there was a significant increase in growth and development of the seedlings by the criteria of increased biomass, leaf development and shoot growth [99].

1.3 Urease Activation in Soybean and in PPFMs

PPFMs associated with urease-negative soybean mutants, which lack functions for insertion of nickel in the plant urease active site, were urease-negative themselves while on the plant [6]. The bacteria were isolated from the leaves of the mutant plants and the urease activity was assayed in these fresh isolates. These bacteria were transiently urease-negative in free-living culture and the reacquisition of urease activity was accelerated by nickel supplementation *in vitro* [6].

Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide. Urease, historically, is a well-studied enzyme. Jack bean urease was the first enzyme crystallized [12] and, nearly 50 years later, was the first reported nickel metalloenzyme [13]. The first report of a biological role for nickel was its requirement in soybean cell cultures

utilizing urea as the sole nitrogen source [14]. Although differing in the number of subunits, plant, fungi and bacterial ureases all show significant sequence similarity [15]. Jack bean urease apoenzyme is a hexamer of a 91-kDa subunit [16, 17] whereas the bacterial counterpart is comprised of three co-linear proteins (UreA, UreB, and UreC) encoded by the genes *ureA*, *ureB*, and *ureC* in *Proteus mirabilis* [18], *Yersinia enterocolitica* [19], and *Klebsiella aerogenes* [20]. These subunits form a trimer of trimers $(\alpha,\beta,\gamma)_3$. In soybean there are two structural genes, the embryo-specific urease, *Eu1*, and the ubiquitous urease, *Eu4* [21, 22]. They share 87% identity and 92% similarity at the amino acid level [133].

The mechanism of urease activation and insertion of nickel into urease is best understood in the bacterium *Klebsiella aerogenes* [25] (Figure 1.3-1). *In vivo* urease activation involves the action of four accessory proteins (UreD, UreE, UreF and UreG) coded by the genes *ureD*, *ureE*, *ureF* and *ureG*, respectively. The genetic organization of the urease gene cluster in *Klebsiella* is shown in Figure 1.3-2. The specific functions of the accessory proteins are being elucidated. For example, deletion of the accessory gene products *ureD*, *ureF*, or *ureG* causes complete loss of urease activity with a concomitant loss of urease-bound nickel. However, deletion of *ureE* only partially reduces the level of urease activity and nickel content [26]. A UreD-apourease complex has been characterized. UreD is speculated to serve as a urease-specific chaperone protein that facilitates the proper assembly of the metallocenter since it binds apourease releasing active urease upon addition of nickel [27]. Little is known about the function of UreF. However, a UreD-UreF-apourease complex has been characterized [28]. This complex

Figure 1.3-1. Model of the urease activation mechanism in *Klebsiella aerogenes*. This figure is taken from Hausinger et al. [25]. UreE functions as a metallochaperone to deliver nickel to urease apoprotein when bound to a protein chaperone complex made up of UreD, UreF, and UreG. Incorporation of nickel and bicarbonate/CO_2 is coupled to the hydrolysis of GTP. A nickel transporter or permease, encoded by gene(s) not present in *K. aerogenes* urease gene cluster, facilitates metal entry into the cell.

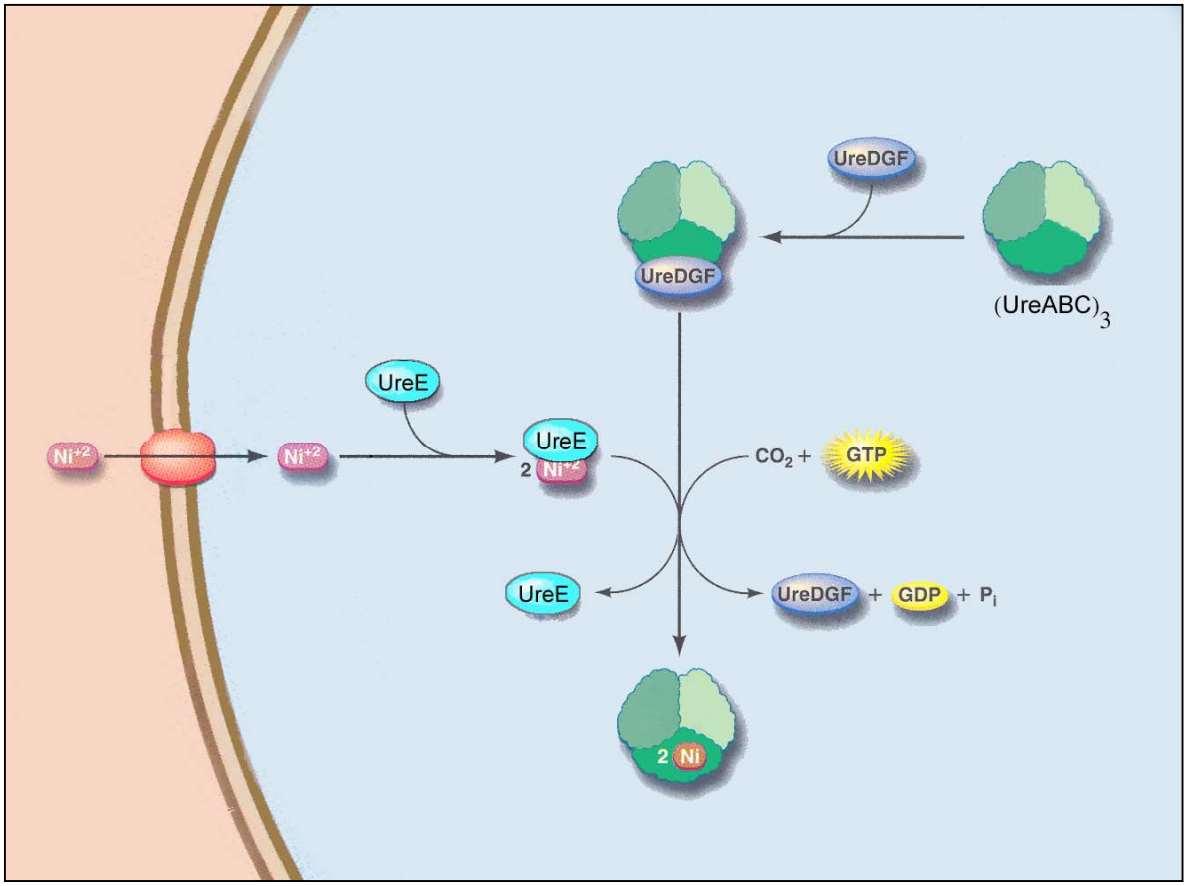
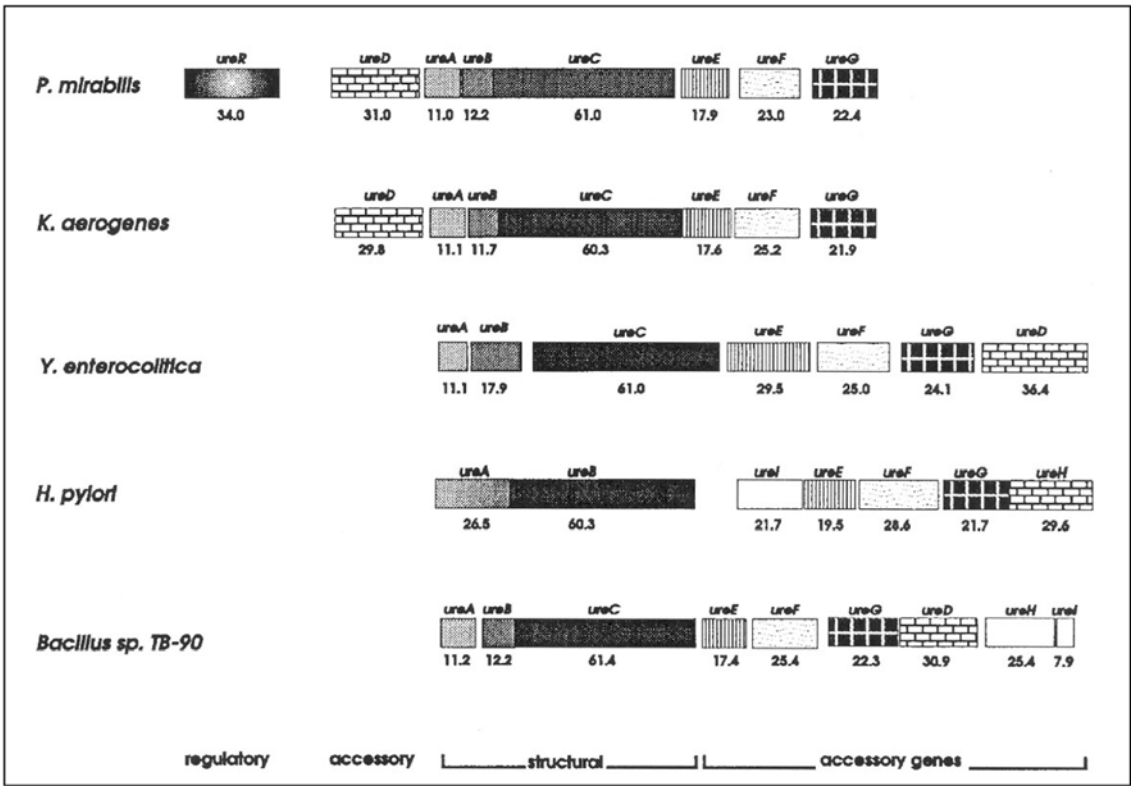

UreDGF
UreDGF
(UreABC)3
UreE
Ni+2
Ni+2
UreE
2 Ni+2
CO2 +
GTP
UreE
UreDGF + GDP + Pi
2 Ni

Figure 1.3-2 Genetic organization of the bacterial urease gene cluster. This diagram is taken from [15] and compares the genetic organization of the urease gene clusters from *Proteus mirabilis, Klebsiella aerogenes, Yersinia enterocolitica, Helicobacter pylori* and *Bacillus* sp. TB-90. The accessory gene *ureD* either precedes the structural genes *ureA*, *ureB*, *ureC* (*ureA* and *ureB* in *H. pylori*) or follows the accessory genes *ureE*, *ureF* and *ureG*.


P. mirabilis
ureR
ureD
ureA
ureB
ureC
ureE
ureF
ureG
34.0
31.0
11.0
12.2
61.0
17.9
23.0
22.4
K. aerogenes
ureD
ureA
ureB
ureC
ureE
ureF
ureG
29.8
11.1
11.7
60.3
17.6
25.2
21.9
Y. enterocolitica
ureA
ureB
ureC
ureE
ureF
ureG
ureD
11.1
17.9
61.0
29.5
25.0
24.1
36.4
H. pylori
ureA
ureB
ureI
ureE
ureF
ureG
ureH
26.5
60.3
21.7
19.5
28.6
21.7
29.6
Bacillus sp. TB-90
ureA
ureB
ureC
ureE
ureF
ureG
ureD
ureH
ureI
11.2
12.2
61.4
17.4
25.4
22.3
30.9
25.4
7.9
regulatory
accessory
structural
accessory genes

differed in activation properties from the UreD-apourease complex and was shown to exclude nickel availability to the active site. It is suggested that the binding of UreF modulates the UreD-apourease activation properties until the complete active complex is formed [28]. UreG contains a nucleotide-binding P-loop essential for *in vivo* activation and is present in a UreD-UreF-UreG-apourease (UreDFG-apourease) complex. This complex is thought to be the key *in vivo* urease activation machinery [29].

In vitro activation of the complex requires GTP and is stimulated by the addition of UreE [30, 31]. UreE contains a histidine (His)-rich C-terminus and can bind six equivalents of nickel. However, a truncated form without the His-rich region binds only two nickel ions but remains functionally active [32]. UreE is speculated to function as a metallochaperone actively delivering nickel to the UreDFG-apourease complex [33-35]. Activation of apourease can be achieved *in vitro* by CO_2 and nickel ions alone [36]. This activation involves CO_2 binding to an active site lysine ε amino group generating a ligand that facilitates productive nickel binding. The activation results and the *K. aerogenes* urease crystal structure are consistent with the formation of a lysine carbamate which bridges the two nickel ions present at the active site [37].

In soybean there are two structural genes, the embryo-specific urease, *Eu1*, and the ubiquitous urease, *Eu4* [21, 22]. Mutation in *Eu1* [21] or *Eu4* [22] affects the activity of only one urease, while the double mutant [22, 38] is essentially urease-negative. Two other genes, *Eu2* and *Eu3*, define a second class in which single gene lesions eliminate the activities of both urease isozymes, with little reduction in the level of the embryo

urease subunit [39]. This second class is analogous to the bacterial accessory genes that are required for the emplacement of nickel on urease for activation (Table 1.3-1). Eu3, which has been identified as UreG, is a nickel binding protein and is required for activation of the ubiquitous urease [23]. Eu3 has a His-rich N-terminus similar to the His-rich C-terminus of bacterial UreE [23, 35]. The enzyme hydrogenase also requires active site Ni. In *Rhizobium leguminosarum* [100] and *Bradyrhizobium japonicum* [101] HypB is involved in the assembly of the hydrogenase Ni metallocenter and is a Ni-binding GTPase with a His-rich N-terminal extension (which is not essential) similar to Eu3. The exact function of Eu2 is currently unknown but it appears that Eu2 encodes neither the accessory proteins UreD nor UreF [102]. It is possible that Eu2 encodes one of the other accessory proteins, such as UreE, and work is ongoing to identify its function.

The structural gene mutant *eu4* repeatedly revealed substantial background urease activity (15-40% of wild-type) in callus cultures and in the unifoliate leaves of seedlings, but not in other tissues that normally contain exclusively the ubiquitous urease [40]. When these plants were cured of their PPFMs, the level of background urease activity was reduced suggesting that this observed activity was bacterial [6]. This background activity did not resemble the ubiquitous urease by three biochemical criteria. However, it did resemble the activity of PPFMs isolated from the plant [6]. PPFMs isolated from soybean mutants *eu2* or *eu3* that have lost the activity of all soybean-encoded ureases, are urease-negative themselves while associated with the plant and transiently urease-

Table 1.3-1. Genes controlling urease production in soybean and in bacteria. The structural genes *Eu1* and *Eu4* in soybean are co-linear with *ureA*-*ureB*-*ureC* in bacteria. *Eu3* is the ortholog of the bacterial accessory gene *ureG*. The exact function of *Eu2* is unknown.

Soybean	Function	Bacteria
Eu1	Structural Gene (Embryo)	*ureA-ureB-ureC*
Eu4	Structural Gene (Ubiquitous)	*ureA-ureB-ureC*
Eu2	Accesory Gene	*unknown*
Eu3	Accesory Gene	*ureG*

negative in free-living culture [6]. The reacquisition of urease activity in culture was accelerated by nickel supplementation (Table 1.3-2).

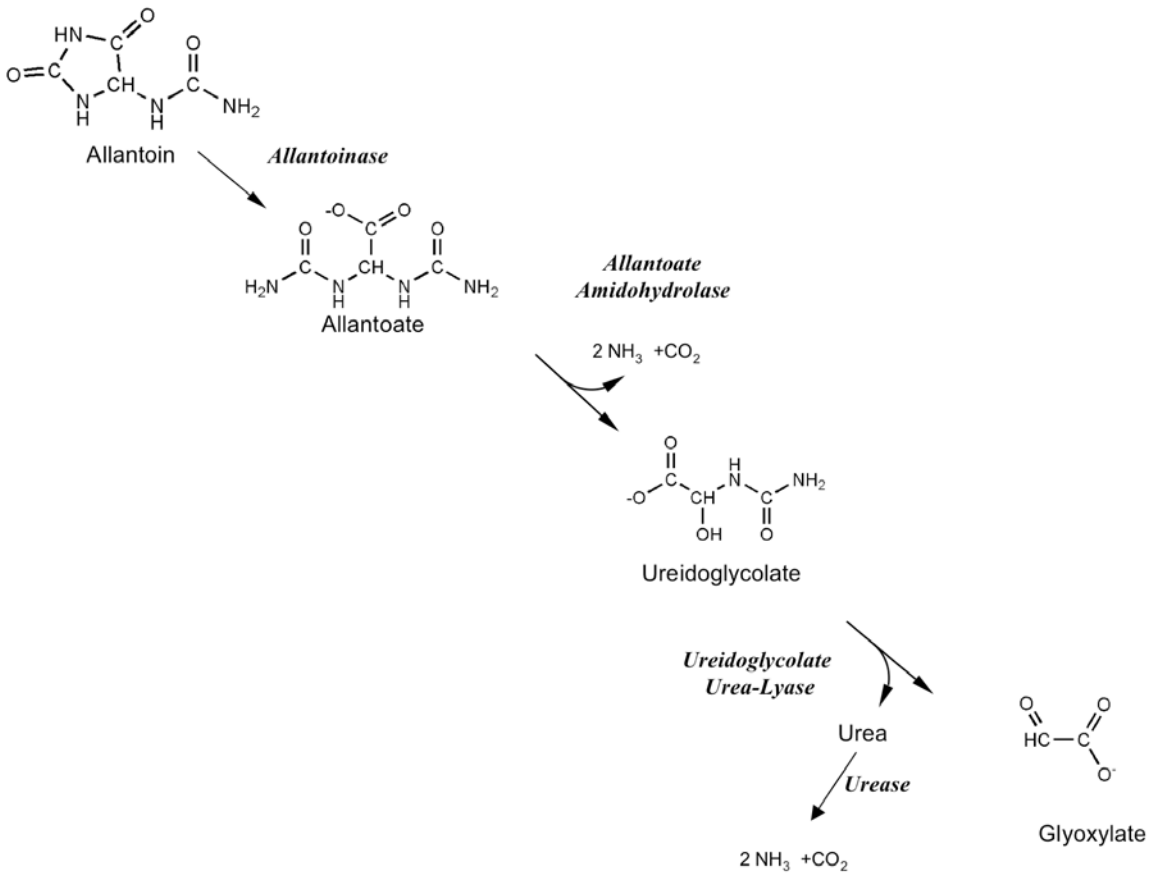
1.4 Ureide Degradation

Nitrogen-fixing legumes transport fixed nitrogen from the nodules to the aerial portions of the plant primarly as the ureides allantoin and allantoic acid [107]. The overall route of ureide degradation in soybean has recently been established [108]. Allantoin is first broken down to allantoate by *allantoinase*. Allantoate then has four possible routes to be broken down ultimately to glyoxylate, NH_3, and CO_2: one route goes through a urea intermediate, and another route releases NH_3 and CO_2 directly. Since at each enzymatic step either route is possible, there are four possible routes of degradation of allantoate. Todd and Polacco [109, 110] have shown that in soybean, *allantoate amidohydrolase* first breaks down allantoate to ureidoglycolate, 2 NH_3, and CO_2 and then in a subsequent step ureidoglycolate is broken down to urea and glyoxylate by *ureidoglycolate urea-lyase*. The enzyme urease then breaks down the urea formed to NH_3, and CO_2 (Figure 1.4-1). Recently, the first plant ammonia-generating *allantoate amidohydrolase* was identified and cloned from *Arabidopsis thaliana*, *AtAAH*, and was functionally expressed in yeast [110]. *Ataah* T-DNA insert lines accumulated higher allantoate levels than wildtype, supporting a block in allantoate catabolism, and results also suggest a possible ureidoglycine intermediate in this step [110]. The breakdown of allantoin follows the same pathway in *E. coli* [111] and *Bacillus subtilis* [112]. In PPFMs, the evidence suggests that the breakdown of ureides produces a urea intermediate at both steps:

Table 1.3-2. Urease activity of PPFMs isolated from the leaves of soybean urease mutants. The data are taken from Holland and Polacco [6]. The sources of PPFMs are: Williams 82 (progenitor), *eu2/eu2*, *eu3-e1/eu3-e1* and *eu4/eu4*. *eu2/eu2* and *eu3-e1/eu3-e1* are both soybean mutants in urease accessory genes responsible for activating soybean apourease and demonstrate pleiotropic urease-negative phenotypes. *eu4/eu4* is defective in the structural gene for the soybean ubiquitous urease.

Source of PPFMs	Urease Activity (nmol urea (min · OD_{550}) $^{-1}$)	
	Minus Ni	Added Ni
Williams 82 (progenitor)	21.0 ± 0.8	17.9 ± 2.4
*eu*2/*eu*2	3.9 ± 0.4	26.8 ± 3.3
*eu*3-e1/*eu*3-e1	4.6 ± 1.1	19.9 ± 0.5
*eu*4/*eu*4	16.3 ± 4.2	16.3 ± 1.4

Figure 1.4-1. Ureide degradation to glyoxylate, NH_3 , and CO_2 in soybean. This figure is adapted from Todd et al. [109]. In the first step of allantoate degradation, NH_3 and CO_2 are evolved directly. In the subsequent step, ureidoglycolate is cleaved to glyoxylate and urea, the latter converted to 2 NH_3 , and CO_2 by urease action.

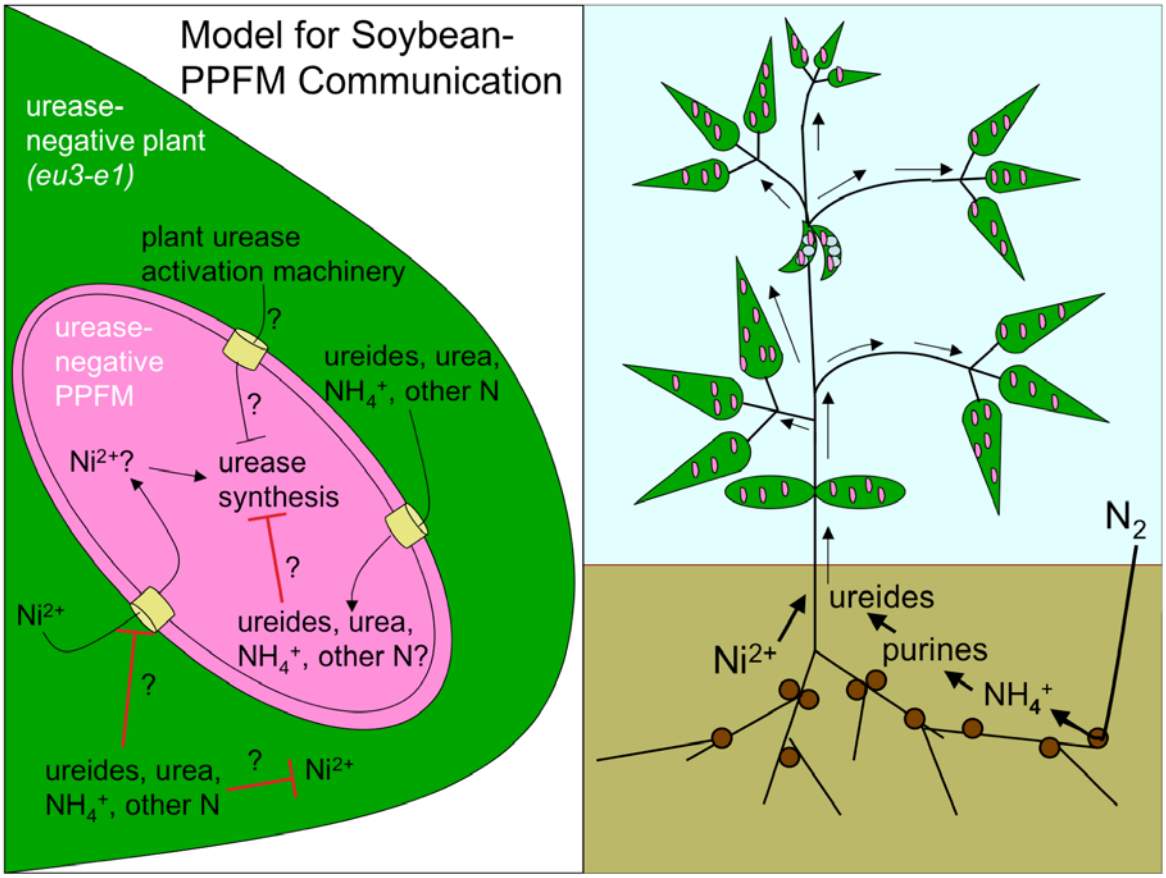

Allantoin
Allantoinase
Allantoate
Allantoate Amidohydrolase
2 NH3 +CO2
Ureidoglycolate
Ureidoglycolate Urea-Lyase
Urea
Urease
2 NH3 +CO2
Glyoxylate

allantoate to ureidogylcolate and ureidoglycolate to gyloxylolate. Growth tests of PPFMs on allantoin (and urea) as the sole nitrogen source +/- the potent urease inhibitor phenylphosphorodiamidate (PPD) [113] revealed growth on allantoin (and urea) minus PPD, but no growth on allantoin (or urea) plus PPD. These results indicate that urease was essential for the growth of PPFMs on allantoin or urea as sole nitrogen source.

2. STATEMENT OF PURPOSE

The purpose of my dissertation project was to investigate the complex association of *Methylobacterium* spp. (PPFMs) with plants. I focused on *Arabidopsis thaliana* and *Glycine max* (soybean), two dicots with which our lab works and which provide valuable mutants. While genetic and genomic analysis of each plant are advancing, little is known about the identity of the PPFMs that associate with the plants. To this end I established phylogenetic relationships of various PPFMs employed in the Polacco lab and elsewhere. Nitrogen metabolism is a point of interaction between PPFMs and the host plants. Especially intriguing is the ability of resident PPFMs to mimic the urease-negative phenotype of two urease-negative soybean host mutants. I developed a working model that suggests that there is some signal from the plant that either inhibits the production of the urease gene products in the associated bacteria or inhibits the function or transport of Ni^{2+} from the plant to the bacteria (Figure 2.1-1). This signal could be a nitrogenous signal (ureides, urea, ammonia) or simply a block in Ni^{2+} transport from the plant or plant cell to the associated bacteria. I attempted the recolonization of plants with PPFMs to determine how this affects urease activity in recovered isolates. Examination of urease expression *in planta* or in culture requires knowledge of the urease genes and the regulation of urease in the PPFMs. Urease expression is directly related to the role of urease in nitrogen assimilation. My studies led to the overall conclusion that urease is essential for assimilation of urea and of ureides, that urease has a constitutive basal level of expression and is "induced" by the ureide allantoin and "repressed" by the preferential nitrogen source, ammonium but that these nitrogenous signals are not responsible for the

Figure 2.1-1 Working Model of Signals Affecting Urease-Status of the Plant-Associated PPFMs. I developed a working model that suggests that there is some signal from the plant that either inhibits the production of the urease gene products in the associated bacteria or inhibits the function or transport of Ni^{2+} from the plant to the bacteria. This signal could be a nitrogenous signal (ureides, urea, ammonia) or simply a block in a transporter required to uptake Ni^{2+} from the plant cell to the associated bacteria.


Model for Soybean-PPFM Communication
urease-negative plant (eu3-e1)
plant urease activation machinery
urease-negative PPFM
ureides, urea, NH4+, other N
Ni2+?
urease synthesis
Ni2+
ureides, urea, NH4+, other N?
ureides, urea, NH4+, other N
Ni2+
N2
ureides
Ni2+
purines
NH4+

urease-negative status of the plant-associated PPFMs. Our working model suggests a block in nickel uptake necessitating examination of nickel content in these bacteria. In the course of these studies it became obvious that the interactions between PPFMs and the host plant is distinguishable between *Arabidopsis* and soybean.

3. MATERIALS AND METHODS

3.1 Bacterial Strains and Growth Conditions

Bacterial strains employed in this study are listed in Table 3.1-1. *Methylobacterium extorquens* AM1 was obtained from Mary Lidstrom and was first described by Peel and Quale [114]. Plant-associated PPFM isolates from soybean, *Arabidopsis*, barley and maize were previously recovered as described by Holland and Polacco [6], by plating ground leaves onto ammonium mineral salts (AMS) medium with methanol as the sole carbon and energy source. The *M.* broccoli isolate was a gift of Mark A. Holland, and is first described here. *M. extorquens*-OR18 was a gift of Mary Lidstrom. The urease-negative mutant (*M.* sp. ex15) is described in detail below. The Km^r leaf-derived strains were constructed by performing a bi-parental mating of the recipient *Methylobacterium* strain and an *E. coli* strain (S17-1) carrying the plasmid pSUP5011 containing mobilizable transposon Tn*5*-mob [115]. The cultures were grown to mid-log phase and the recipient *Methylobacterium* strain and the *E.coli* strain carrying the plasmid pSUP5011 were filtered sequentially onto a sterile 0.45 μm Metricel® membrane filter (Pall Life Sciences) in a 5:1 (v/v) ratio. The filter was incubated at 30° C for 18 h on solidified nutrient broth (NB) (Difco Laboratories). The filters were then vortexed in 5 mL AMS media and dilutions were plated onto AMS medium with kanamycin (50 μg/mL) for selection of Km^r exconjugants. Putative exconjugants were confirmed by single-colony transfer to AMS with kanamycin to ensure the absence of *E. coli* donor cells. The leaf isolate from the urease-negative soybean mutant *eu3-e1/eu3-e1* was selected as previously described [6] on AMS containing 30 μg/mL cycloheximide (to

Table 3.1-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or Reference
Bacteria		
E.coli strains		
DH5α	F$^-$Δ(*lacZYA-argF*)U169 *supE44 hsdR17*(r_B^- m_B^+) *recA1 gyrA96 endA1 thi-1 relA1 deoR* ϕ80d(*lacΔZ*)M15 lamda$^-$	
DH10B	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara,leu*) 7967 *ara*D139 *gal*U *gal*K *nup*G *rps*L	
Methylobacterium strains		
M. extorquens	ATCC type strain #43645	ATCC[1]
M. extorquens AM1	Wild type, Rif^r	[48, 114]
M. sp. soyleaf2	Soybean leaf isolate 2	[6]
M. sp. ex15	*ureC*::pAYC61, Tc^r	This study
M. sp. *At*leaf1	Arabidopsis leaf isolate 1	[7]
M. sp. barley1	Barley leaf isolate 1	[7]
M. sp. maize1	Maize leaf isolate 1	[7]
M. sp. broccoli1	Broccoli isolate 1	Holland, M.A.
M. sp. *At*leaf1-65	Arabidopsis leaf isolate 1, Km^r, Tn*5*-mob	Polacco, J.C.
M. extorquens-OR18	Km^r	Lidstrom, M.E.
M. sp. soyleaf2-140	Soybean leaf isolate 2, Km^r, Tn*5*-mob	This Study
M. sp. *eu3-e1*leaf	Leaf isolate from *eu3-e1/eu3-e1*	This Study
M. sp. *eu3-e1*leaf-C4	Leaf isolate from *eu3-e1/eu3-e1*, Km^r, Tn*5*-mob	This Study
M. sp. *eu3-e1*leaf-C5	Leaf isolate from *eu3-e1/eu3-e1*, Km^r, Tn*5*-mob	This Study
Plasmids		
pGEM-T-easy	Ap^r *lacZ*'	Promega, Inc.
pSBW1	pGEM-T-easy::593bp *ureC* fragment	This study
pAYC61	Ap^r, Tc^r, *mob*$^+$ IncColE1	[42]
pSBW4	pAYC61::593bp *ureC* fragment	This study
pRK2013	ColE1-Tra (RK2)$^+$ Km^r	[136]
pSUP5011	Ap^r, Cm^r, Kan^r, Tn*5*-mob	[115]
pACYC184	Tc^r, Cm^r	New England Biolabs, Inc.

[1]American Type Culture Collection (ATCC), Manassas, VA

prevent fungal contamination). PPFM liquid cultures were started from a single colony and grown at 30° C, 250 rpm in 50 mL AMS in 250 mL baffle flasks to stationary phase. Antibiotics were used in the following concentrations: tetracycline, 15 μg/mL; rifampicin, 100 μg/mL; kanamycin, 50 μg/mL. When the nitrogen (N) source was varied, the various media always contained equal milliequivalents N/L (9.3 meq N/L). Urea, allantoin, arginine and hydantoin were all filter sterilized. CitB (10 mM potassium citrate, 10 μM $NiSO_4$, pH 6.0) was used where nickel supplementation was tested.

E. coli cultures were grown on solidified Luria broth (LB) medium [117] or liquid LB medium at 37° C and 250 rpm. Overnight cultures were 5 mL/16x150 mm tubes and larger cultures were 100 mL/500 mL flask. Antibiotics were used in the following concentrations: tetracycline, 15 μg/mL; kanamycin, 50 μg/mL; ampicillin, 100 μg/mL; chloramphenicol, 40 μg/mL.

E. coli was transformed by the heat shock method as described by Sambrook et al. [117]. Briefly, DNA (usually 5 μL of a ligation reaction) was added to 100 μL of chemically competent cells and incubated on ice for 20 minutes. The cells were heat shocked at 42° C for 2 min and placed back on ice for 1-2 min. Pre-warmed (37° C) SOC medium (850 μL) was added and the cells were incubated at 37° C at 250 rpm, 1 h. Dilutions were plated to LB medium with the appropriate antibiotic. Plasmids were transferred from *E. coli* into *Methylobacterium* by tri-parental mating as described in [42]. *Tra* and *Mob* functions were carried on pRK2013 and pAYC61, respectively (Table 3.1-1). The cultures were grown to mid-log phase and the recipient *Methylobacterium* strain, an *E.*

coli strain carrying the mobilization helper plasmid pRK2013, and the *E. coli* donor strain carrying the suicide plasmid pAYC61 were filtered sequentially onto a sterile 0.45 µm Metricel® membrane filter (Pall Life Sciences) in a 5:1:1 ratio. The filter was incubated at 30° C for 18 h on nutrient broth (NB) media (Difco Laboratories). The filters were vortexed in 5 mL AMS medium and dilutions were plated onto AMS medium with tetracycline for selection. Putative exconjugants were confirmed by single-colony transfer to AMS with tetracycline to ensure the absence of donor cells which do not grow on AMS.

3.2 Plant Material and Growth Conditions

Wildtype soybean (*Glycine max*) *cv* Williams 82 was obtained from commercial sources. The urease mutants *eu4/eu4* [40], *eu2/eu2* and *eu3-e1/eu3-e1* [39] were previously described. Seeds were germinated at 27° C in the dark in rolled germination paper in distilled water and planted in a 50:50 Promix:soil mixture under greenhouse conditions 26/21° C day/night with a 16 h photoperiod. Wildtype *Arabidopsis thaliana* was ecotype Columbia. Urease-negative *Arabidopsis* mutants *At-ure-1, At-ureD-1, At-ureF-1* and *At-ureG-2* were a gift of Claus-Peter Witte [116]. *Arabidopsis* was germinated on solidified ½ MS medium and grown at 22° C with a 16 h photoperiod. Soybean callus was induced from surface-sterilized hypocotyls on R3 medium as described [40] and transferred for maintenance on S3 medium in which hormones were 5.5×10^{-4} mg/L each 6-benzylaminopurine and 2,4-dichlorophenoxyacetic acid.

3.3 DNA Manipulations

Plasmids employed in this study are listed in Table 3.1-1. Large-scale plasmid preparations were performed as described by Sambrook et al. [117] with slight modifications. Cells from a 100 mL culture were pelleted by centrifugation and washed in ice-cold STE (0.1 M NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The washed pellet was then suspended in 3.6 mL GTE (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA, pH 8.0) to which 0.4 mL freshly prepared 10 mg/mL lysozyme (in 10mM Tris-Cl, pH 8.0) solution was added. Eight milliliters 0.2 N NaOH/1% (w/v) SDS (sodium dodecyl sulfate) was added and contents were mixed by gentle inversion and incubated at room temperature for 10 min. Four milliliters of an ice-cold potassium acetate solution (3 M with respect to potassium and 5 M with respect to acetate) was added, the contents were mixed by shaking and placed on ice for 10 min. Upon centrifugation the supernatant was filtered through cheesecloth. Isopropanol (0.6 vol) was added to the filtered supernatant, mixed by inversion and incubated for 10 min at room temp. Nucleic acids were recovered by centrifugation, washed with 70% ethanol, air-dried and finally dissolved in 0.6 mL TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The nucleic acid was treated with RNase A (1.2 uL of a 10 mg/mL stock) at 37° C for 30 min. The preparation was extracted once with an equal volume phenol/chloroform, then again with an equal volume of chlorform and precipitated by adding 1/10 vol of 3 M NaOAc, pH 5.5 and 2 vol ice-cold 95% ethanol. DNA was pelleted by centrifugation, washed with 70% ethanol, air-dried and suspended in TE. Small-scale plasmid preparations were prepared with the Wizard Plus SV miniprep kit (Promega Inc.), the FastPlasmid miniprep kit (Eppendorf)

or the QIAprep spin miniprep kit (Qiagen Sciences) following the manufactures directions.

Genomic DNA was prepared from PPFMs as follows: Cells from 100 mL culture in stationary phase were pelleted by centrifugation and resuspended in 1 mL GTE. Then 750 µL 1-butanol was added, mixed by vortexing and incubated for 5 min. Cells were again pelleted and washed in TE, then resuspended in 1 mL GTE to which 80 µL fresh 100 mg/mL lysozyme solution was added along with 2 µL 10 mg/mL RNase A. After 1-2 h incubation at 37° C, 120 µL 20% (w/v) SDS and 100 µL 20 mg/mL Proteinase K were added and the preparation was incubated for an additional 30-60 min at 50° C. The preparation was extracted once with equal volume chloroform and then brought to 100 mM NH_4OAc by addition of 7.5 M NH_4OAc, pH 7.0. DNA was precipitated with 2/3 vol isopropanol, spooled out with a glass hook, rinsed in 70% ethanol, air-dried and resuspended in 500 µL TE. It was extracted once with equal volume phenol/chloroform, then again with equal volume chloroform and reprecipitated with 2 vol ice-cold 95% ethanol and 0.3 mM NaOAc, pH 5.5. The yield was approximately 100 µg PPFM DNA per 100 mL culture and the DNA was stored as a 1 µg/µL solution in TE at -20° C.

Restriction digests were performed by the manufacturers' instructions (Promega, Inc.; New England Biolabs; Fermentas). Fragments were analyzed by horizontal agarose gel electrophoresis in 1% (w/v) agarose in TAE (0.4 M Tris-acetate, 0.001 M EDTA, pH 8.5) buffer with 0.5 µg/mL ethidium bromide for visualization of the nucleic acids under ultraviolet light. Restriction fragments were recovered from agarose using either the

GibcoBRL Concert Matrix Gel extraction system (Gibco life technologies) or the UltraFree-DA Centrifugal Filter Device (Millipore Corp.). Ligations were performed following the manufacturer's instructions using T4 DNA ligase (Promega Inc.; Fermentas Life Sciences).

3.4 PCR amplification

Reactions were carried out under the following conditions: 50 mM KCl, 10 mM Tris-Cl, 2 mM $MgCl_2$, 1X TaqMaster PCR enhancer (Eppendorf, Inc.), *Taq* Polymerase following the manufacturers' instructions (Promega, Inc., Eppendorf, Inc., Sigma-Aldrich, Inc., Takara Bio Inc.), 0.25 mM deoxynucleotide (dNTP) mix and 0.6-1 mM of each primer. Reaction cycles were as follows: 96° C for 5 min followed by 35 cycles of 96° C for 1 min, 55° C for 1 min, 72° C for 1.5 min and a final extension at 72°C for 10 min. Products were analyzed by agarose gel electrophoresis and fragments of interest were gel purified as above (chapter 3.3) and cloned into the plasmid pGEMT-easy (Promega, Inc.) for replication in *E. coli.*

3.5 DNA sequencing and analysis

DNA was sequenced at the University of Missouri DNA Core Facility by using a 3730 96-capillary DNA Analyzer with ABI Big Dye Terminator cycle sequencing chemistry (Applied Biosystems, Inc.). The resultant sequence was analyzed *in silico* using VectorNTI suite version 7.1 (InforMax, Inc.).

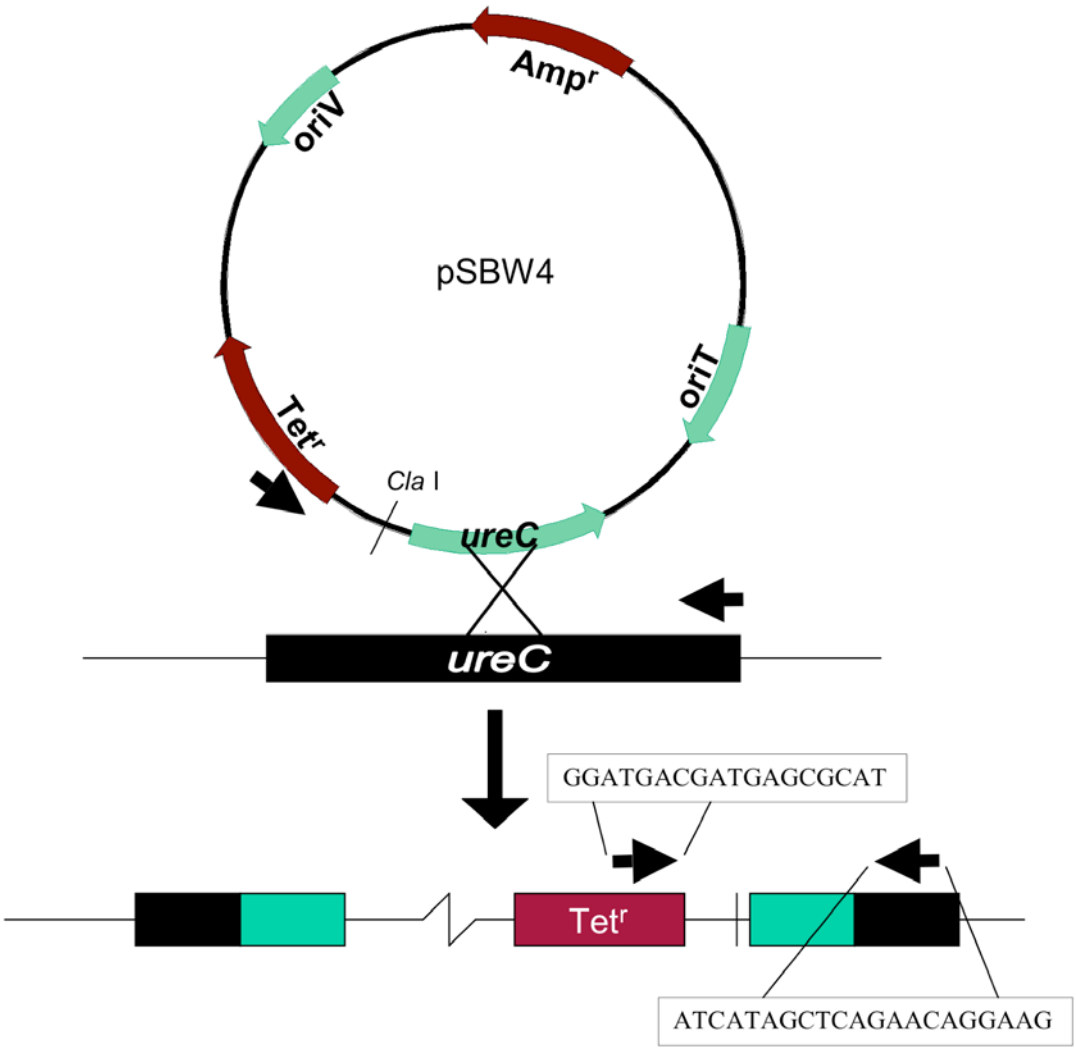
3.6 Generation of urease-negative PPFM

A 593 bp internal fragment of the *Methylobacterium* structural gene *ureC* was generated by PCR using primers (sense primer sequence: TCCGACAGTCAGGCGAT; antisense primer sequence: TCGCAGACCAGCAATTCG) designed from the *Methylobacterium extorquens* AM1 genome sequence [118]. The resultant fragment was first ligated into pGEMT-easy to generate plasmid pSBW1, then excised with *Eco*RI and cloned into the *Eco*RI site of the multiple cloning region of the suicide plasmid pAYC61 to generate plasmid pSBW4 (Figure 3.6-1). The plasmid pSBW4 was mobilized into *Methylobacterium* sp. Soyleaf2 by tri-parental mating described above. Tetracycline-resistant exconjugates were isolated and integration of the plasmid into the *ureC* gene by single homologous crossover was confirmed by PCR analysis using primers to the vector (GGATGACGATGAGCGCAT) and to the genomic region of *ureC* outside of the internal fragment (ATCATAGCTCAGAACAGGAAG) (Figure 3.6-1).

3.7 Urease Assay

A qualitative urease assay for bacterial cells was adapted from the seed chip assay previously described [39]. Briefly, a loop or 0.1 mL pelleted cells was incubated in a solution of cresol red in phosphate buffer (0.1 M urea, 5 μg/mL cresol red, 0.02% (w/v) sodium azide, 10 mM potassium phosphate, 1 mM EDTA), pH 7.0. After 18 h at 30° C, urease-positive bacteria turn the solution from yellow to pink by alkalinization due to ammonia production. Quantitative urease assays were performed as described [119] based on the release of $^{14}CO_2$ from [^{14}C]urea. PPFM cells were pelleted by centrifugation, washed, resuspended in 0.1 M Tris-maleate, 1 mM EDTA, pH 7.0 and

Figure 3.6-1 Diagram outlining the strategy for generation of *ureC* interruption mutant in *M.* sp. soyleaf2. Integration of the plasmid will result from a single homologous recombination event. Arrows indicate PCR primers used to confirm interruption of the gene in tetracycline-resistant exconjugants. The sequence of these primers are shown.


Amp^r
oriV
pSBW4
oriT
Tet^r
Cla I
ureC
ureC
GGATGACGATGAGCGCAT
Tet^r
ATCATAGCTCAGAACAGGAAG

sonicated 1 min at 3 Watts on a Branson Digital Sonifier (VWR Scientific). Triplicate aliquots of 0.1 ml PPFM cells were incubated in 1 mL 10 mM [^{14}C]urea (30 uCi•mmol^{-1}), 0.1 M Tris-maleate, 1 mM EDTA, pH 7.0 at 37° C, 1 h. Specific activity was expressed as nmoles urea hydrolyzed (OD_{550} h)$^{-1}$.

3.8 Allantoin Utilization

To determine the amount of allantoin utilized by PPFMs in culture, an alkaline/acid hydrolysis reaction followed by a colorimetric determination of glyoxylate was performed based on Vogels and Van Der Drift [120]. The supernatant from PPFM cultures in early stationary phase was collected by centrifugation and 0.05 mL was added to 0.950 mL sterile distilled water to which 0.25 mL 0.5 M NaOH was added. The sample was heated in a boiling water bath 8 min, allowed to cool to room temp and 0.25 mL 0.65 M HCl was added, heated in a boiling water bath 4 min and allowed to cool to room temp. To this mixture, 0.25 mL 1 M potassium phosphate buffer, pH 7.0 and 0.25 mL (w/v) 0.33% phenylhydrazine was added and incubated 10 min. After incubation, 1 mL cHCl and 0.25 mL 1.67% (w/v) potassium ferricyanide were sequentially added and mixed. The mixture was incubated for 10 min, and the absorption at 520 nm was compared to standard curves of known allantoin values (0-100 nmol) treated in the same manner.

3.9 Plant Inoculation with PPFMs

Axenic callus cultures were inoculated with PPFMs by coating newly transferred callus with 20 μL of an early stationary-phase culture. Seeds were imbibed in an early-stationary phase PPFM culture at room temperature for 5 hours with gentle shaking as described [8]. After imbibition, seeds were drained and allowed to germinate 5 d on germination paper at 27° C in the dark.

3.10 Seed Sterilization Treatments

To remove PPFMs and other microorganisms from seeds, the following sterilization treatments were performed. For soybean, the seeds were placed in a dry-heat oven at 50° C 48 h as described [6]. For *Arabidopsis* seeds, dry-heat treatments at 50, 65, 85 and 100° C for 1-12 d were used. Surface sterilization treatments were adapted from Clough and Bent [103]. Briefly, seeds were treated with either ethanol/bleach (70% ethanol, 5 min/50% bleach 3 min, rinsed at least 5 times with sterile distilled water), bleach alone (50% bleach 5 min rinsed at least 5 times with sterile distilled water) and the chlorine vapor method. In the latter, *Arabidopsis* seeds were placed in an open centrifuge tube in a glass jar with a 250 mL beaker containing 100 mL bleach to which 3 mL cHCl was added and the jar quickly sealed with a lid wrapped in parafilm. Seeds were sterilized by the vapor method for 4-16 h. Sterilization by microwave treatment was adapted from Holland and Polacco [3]. *Arabidopsis* seeds were placed in a paper envelope inside a glass tray and microwaved on full power for 10 to 180 min.

3.11 16S rDNA Analysis

Primers were designed based on Weisburg et al. [121] to amplify near-full length 16S ribosomal DNA (rDNA) from the *Methylobacterium* strains listed in Table 3.1-1. Primers fD1 and rD1 were modified so as not to include the linker sequences containing restriction sites (sense primer sequence: AGAGTTTGATCCTGGCTCAG; antisense primer sequence: AAGGAGGTGATCCAGCC, respectively). The fragments were amplified by PCR, cloned into pGEMT-easy and sequenced at the University of Misouri DNA Core Facility as described above. Sequences were compared to known *Methylobacterium* 16s rDNAs deposited in GenBank (Genus, species, strain (GenBank accession number)): *M*. sp. CM4 (AF198624); *M. nodulans*, strain ORS 1924 (AF220762); *M. nodulans*, strain ORS 2060 (AF220763); *M. dichlorometanicum* (AF227128); *M. podarium* (AF514774); *M. fujisawaense*, strain DSM 5686 (AJ250801); *M. mesophilicum*, strain JCM 2829 (partial) (AJ400919); *M. portugalicum* (AY009403); *M. suomicum* (partial) (AY009404); *M. extorquens*, strain JCM 2802 (D32224); *M. mesophilicum*, strain JCM 2829 (D32225); *M. organophilum*, strain JCM 2833 (D32226); *M. radiotolerans*, strain JCM 2831 (D32227); *M. rhodesianum*, strain JCM 2810 (D32228); *M. rhodinum*, strain JCM 2811 (D32229); *M. zatmanii*, strain JCM 2819 (D32230); *M*. sp. F48 (D32236); *M. extorquens* (M29027); *M. organophilum* (M29028); *M*. sp. (M29029) *in silico* using VectorNTI suite version 7.1 (InforMax, Inc.).

4. RESULTS AND DISCUSSION

4.1 *Methylobacterium* spp. phylogeny

Methylobacterium isolates, specifically PPFMs, are identified by their pink color and growth with methanol as sole carbon and energy source. Sequencing of 16S ribosomal DNA is often used to classify the bacterial isolates into genus and species [86, 87, 124, 125, 126, 127]. However, I have found that there are many errors in the sequences deposited in GenBank, and they can affect how the organisms are classified. To this end, I set out to sequence near full length 16S rDNA from the *Methylobacterium* strains listed in Table 3.1-1 to classify the strains used in this study and to assist the community in the proper species classification. Sequences of 16S rDNA of well-characterized *M. extorquens* and *M. extorquens* AM1, as well as lab isolates *M*. sp. soyeaf2, *M*. sp. *At*leaf1, *M*. sp. barley1, *M*. sp. maize1, and *M*. sp. broccoli1 were compared and a phylogenetic tree was generated (Figure 4.1-1). The sequences were 99.6% similar with 93.8% identity (Figure 4.1-2). Within these seven isolates, it appears that the soyleaf2 isolate clustered with *M. extorquens* and *M. extorquens* AM1 and the barley, maize and broccoli isolates all clustered together. These seven isolates were aligned with 15 isolates representing 12 different species whose sequences in GenBank were complete and without obvious errors. The resultant phylogenetic tree is presented in Figure 4.1-3. *At*leaf1 and soyleaf2 clustered with *extorquens* AM1 and *extorquens* clustered with *zatmanii* and sp. CM4 suggesting that these four strains are closely related and may all be best fit into the species '*extorquens*' with strain designations. The barley1, maize1 and broccoli1 sequences all clustered with *mesophilicum, radiotolerans* and *fujisawaense*

Figure 4.1-1 Phylogenetic Tree of *Methylobacterium* strains based on 16S rDNA sequences. 16s rDNA sequences of *M. extorquens*, *M. extorquens* AM1, *M*. sp. soyeaf2, *M*. sp. *At*leaf1, *M*. sp. barley1, *M*. sp. maize1, and *M*. sp. broccoli1 were compared. The tree was generated using VectorNTI v.10.3 that utilizes the Neighbor Joining algorithm of Saitou and Nei [137] based on a sequence distance method. Distance values, based on nucleotide substitutions, are provided in parenthesis.

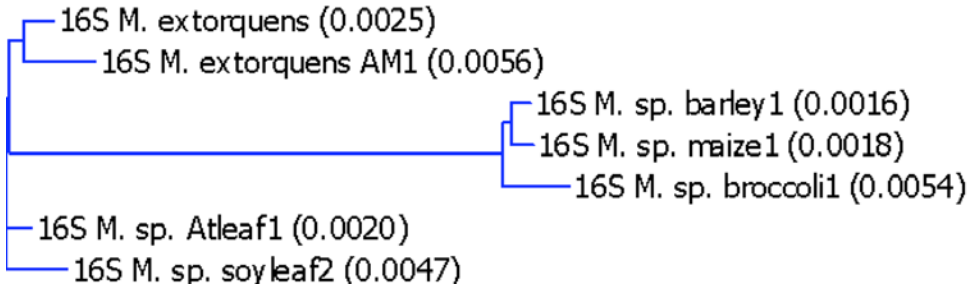

16S M. extorquens (0.0025)
16S M. extorquens AM1 (0.0056)
16S M. sp. barley1 (0.0016)
16S M. sp. maize1 (0.0018)
16S M. sp. broccoli1 (0.0054)
16S M. sp. Atleaf1 (0.0020)
16S M. sp. soyleaf2 (0.0047)

Figure 4.1-2 Multiple sequence alignment of 16S rDNA sequences from *Methylobacterium* strains. 16S rDNA sequences of *M. extorquens, M. extorquens* AM1, *M*. sp. soyeaf2, *M*. sp. *At*leaf1, *M*. sp. barley1, *M*. sp. maize1, and *M*. sp. broccoli1 were compared using the Multiple Alignment algorithm in VectorNTI v.10.3.

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                                1                                                 50
    16S M. extorquens     (1) AGAGTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCAGGCTTAACACATG
16S M. extorquens AM1     (1) AGAGTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCAGGCTTAACACATG
   16S M. sp. barley1     (1) AGAGTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCAGGCTTAACACATG
    16S M. sp. maize1     (1) AGAGTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCAGGCTTAACACATG
 16S M. sp. broccoli1     (1) AGAGTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCAGGCTTAACACATG
   16S M. sp. Atleaf1     (1) AGAGTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCAGGCTTAACACATG
  16S M. sp. soyleaf2     (1) AGAGTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCAGGCTTAACACATG
            Consensus     (1) AGAGTTTGATCCTGGCTCAGAGCGAACGCTGGCGGCAGGCTTAACACATG
                               51                                                100
    16S M. extorquens    (51) CAAGTCGAACGGGCACCTTCGGGTGTCAGTGGCAGACGGGTGAGTAACAC
16S M. extorquens AM1    (51) CAAGTCGAACGGGCTTCTTCGGAAGTCAGTGGCAGACGGGTGAGTAACAC
   16S M. sp. barley1    (51) CAAGTCGAGCGGGC-CCTTCGGG-GTCAGCGGCGGACGGGTGAGTAACGC
    16S M. sp. maize1    (51) CAAGTCGAGCGGAC-CTTTCGGG-GTCAGCGGCGGACGGGTGAGTAACGC
 16S M. sp. broccoli1    (51) CAAGTCGAGCGGGC-CCTTCGGG-GTCAGCGGCGGACGGGTGAGTAACGC
   16S M. sp. Atleaf1    (51) CAAGTCGAACGGGCTTCTTCGGAAGTCAGTGGCAGACGGGTGAGTAACAC
  16S M. sp. soyleaf2    (51) CAAGTCGAACGGGCACCTTCGGGTGTCAGTGGCAGACGGGTGAGTAACAC
            Consensus    (51) CAAGTCGAACGGGC CCTTCGGG GTCAGTGGCAGACGGGTGAGTAACAC
                              101                                               150
    16S M. extorquens   (101) GTGGGAACGTACCCTTCGGTTCGGAATAACTCAGGGAAACTTGAGCTAAT
16S M. extorquens AM1   (101) GTGGGAACGTGCCCTTCGGTTCGGAATAACTCAGGGAAACTTGAGCTAAT
   16S M. sp. barley1    (99) GTGGGAACGTGCCTTCCGGTTCGGAATAACCCTGGGAAACTAGGGCTAAT
    16S M. sp. maize1    (99) GTGGGAACGTGCCTTCCGGTTCGGAATAACCCTGGGAAACTAGGGCTAAT
 16S M. sp. broccoli1    (99) GTGGGAACGTGCCTTCTGGTTCGGAATAACCCTGGGAAACTAGGGCTAAT
   16S M. sp. Atleaf1   (101) GTGGGAACGTGCCCTTCGGTTCGGAATAACTCAGGGAAACTTGAGCTAAT
  16S M. sp. soyleaf2   (101) GTGGGAACGTGCCCTTCGGTTCGGAATAACTCAGGGAAACTTGAGCTAAT
            Consensus   (101) GTGGGAACGTGCCCTTCGGTTCGGAATAACTCAGGGAAACTTGAGCTAAT
                              151                                               200
    16S M. extorquens   (151) ACCGGATACGCCCTTTTGGGGAAAGGTTTACTGCCGAAGGATCGGCCCGC
16S M. extorquens AM1   (151) ACCGGATACGCCCTTATGGGGAAAGGTTTACTGCCGAAGGATCGGCCCGC
   16S M. sp. barley1   (149) ACCGGATACGCCCTTTTGGGGAAAGGTTTACTGCCGGAAGATCGGCCCGC
    16S M. sp. maize1   (149) ACCGGATACGCCCTTATGGGGAAAGGTTTACTGCCGGAAGATCGGCCCGC
 16S M. sp. broccoli1   (149) ACCGGATACGCCCTTTTGGGGAAAGGTTTACTGCCGGAAGATCGGCCCGC
   16S M. sp. Atleaf1   (151) ACCGGATACGCCCTTATGGGGAAAGGTTTACTGCCGAAGGATCGGCCCGC
  16S M. sp. soyleaf2   (151) ACCGGATACGCCCTTACGGGGAAAGGTTTACTGCCGAAGGATCGGCCCGC
            Consensus   (151) ACCGGATACGCCCTTATGGGGAAAGGTTTACTGCCGAAGGATCGGCCCGC
                              201                                               250
    16S M. extorquens   (201) GTCTGATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGT
16S M. extorquens AM1   (201) GTCTGATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGT
   16S M. sp. barley1   (199) GTCTGATTAGCTAGTTGGTGGGGTAACGGCCCACCAAGGCGACGATCAGT
    16S M. sp. maize1   (199) GTCTGATTAGCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGT
 16S M. sp. broccoli1   (199) GTCTGATTAGCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGT
   16S M. sp. Atleaf1   (201) GTCTGATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGT
  16S M. sp. soyleaf2   (201) GTCTGATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGT
            Consensus   (201) GTCTGATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCGACGATCAGT
                              251                                               300
    16S M. extorquens   (251) AGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAG
16S M. extorquens AM1   (251) AGCTGGTCTGAGAGGATGATCAGCCGCACTGGGACTGAGACACGGCCCAG
   16S M. sp. barley1   (249) AGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAG
    16S M. sp. maize1   (249) AGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAG
 16S M. sp. broccoli1   (249) AGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAG
   16S M. sp. Atleaf1   (251) AGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAG
  16S M. sp. soyleaf2   (251) AGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAG
            Consensus   (251) AGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAG
                              301                                               350
    16S M. extorquens   (301) ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCT
16S M. extorquens AM1   (301) ACTCCTACGGGGGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCT
   16S M. sp. barley1   (299) ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCT
    16S M. sp. maize1   (299) ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCT
 16S M. sp. broccoli1   (299) ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCT
   16S M. sp. Atleaf1   (301) ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCT
  16S M. sp. soyleaf2   (301) ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCT
            Consensus   (301) ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCT
                              351                                               400
    16S M. extorquens   (351) GATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCT
16S M. extorquens AM1   (351) GATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCT
   16S M. sp. barley1   (349) GATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCT
    16S M. sp. maize1   (349) GATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCT
 16S M. sp. broccoli1   (349) GATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCT
   16S M. sp. Atleaf1   (351) GATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCT
  16S M. sp. soyleaf2   (351) GATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCT
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           Consensus   (351) GATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCT
                             401                                             450
    16S M. extorquens  (401) TTTGTCCGGGACGATAATGACGGTACCGGAAGAATAAGCCCCGGCTAACT
16S M. extorquens AM1  (401) TTTGTCCGGGACGATAATGACGGTACCGGAGGAATAAGCCCCGGCTAACT
   16S M. sp. barley1  (399) TTTATCCGGGACGATAATGACGGTACCGGAGGAATAAGCCCCGGCTAACT
    16S M. sp. maize1  (399) TTTATCCGGGACGATAATGACGGTACCGGAGGAATAAGCCCCGGCTAACT
 16S M. sp. broccoli1  (399) TTTATCCGGGACGATAATGACGGTACCGGAGGAATAAGCCCCGGCTAACT
   16S M. sp. Atleaf1  (401) TTTGTCCGGGACGATAATGACGGTACCGGAAGAATAAGCCCCGGCTAACT
  16S M. sp. soyleaf2  (401) TTTGTCCGGGACGATAATGACGGTACCGGAAGAATAAGCCCCGGCTAACT
           Consensus   (401) TTTGTCCGGGACGATAATGACGGTACCGGAGGAATAAGCCCCGGCTAACT
                             451                                             500
    16S M. extorquens  (451) TCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATC
16S M. extorquens AM1  (451) TCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATC
   16S M. sp. barley1  (449) TCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATC
    16S M. sp. maize1  (449) TCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATC
 16S M. sp. broccoli1  (449) TCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATC
   16S M. sp. Atleaf1  (451) TCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATC
  16S M. sp. soyleaf2  (451) TCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATC
           Consensus   (451) TCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATC
                             501                                             550
    16S M. extorquens  (501) ACTGGGCGTAAAGGGCGCGTAGGCGGCCGATTAAGTCGGGGGTGAAAGCC
16S M. extorquens AM1  (501) ACTGGGCGTAAAGGGCACGTAGGCGGCCGATTAAGTCGGGGGTGAAAGCC
   16S M. sp. barley1  (499) ACTGGGCGTAAAGGGCGCGTAGGCGGCGTTTTAAGTCGGGGGTGAAAGCC
    16S M. sp. maize1  (499) ACTGGGCGTAAAGGGCGCGTAGGCGGCGTTTTAAGTCGGGGGTGAAAGCC
 16S M. sp. broccoli1  (499) ACTGGGCGTAAAGGGCGCGTAGGCGGCGTTTTAAGTCGGGGGTGAAAGCC
   16S M. sp. Atleaf1  (501) ACTGGGCGTAAAGGGCGCGTAGGCGGCCGATTAAGTCGGGGGTGAAAGCC
  16S M. sp. soyleaf2  (501) ACTGGGCGTAAAGGGCGCGTAGGCGGCCGATTAAGTCGGGGGTGAAAGCC
           Consensus   (501) ACTGGGCGTAAAGGGCGCGTAGGCGGCCGATTAAGTCGGGGGTGAAAGCC
                             551                                             600
    16S M. extorquens  (551) TGTGGCTCAACCACAGAATTGCCTTCGATACTGGTTGGCTTGAGACCGGA
16S M. extorquens AM1  (551) TGTGGCTCAACCACAGAATTGCCTTCGATACTGGTTGGCTTGAGACCGGA
   16S M. sp. barley1  (549) TGTGGCTCAACCACAGAATGGCCTTCGATACTGGGACGCTTGAGTATGGT
    16S M. sp. maize1  (549) TGTGGCTCAACCACAGAATGGCCTTCGATACTGGGACGCTTGAGTATGGT
 16S M. sp. broccoli1  (549) TGTGGCTCAACCACAGAATGGCCTTCGATACTGGGACGCTTGAGTATGGT
   16S M. sp. Atleaf1  (551) TGTGGCTCAACCACAGAATTGCCTTCGATACTGGTTGGCTTGAGACCGGA
  16S M. sp. soyleaf2  (551) TGTGGCTCAACCACAGAATTGCCTTCGATACTGGTTGGCTTGAGACCGGA
           Consensus   (551) TGTGGCTCAACCACAGAATTGCCTTCGATACTGGTTGGCTTGAGACCGGA
                             601                                             650
    16S M. extorquens  (601) AGAGGACAGCGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCA
16S M. extorquens AM1  (601) AGAGGACAGCGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCA
   16S M. sp. barley1  (599) AGAGGTTGGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCA
    16S M. sp. maize1  (599) AGAGGTTGGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCA
 16S M. sp. broccoli1  (599) AGAGGTTGGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCA
   16S M. sp. Atleaf1  (601) AGAGGACAGCGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCA
  16S M. sp. soyleaf2  (601) AGAGGACAGCGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCA
           Consensus   (601) AGAGGACAGCGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCA
                             651                                             700
    16S M. extorquens  (651) AGAACACCAGTGGCGAAGGCGGCTGTCTGGTCCGGTT-CTGACGCTGAGG
16S M. extorquens AM1  (651) AGAACGCCAGTGGCGAAGGCGGCTGTCTGGTCCGGTT-CTGACGCTGAGG
   16S M. sp. barley1  (649) AGAACACCGGTGGCGAAGGCGGCTGTCTGGTCCGGTTACTGACGCTGAGG
    16S M. sp. maize1  (649) AGAACACCGGTGGCGAAGGCGGCTGTCTGGTCCGGTT-CTGACGCTGAGG
 16S M. sp. broccoli1  (649) AGAACACCGGTGGCGAAGGCGGCCAACTGGACCA-TTACTGACGCTGAGG
   16S M. sp. Atleaf1  (651) AGAACACCAGTGGCGAAGGCGGCTGTCTGGTCCGGTT-CTGACGCTGAGG
  16S M. sp. soyleaf2  (651) AGAACACCAGTGGCGAAGGCGGCTGTCTGGTCCGGTT-CTGACGCTGAGG
           Consensus   (651) AGAACACCAGTGGCGAAGGCGGCTGTCTGGTCCGGTT CTGACGCTGAGG
                             701                                             750
    16S M. extorquens  (700) CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
16S M. extorquens AM1  (700) CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
   16S M. sp. barley1  (699) CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
    16S M. sp. maize1  (698) CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
 16S M. sp. broccoli1  (698) CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
   16S M. sp. Atleaf1  (700) CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
  16S M. sp. soyleaf2  (700) CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
           Consensus   (701) CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
                             751                                             800
    16S M. extorquens  (750) GTAAACGATGAATGCCAGCCGTTGGCCTGCTTGCAGGTCAGTGGCGCCGC
16S M. extorquens AM1  (750) GTAAACGATGAATGCCAGCCGTTGGCCTGCTTGCAGGTCAGTGGCGCCGC
   16S M. sp. barley1  (749) GTAAACGATGAATGCCAGCTGTTGGGGTGCTTGCACCGCAGTAGCGCAGC
    16S M. sp. maize1  (748) GTAAACGATGAATGCCAGCTGTTGGGGTGCTTGCACCGCAGTAGCGCAGC
 16S M. sp. broccoli1  (748) GTAAACGATGAATGCCAGCTGTTGGGGTGCTTGCACCGCAGTAGCGCAGC
   16S M. sp. Atleaf1  (750) GTAAACGATGAATGCCAGCCGTTGGCCTGCTTGCAGGTCAGTGGCGCCGC
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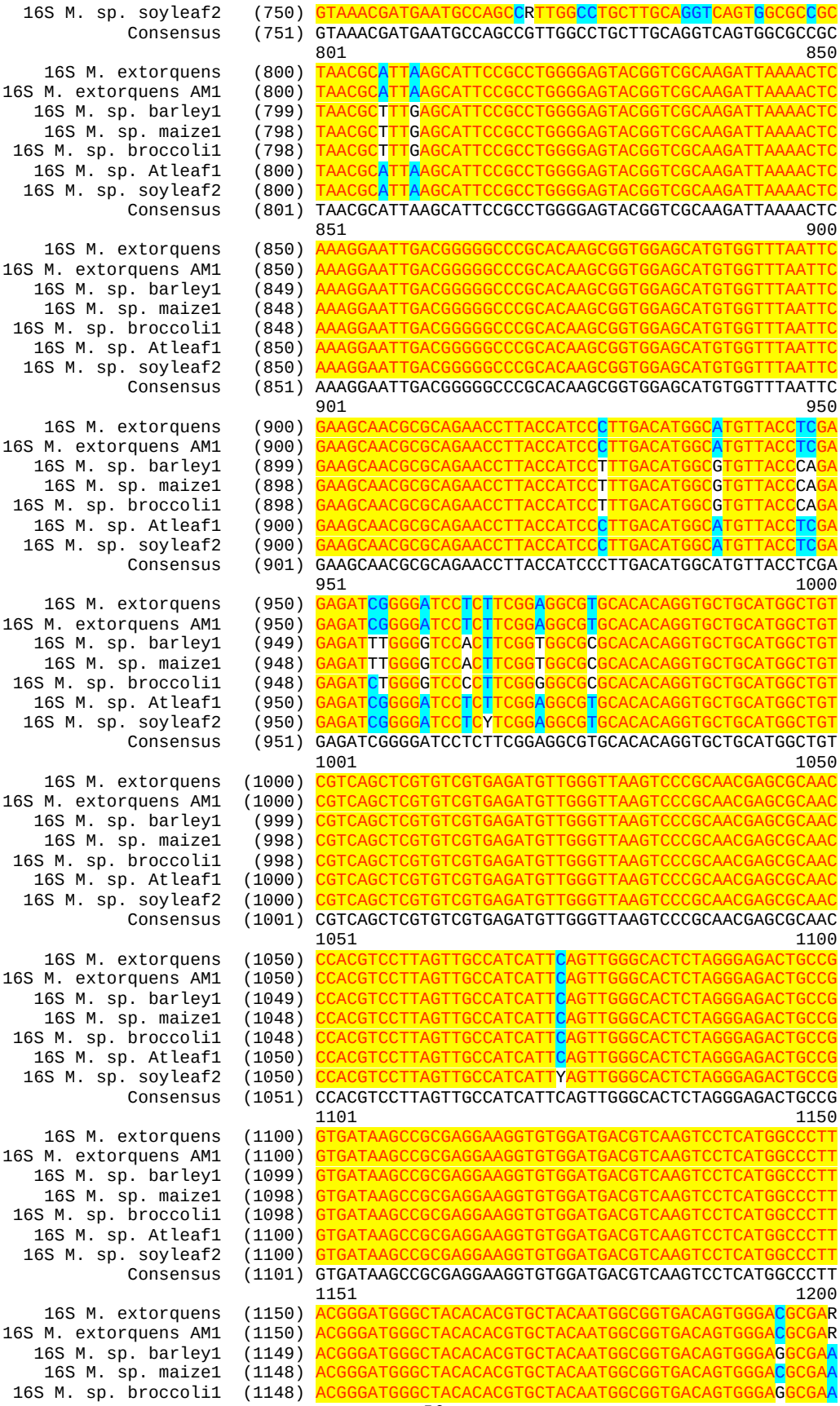
16S M. sp. soyleaf2 (750) GTAAACGATGAATGCCAGCCRTTGGCCTGCTTGCAGGTCAGTGGCGCCGC
Consensus (751) GTAAACGATGAATGCCAGCCGTTGGCCTGCTTGCAGGTCAGTGGCGCCGC
801 850
16S M. extorquens (800) TAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
16S M. extorquens AM1 (800) TAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
16S M. sp. barley1 (799) TAACGCTTTGAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
16S M. sp. maize1 (798) TAACGCTTTGAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
16S M. sp. broccoli1 (798) TAACGCTTTGAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
16S M. sp. Atleaf1 (800) TAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
16S M. sp. soyleaf2 (800) TAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
Consensus (801) TAACGCATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTC
851 900
16S M. extorquens (850) AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
16S M. extorquens AM1 (850) AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
16S M. sp. barley1 (849) AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
16S M. sp. maize1 (848) AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
16S M. sp. broccoli1 (848) AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
16S M. sp. Atleaf1 (850) AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
16S M. sp. soyleaf2 (850) AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
Consensus (851) AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
901 950
16S M. extorquens (900) GAAGCAACGCGCAGAACCTTACCATCCCTTGACATGGCATGTTACCTCGA
16S M. extorquens AM1 (900) GAAGCAACGCGCAGAACCTTACCATCCCTTGACATGGCATGTTACCTCGA
16S M. sp. barley1 (899) GAAGCAACGCGCAGAACCTTACCATCCTTTGACATGGCGTGTTACCCAGA
16S M. sp. maize1 (898) GAAGCAACGCGCAGAACCTTACCATCCTTTGACATGGCGTGTTACCCAGA
16S M. sp. broccoli1 (898) GAAGCAACGCGCAGAACCTTACCATCCTTTGACATGGCGTGTTACCCAGA
16S M. sp. Atleaf1 (900) GAAGCAACGCGCAGAACCTTACCATCCCTTGACATGGCATGTTACCTCGA
16S M. sp. soyleaf2 (900) GAAGCAACGCGCAGAACCTTACCATCCCTTGACATGGCATGTTACCTCGA
Consensus (901) GAAGCAACGCGCAGAACCTTACCATCCCTTGACATGGCATGTTACCTCGA
951 1000
16S M. extorquens (950) GAGATCGGGGATCCTCTTCGGAGGCGTGCACACAGGTGCTGCATGGCTGT
16S M. extorquens AM1 (950) GAGATCGGGGATCCTCTTCGGAGGCGTGCACACAGGTGCTGCATGGCTGT
16S M. sp. barley1 (949) GAGATTTGGGGTCCACTTCGGTGGCGCGCACACAGGTGCTGCATGGCTGT
16S M. sp. maize1 (948) GAGATTTGGGGTCCACTTCGGTGGCGCGCACACAGGTGCTGCATGGCTGT
16S M. sp. broccoli1 (948) GAGATCTGGGGTCCCCTTCGGGGGCGCGCACACAGGTGCTGCATGGCTGT
16S M. sp. Atleaf1 (950) GAGATCGGGGATCCTCTTCGGAGGCGTGCACACAGGTGCTGCATGGCTGT
16S M. sp. soyleaf2 (950) GAGATCGGGGATCCTCYTCGGAGGCGTGCACACAGGTGCTGCATGGCTGT
Consensus (951) GAGATCGGGGATCCTCTTCGGAGGCGTGCACACAGGTGCTGCATGGCTGT
1001 1050
16S M. extorquens (1000) CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
16S M. extorquens AM1 (1000) CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
16S M. sp. barley1 (999) CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
16S M. sp. maize1 (998) CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
16S M. sp. broccoli1 (998) CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
16S M. sp. Atleaf1 (1000) CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
16S M. sp. soyleaf2 (1000) CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
Consensus (1001) CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
1051 1100
16S M. extorquens (1050) CCACGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCG
16S M. extorquens AM1 (1050) CCACGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCG
16S M. sp. barley1 (1049) CCACGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCG
16S M. sp. maize1 (1048) CCACGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCG
16S M. sp. broccoli1 (1048) CCACGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCG
16S M. sp. Atleaf1 (1050) CCACGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCG
16S M. sp. soyleaf2 (1050) CCACGTCCTTAGTTGCCATCATTYAGTTGGGCACTCTAGGGAGACTGCCG
Consensus (1051) CCACGTCCTTAGTTGCCATCATTCAGTTGGGCACTCTAGGGAGACTGCCG
1101 1150
16S M. extorquens (1100) GTGATAAGCCGCGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTT
16S M. extorquens AM1 (1100) GTGATAAGCCGCGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTT
16S M. sp. barley1 (1099) GTGATAAGCCGCGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTT
16S M. sp. maize1 (1098) GTGATAAGCCGCGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTT
16S M. sp. broccoli1 (1098) GTGATAAGCCGCGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTT
16S M. sp. Atleaf1 (1100) GTGATAAGCCGCGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTT
16S M. sp. soyleaf2 (1100) GTGATAAGCCGCGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTT
Consensus (1101) GTGATAAGCCGCGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTT
1151 1200
16S M. extorquens (1150) ACGGGATGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGACGCGAR
16S M. extorquens AM1 (1150) ACGGGATGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGACGCGAR
16S M. sp. barley1 (1149) ACGGGATGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGAGGCGAA
16S M. sp. maize1 (1148) ACGGGATGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGACGCGAA
16S M. sp. broccoli1 (1148) ACGGGATGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGAGGCGAA

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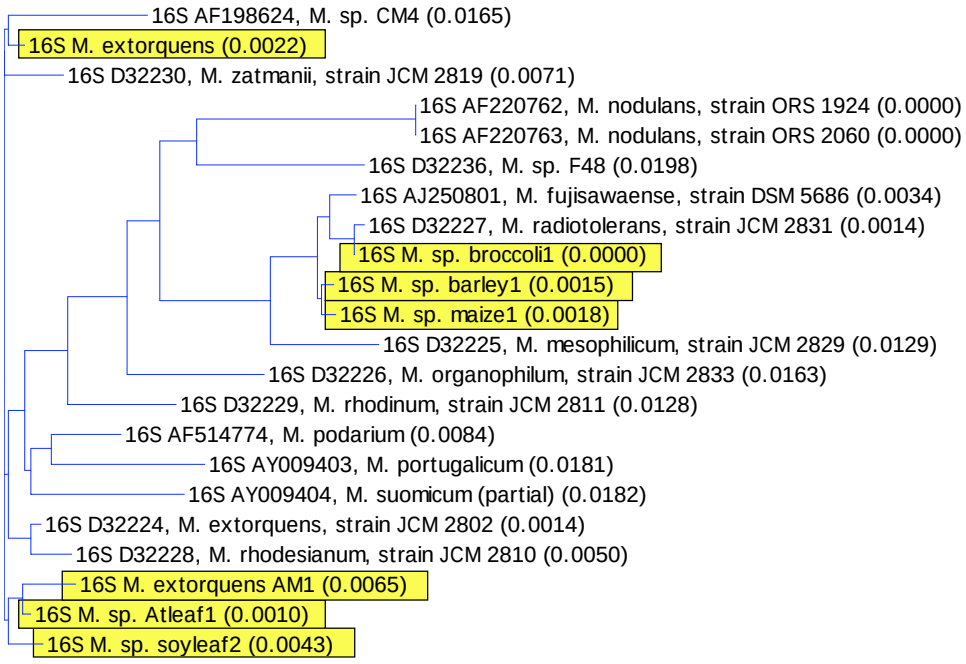
   16S M. sp. Atleaf1  (1150) ACGGGATGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGACGCGAA
  16S M. sp. soyleaf2  (1150) ACGGGATGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGACGCGAA
            Consensus  (1151) ACGGGATGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGACGCGAA
                               1201                                          1250
    16S M. extorquens  (1200) RCCGCGAGGTKGAGCAAATCCCCAAAARCCGTCTCAGTTCGGATTGCACT
16S M. extorquens AM1  (1200) RCCGCGAGGTKGAGCAAATCCCCAAAARCCGTCTCAGTTCGGATTGCACT
   16S M. sp. barley1  (1199) GGAGCGATCTGGAGCAAATCCCCAAAAGCCGTCTCAGTTCGGATTGCACT
    16S M. sp. maize1  (1198) GGAGCGATCTGGAGCAAATCCCCAAAAGCCGTCTCAGTTCGGATTGCACT
 16S M. sp. broccoli1  (1198) GGAGCGATCTGGAGCAAATCCCCAAAAGCCGTCTCAGTTCGGATTGCACT
   16S M. sp. Atleaf1  (1200) ACCGCGAGGTTGAGCAAATCCCCAAAAGCCGTCTCAGTTCGGATTGCACT
  16S M. sp. soyleaf2  (1200) ACCGCGAGGTTGAGCAAATCCCCAAAAGCCGTCTCAGTTCGRATTGCACT
            Consensus  (1201)  CCGCGAGGT GAGCAAATCCCCAAAAGCCGTCTCAGTTCGGATTGCACT
                               1251                                          1300
    16S M. extorquens  (1250) CTGCAACTCGGGTGCATGAAGGCGGAATCGCTAGTAATCGTGGATCAGCA
16S M. extorquens AM1  (1250) CTGCAACTCGGGTGCATGAAGGCGGAATCGCTAGTAATCGTGGATCAGCA
   16S M. sp. barley1  (1249) CTGCAACTCGAGTGCATGAAGGCGGAATCGCTAGTAATCGTGGATCAGCA
    16S M. sp. maize1  (1248) CTGCAACTCGAGTGCATGAAGGCGGAATCGCTAGTAATCGTGGATCAGCA
 16S M. sp. broccoli1  (1248) CTGCAACTCGAGTGCATGAAGGCGGAATCGCTAGTAATCGTGGATCAGCA
   16S M. sp. Atleaf1  (1250) CTGCAACTCGGGTGCATGAAGGCGGAATCGCTAGTAATCGTGGATCAGCA
  16S M. sp. soyleaf2  (1250) CTGCAACTCGGGTGCATGAAGGCGGAATCGCTAGTAATCGTGGATCAGCA
            Consensus  (1251) CTGCAACTCGGGTGCATGAAGGCGGAATCGCTAGTAATCGTGGATCAGCA
                               1301                                          1350
    16S M. extorquens  (1300) CGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
16S M. extorquens AM1  (1300) CGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
   16S M. sp. barley1  (1299) TGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
    16S M. sp. maize1  (1298) TGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
 16S M. sp. broccoli1  (1298) TGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
   16S M. sp. Atleaf1  (1300) CGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
  16S M. sp. soyleaf2  (1300) CGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
            Consensus  (1301) CGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
                               1351                                          1400
    16S M. extorquens  (1350) TGGGAGTTGGTCTTACCCGA-CGGCGCTGCGCCAACCGCAAGGRGGCAGG
16S M. extorquens AM1  (1350) TGGGAGTTGGTCTTACCCGA-CGGCGCTGCGCCAACCGCAAGGRGGCAGG
   16S M. sp. barley1  (1349) TGGGAGTTGGTCTTACCCGA-CGGCGCTGCGCCAACCGCAAGGAGGCAGG
    16S M. sp. maize1  (1348) TGGGAGTTGGTCTTACCCGA-CGGCGCTGCGCCAACCGCAAGGAGGCAGG
 16S M. sp. broccoli1  (1348) TGGGAGTTGGTCTTACCCGA-CGGCGCTGCGCCAACCGCAAGGAGGCAGG
   16S M. sp. Atleaf1  (1350) TGGGAGTTGGTCTTACCCGA-CGGCGCTGCGCCAACCGCAAGGAGGCAGG
  16S M. sp. soyleaf2  (1350) TGGGAGTTGGTCTTACCCGATCGGCGCTGCGCCAACCGCAAGGRGGCAGG
            Consensus  (1351) TGGGAGTTGGTCTTACCCGA CGGCGCTGCGCCAACCGCAAGGAGGCAGG
                               1401                                          1450
    16S M. extorquens  (1399) CGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
16S M. extorquens AM1  (1399) YGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
   16S M. sp. barley1  (1398) CGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
    16S M. sp. maize1  (1397) CGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
 16S M. sp. broccoli1  (1397) CGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
   16S M. sp. Atleaf1  (1399) CGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
  16S M. sp. soyleaf2  (1400) CGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
            Consensus  (1401) CGACCACGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAGCCGT
                               1451                    1479
    16S M. extorquens  (1449) AGGGGAACCTGCGGCTGGATCACCTCCTT
16S M. extorquens AM1  (1449) AGGGGAACCTGCGGCTGGATCACCTCCTT
   16S M. sp. barley1  (1448) AGGGGAACCTGCGGCTGGATCACCTCCTT
    16S M. sp. maize1  (1447) AGGGGAACCTGCGGCTGGATCACCTCCTT
 16S M. sp. broccoli1  (1447) AGGGGAACCTGCGGCTGGATCACCTCCTT
   16S M. sp. Atleaf1  (1449) AGGGGAACCTGCGGCTGGATCACCTCCTT
  16S M. sp. soyleaf2  (1450) AGGGGAACCTGCGGCTGGATCACCTCCTT
            Consensus  (1451) AGGGGAACCTGCGGCTGGATCACCTCCTT
```

Figure 4.1-3 Phylogenetic Tree of *Methylobacterium* strains based on 16S rDNA sequences. 16S rDNA sequences of *M. extorquens*, *M. extorquens* AM1, *M*. sp. soyeaf2, *M*. sp. *At*leaf1, *M*. sp. barley1, *M*. sp. maize1, and *M*. sp. broccoli1 were compared to known *Methylobacterium* 16s rDNAs deposited in GenBank (Genus, species, strain (GenBank accession number)): *M*. sp. CM4 (AF198624); *M. nodulans*, strain ORS 1924 (AF220762); *M. nodulans*, strain ORS 2060 (AF220763); *M. podarium* (AF514774); *M. fujisawaense*, strain DSM 5686 (AJ250801); *M. portugalicum* (AY009403); *M. suomicum* (partial) (AY009404); *M. extorquens*, strain JCM 2802 (D32224); *M. mesophilicum*, strain JCM 2829 (D32225); *M. organophilum*, strain JCM 2833 (D32226); *M. radiotolerans*, strain JCM 2831 (D32227); *M. rhodesianum*, strain JCM 2810 (D32228); *M. rhodinum*, strain JCM 2811 (D32229); *M. zatmanii*, strain JCM 2819 (D32230); *M*. sp. F48 (D32236). The tree was generated using VectorNTI v.10.3 that utilizes the Neighbor Joining algorithm of Saitou and Nei [137] based on a sequence distance method. Distance values, based on nucleotide substitutions, are provided in parenthesis.


16S AF198624, M. sp. CM4 (0.0165)
16S M. extorquens (0.0022)
16S D32230, M. zatmanii, strain JCM 2819 (0.0071)
16S AF220762, M. nodulans, strain ORS 1924 (0.0000)
16S AF220763, M. nodulans, strain ORS 2060 (0.0000)
16S D32236, M. sp. F48 (0.0198)
16S AJ250801, M. fujisawaense, strain DSM 5686 (0.0034)
16S D32227, M. radiotolerans, strain JCM 2831 (0.0014)
16S M. sp. broccoli1 (0.0000)
16S M. sp. barley1 (0.0015)
16S M. sp. maize1 (0.0018)
16S D32225, M. mesophilicum, strain JCM 2829 (0.0129)
16S D32226, M. organophilum, strain JCM 2833 (0.0163)
16S D32229, M. rhodinum, strain JCM 2811 (0.0128)
16S AF514774, M. podarium (0.0084)
16S AY009403, M. portugalicum (0.0181)
16S AY009404, M. suomicum (partial) (0.0182)
16S D32224, M. extorquens, strain JCM 2802 (0.0014)
16S D32228, M. rhodesianum, strain JCM 2810 (0.0050)
16S M. extorquens AM1 (0.0065)
16S M. sp. Atleaf1 (0.0010)
16S M. sp. soyleaf2 (0.0043)

suggesting that these six isolates may best fit into the species '*mesophilicum*', '*radiotolerans*' or '*fujisawaense*' with strain designations. GenBank can be a useful resource, but the sequence data deposited are not always reliable. It is important to have complete reliable sequence available when classifying organisms to genus and species.

4.2 Characterization of *Methylobacterium* urease-negative mutant, *M*. sp. ex15.

My goal was to produce disruptions in urease structural and accessory genes of *Methylobacterium* spp. because I wanted to investigate why a commensal bacterium mimics the phenotype of the host plant, i.e. why PPFMs from urease-negative *eu3-e1/eu3-e1* are urease-negative while on the plant [6]. A 593 bp internal fragment of the urease structural gene ureC was amplified by PCR and cloned into the suicide vector pAYC61 to generate the plasmid pSBW4 (Figure 3.6-1). This plasmid was mobilized into *M.* sp. soyleaf2 by tri-parental mating. The resultant tetracycline-resistant exconjugants were screened by colony PCR using the strategy outlined in Figure 3.6-1. ex15 revealed a band of the expected size suggesting that pSBW4 inserted into ureC in the chromosome (Figure 4.2-1). Three additional exconjugants (ex31, ex32 and ex33) from the screen are shown and suggest lack of insertion into ureC. The exconjugants were further screened in a qualitative assay for the enzyme urease based on the seed chip assay [39] whereby urease-positive bacteria turn the assay solution from yellow to pink by alkalization due to urea-dependent ammonia production. The exconjugant ex15 appeared to be urease-negative (Figure 4.2-2). To confirm, a quantitative urease assay based on the release $^{14}CO_2$ from [^{14}C]urea was used to compare the urease activity of *M.* sp. ex15 to the soyleaf2 isolate. The potent urease inhibitor phenylphosphorodiamidate

Figure 4.2-1 Colony PCR of tetracycline-resistant exconjugates. To confirm the insertion of the plasmid pSBW4 into the ureC structural gene in *Methylobacterium* sp. soyleaf2, tetracycline-resistant exconjugants were screened by colony PCR as outlined in figure 3.6-2. Primers to the vector and to a genomic region of ureC outside of the internal fragment were used for ex15, ex31, ex32 and ex33. *E. coli* carrying pSBW4 was used as a control with primers to the internal fragment of ureC.

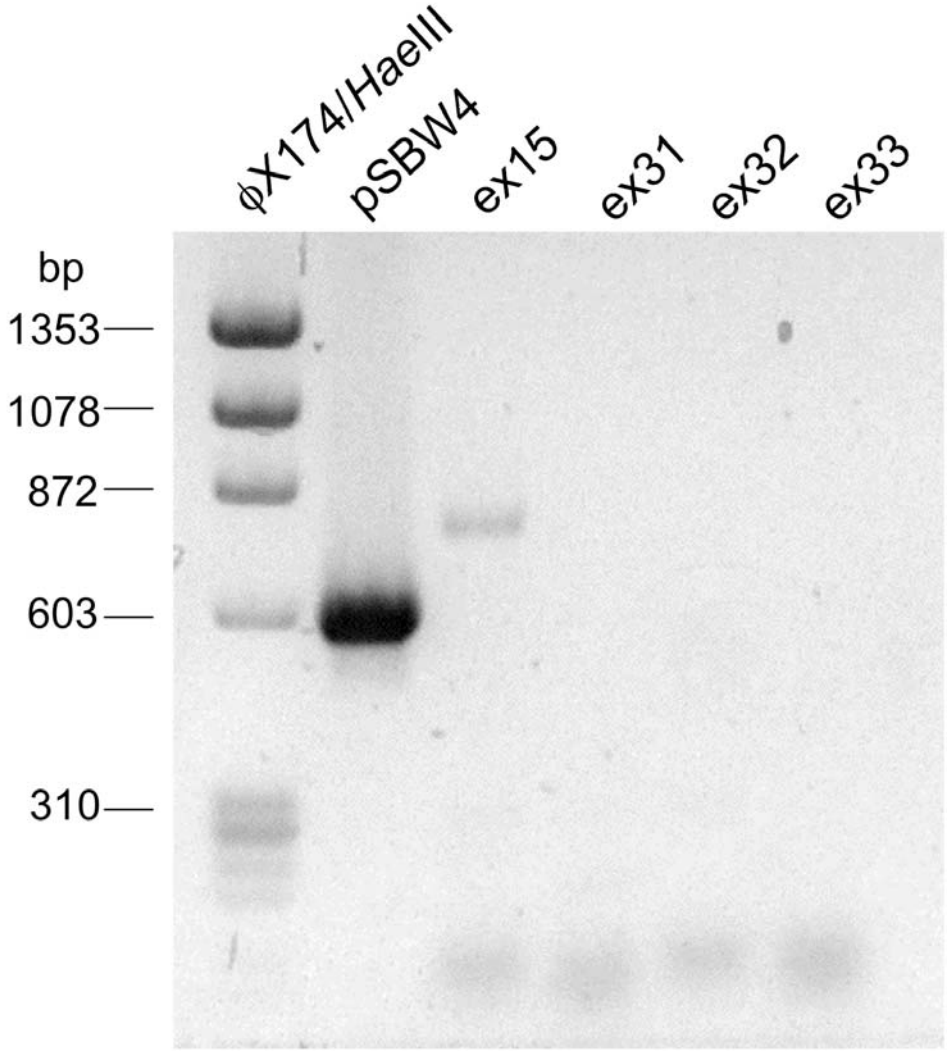
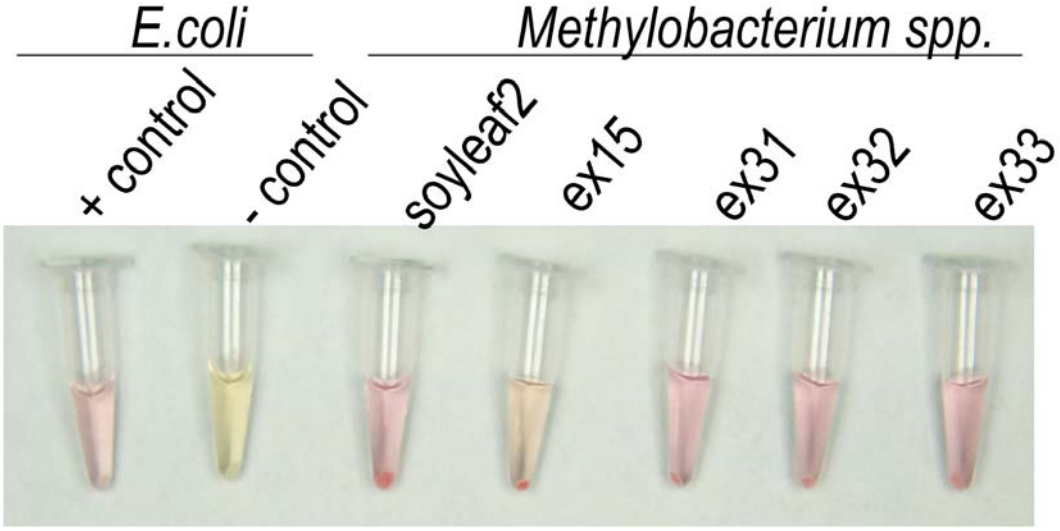

φX174/HaeIII
pSBW4
ex15
ex31
ex32
ex33
bp
1353
1078
872
603
310

Figure 4.2-2 Bacteria "chip" assay. A qualitative assay for the enzyme urease was developed based on the seed chip assay [39]. A loop of bacterial cells was incubated in a solution of cresol red in phosphate buffer, pH 7.0 containing urea. After 18 h at 30° C, urease-positive bacteria turn the solution from yellow to pink by alkalization due to ammonia production. Tetracycline-resistant exconjugants ex15, ex31, ex32 and ex33 were compared to the soyleaf2 isolate as well as urease-positive and urease-negative *E. coli* strains containing the plasmids pKAU17 and pKAU17ΔF [26] respectively.


E.coli
Methylobacterium spp.
+ control
- control
soyleaf2
ex15
ex31
ex32
ex33

(PPD) was used to inhibit completely the urease activity of the progenitor cells. The exconjugant ex15 is urease-negative (Figure 4.2-3). This mutant was used in experiments below testing the regulation of urease as well as in the determination of the pathway for ureide degradation in *Methylobacterium*.

Our working model suggests that there is some signal from the plant that either inhibits the production of the urease gene products in the associated bacteria or inhibits the function or transport of Ni^{2+} from the plant to the bacteria. This signal could be a nitrogenous signal (among them ureides, urea, ammonia) or simply a block in a transporter required to take up Ni^{2+} from the plant cell to the associated bacteria. To begin to address the model we wanted first to test whether a nitrogenous signal produced by the plant could be a factor in the urease-negative status of the PPFMs associated with the urease-negative soybean mutants. We approached this by varying N sources in free-living cultures and examining the effects on growth, urease activity and ureide utilization.

4.3 Regulation of urease in *Methylobacterium* spp.

The leaves of urease-negative soybean mutant *eu3-e1/eu3-e1* accumulate urea [56], an accumulation also evident in necrotic leaf-tip urea-burn of nickel-free, urease-negative plants [134-135]. I tested the hypothesis that urease expression in PPFMs was under the control of some nitrogenous compound (urea?) that accumulates in eu3-e1 plants but not in eu4 plants. Growth of the urease-negative ex15 strain (ex15) was compared to the soyleaf2 isolate (WT) on various nitrogen (N) sources. Both strains were able to grow on AMS (NH_4^+) in the presence or absence of the urease inhibitor PPD (Figure 4.3-1).

Figure 4.2-3 *Methylobacterium* sp. ex15 is urease-negative. A quantitative urease assay assay [119] based on the release of $^{14}CO_2$ from [^{14}C]urea was used to compare the urease activity of *M*. sp ex15 (ex15) to the soyleaf2 isolate (WT). The urease inhibitor phenylphosphorodiamidate (PPD) was added at 50 μM.

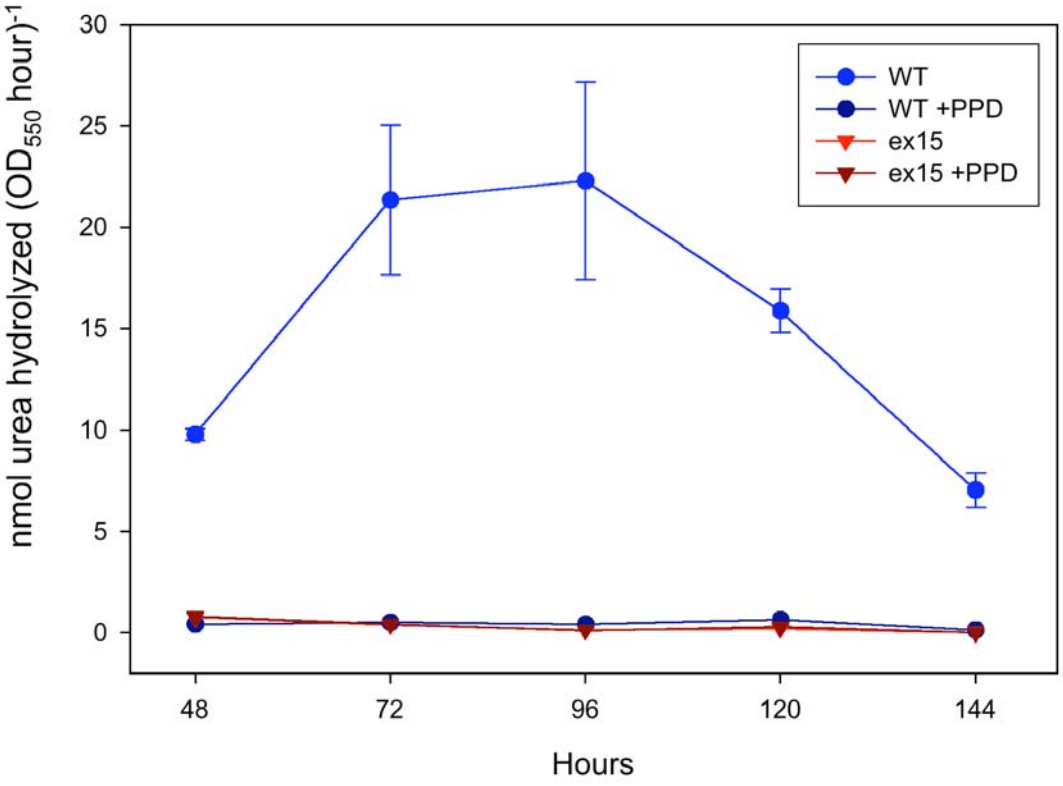
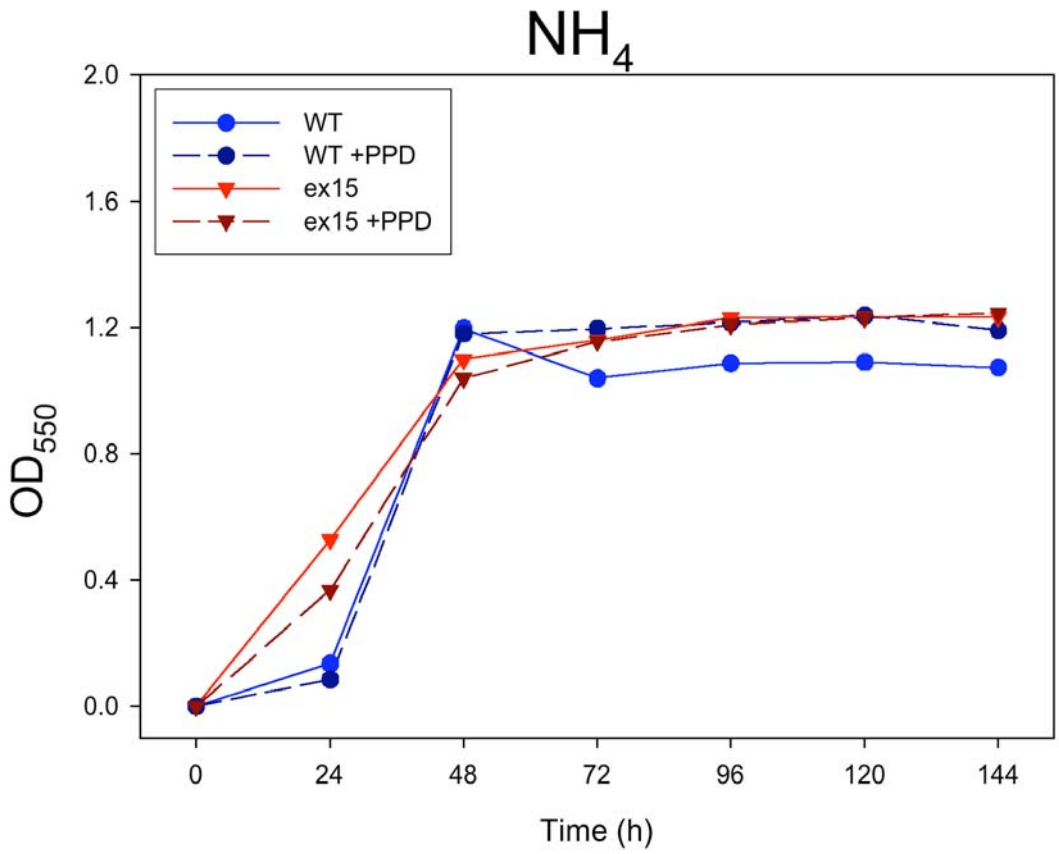

nmol urea hydrolyzed (OD550 hour)-1
30
25
20
15
10
5
0
48
72
96
120
144
Hours
WT
WT +PPD
ex15
ex15 +PPD

Figure 4.3-1 Growth of *Methylobacterium* on Ammonium Mineral Salts. *M*. sp. soyleaf2 (WT) and *M*. sp. ex15 (ex15) were grown on Ammonium Mineral Salts (AMS) medium in the absence or presence of the urease inhibitor phenylphosphorodiamidate (PPD) (50 μM). Nitrogen was supplied at 9.3 meq/L.


NH4
WT
WT +PPD
ex15
ex15 +PPD
OD550
2.0
1.6
1.2
0.8
0.4
0.0
0
24
48
72
96
120
144
Time (h)

There was no difference in growth among WT, WT+PPD and ex15, demonstrating that urease activity is not required for growth on NH_4^+. The soyleaf2 isolate grew with urea as the sole N-source and this growth was inhibited by PPD (Figure 4.3-2). The ex15 strain did not grow with urea as the sole N-source further supporting a lack of urease activity in that strain (Figure 4.3-2). Growth of these strains on allantoin as the sole N-source was similar in that WT was able to utilize allantoin and growth was inhibited by PPD indicating that allantoin N is "funneled" exclusively through urea intermediates (Figure 4.3-3). The ex15 strain could not utilize allantoin as the sole N-source supporting the breakdown of all allantoin N to ammonia via two urea intermediates (Figure 4.3-3). Allantoin is a substituted hydantoin, and arginine is a source of urea in plants. Neither the soyleaf2 isolate nor ex15 could utilize arginine or hydantoin as a sole-N source suggesting that *Methylobacterium* lacks a functional arginase or hydantoinase (Figure 4.3-4). Mining the *M. extorquens* AM1 genome database supports this hypothesis since there was no annotated sequence corresponding to either arginase or hydantoinase [118]. Additionally, PPD alone cannot support the growth of *Methylobacterium* (Figure 4.3-4).

The hypothesis to be tested was that regulation of urease in *Methylobacterium* was under nitrogen control, so urease activity was measured in cultures growing on various N-sources. Rosenstein et al. [53] examined the effect of the urease inhibitor acetohydroxamic acid (AHA) on the regulation of urease in *Proteus morganii*. They observed no induction by urea showing that urease in *P. morganii* was synthesized constitutively. Urease was also expressed constitutively in the soil bacterium *Bacillus pasteurii* [122]. Urease in *P. mirablis*, *P. vulgaris* and *P. rettgeri*, however, was

Figure 4.3-2 Growth of *Methylobacterium* on urea as sole nitrogen source. *M*. sp. soyleaf2 (WT) and *M*. sp. ex15 (ex15) were grown on urea-substituted Ammonium Mineral Salts (Urea-MS) medium in the absence or presence of the urease inhibitor phenylphosphorodiamidate (PPD) (50 μM). Nitrogen was supplied at 9.3 meq/L.

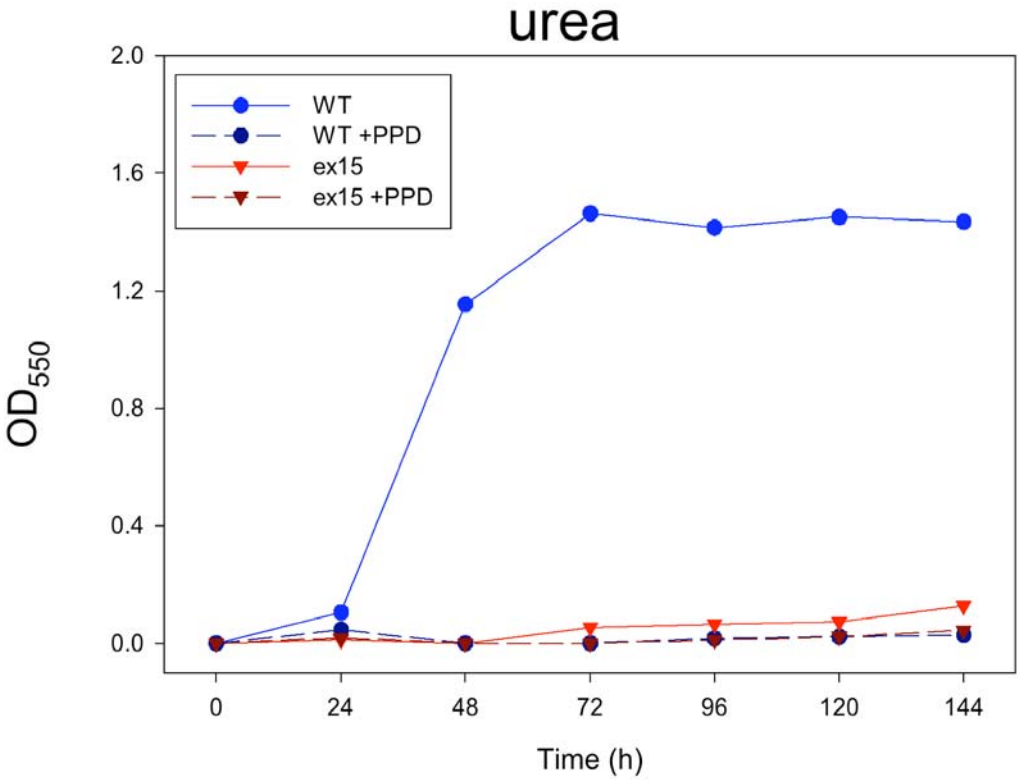

urea
WT
WT +PPD
ex15
ex15 +PPD
OD550
2.0
1.6
1.2
0.8
0.4
0.0
0
24
48
72
96
120
144
Time (h)

Figure 4.3-3 Growth of *Methylobacterium* on allantoin as sole nitrogen source. *M*. sp. soyleaf2 (WT) and *M*. sp. ex15 (ex15) were grown on allantoin-substituted Ammonium Mineral Salts (Allantoin-MS) medium in the absence or presence of the urease inhibitor phenylphosphorodiamidate (PPD) (50 μM). Nitrogen was supplied at 9.3 meq/L.

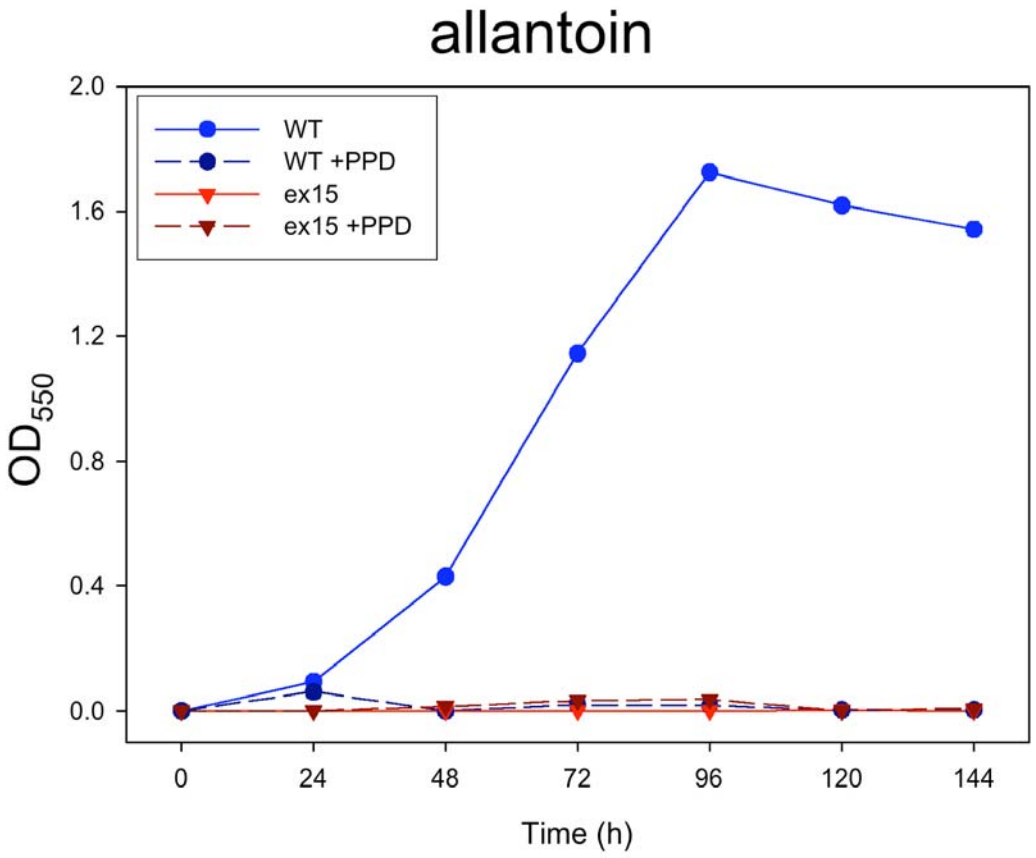
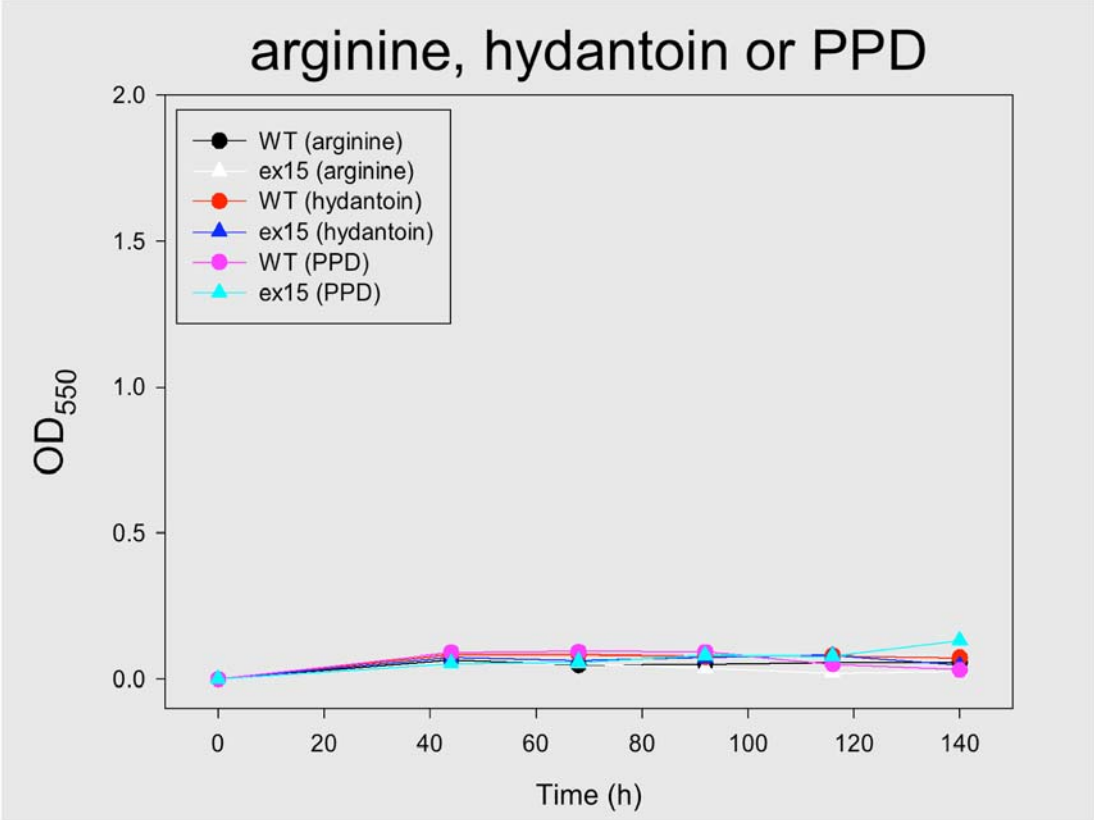

allantoin
WT
WT +PPD
ex15
ex15 +PPD
OD550
2.0
1.6
1.2
0.8
0.4
0.0
0
24
48
72
96
120
144
Time (h)

Figure 4.3-4 Growth of *Methylobacterium* on arginine, hydantoin or PPD as sole nitrogen source. *M*. sp. soyleaf2 (WT) and *M*. sp. ex15 (ex15) were grown on arginine, hydantoin or PPD-substituted Ammonium Mineral Salts (Arginine-MS, Hydantoin-MS and PPD-MS, respectively) medium. Nitrogen was supplied to Arginine-MS and Hydantoin-MS at 9.3 meq/L, PPD was 50 μM.

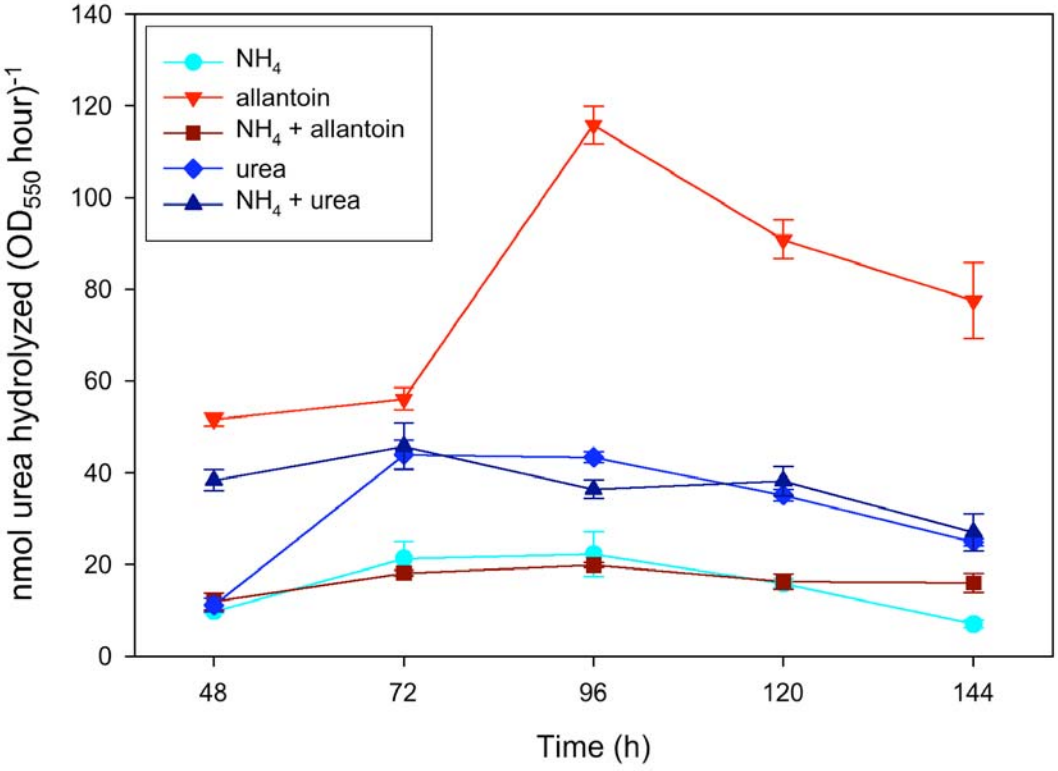

arginine, hydantoin or PPD
WT (arginine)
ex15 (arginine)
WT (hydantoin)
ex15 (hydantoin)
WT (PPD)
ex15 (PPD)
OD550
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
140
Time (h)

inducible by urea and induction was increased when both urea and AHA were present [53]. AHA alone did not induce urease. However, it minimized the pH increases due to hydrolysis of urea and allowed urea to act as an inducer instead of a substrate [53]. In contrast, urease was repressed in *K. aerogenes* when grown in ammonia or other nitrogen-rich compounds and synthesis was de-repressed in nitrogen limiting conditions [54]. The urease activity of the *M*. sp. soyleaf2 isolate grown on allantoin as the sole N-source was 5-11 fold higher than when grown on ammonia (Figure 4.3-5). However, cultures grown in the presense of allantoin+ammonia had only the urease activity of ammonia-grown cultures suggesting ammonia-repression (Figure 4.3-5). There was a 2-fold increase in urease activity when the soyleaf2 isolate was grown on urea (Figure 4.3-5). However, this increase in activity was not repressed when ammonia was present since there was probably lots of ammonia already present from urea (Figure 4.3-5). There is urease activity in *Methylobacterium* grown on "N-rich" ammonium (Figure 4.3-4). This suggests that urease expression is constitutive in *Methylobacterium*, at least to a basal level, and may be repressed by ammonia in the presence of other N-sources such as allantoin (Figure 4.3-4).

4.4 Allantoin degradation in *Methylobacterium* spp.

Since *M*. sp. soyleaf2 was able to grow on allantoin as a sole N-source (Figure 4.3-3) and given that the activity of urease was increased when grown on allantoin but not allantoin+ammonia, I investigated the effect of ammonium on allantoin degradation by PPFMs in culture. Allantoin was utilized by the soyleaf2 isolate when allantoin is the sole N-source as evidenced by growth (Figure 4.3-3). Consistent with the allantoin-

Figure 4.3-5 Urease Activity of *Methylobacterium* sp. soyleaf2 on various nitrogen sources. *M*. sp. soyleaf2 was grown in liquid culture on NH_4, allantoin or urea as sole nitrogen source or in an NH_4/allantoin or NH_4/urea combination. Quantitative urease assays were performed as described [119] based on the release of $^{14}CO_2$ from [^{14}C]urea.


140
120
100
80
60
40
20
0
nmol urea hydrolyzed (OD550 hour)-1
NH4
allantoin
NH4 + allantoin
urea
NH4 + urea
48
72
96
120
144
Time (h)

supported growth, allantoin disappeared in the culture supernatant (Figure 4.4-1). However, when ammonia was present allantoin was not utilized by either the soyleaf2 or the urease-negative mutant ex15 (Figure 4.4-1). These data, together with the urease activity data, suggest that PPFMs preferentially utilize ammonia as N-source when both ammonia and allantoin are available.

Nitrogen-fixing tropical and warm weather legumes transport fixed nitrogen from the nodules to the aerial portions of the plant primarily as the ureides allantoin and allantoate [107]. In soybean, the overall route of ureide degradation has recently been established [108] (Figure 1.4-1). Allantoate has four possible routes to be broken down ultimately to glyoxylate, NH_3, and CO_2: each ureido group can be liberated either as urea or by direct release of NH_3 and CO_2. Since at each enzymatic step catalyzing ureido 'excision' either alternative is possible, there are four possible routes of degradation of allantoate. Allantoin cannot serve as a nitrogen source for the urease-negative mutant ex15 (Figure 4.3-3) indicating that urease activity was essential for growth on allantoin. Additionally, growth of the soyleaf2 isolate on allantoin was inhibited by the urease inhibitor PPD (Figure 4.3-3 and Polacco and Holland [113]). Taken together, the results are strong evidence for allantoin degradation proceeding through two urea intermediates described above: allantoin to ureidoglycolate and urea; and ureidoglycolate to glyoxylate and urea. This ureide degradation pathway in *Methylobacterium* is shown in Figure 4.4-2. It agrees with that in budding yeast (*Saccharomyces cerevisiae*) [129] and in fission yeast (*Schizosaccharomyces pombe*) [130].

Figure 4.4-1 Allantoin utilization in *Methylobacterium*. *M*. sp. soyleaf 2 (WT) and *M*. sp. ex15 (ex15) were grown in liquid culture with allantoin as the sole nitrogen source or with allantoin in the presence of NH_4. To determine the amount of allantoin utilized by the *Methylobacterium* strains in culture, an alkaline/acid hydrolysis reaction followed by a colorimetric determination of glyoxylate was performed based on Vogels and Van Der Drift [120].

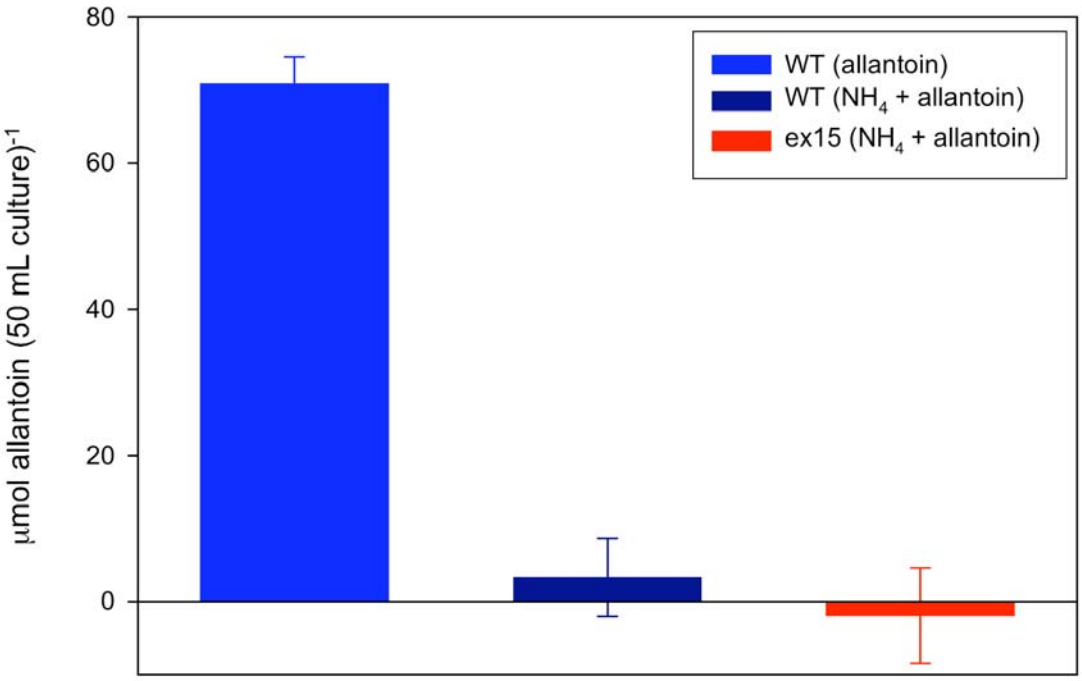
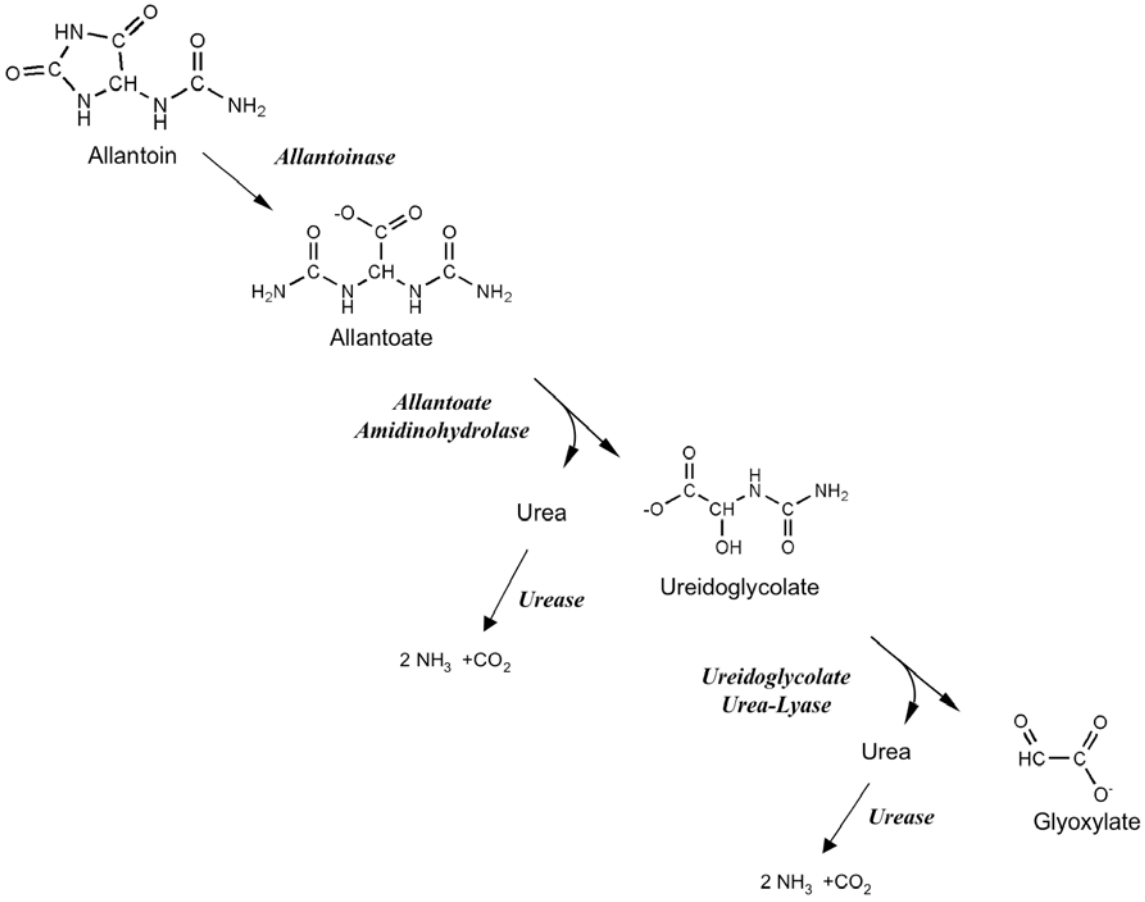

80
60
40
20
0
µmol allantoin (50 mL culture)-1
WT (allantoin)
WT (NH4 + allantoin)
ex15 (NH4 + allantoin)

Figure 4.4-2 Ureide degradation pathway in *Methylobacterium*. The overall conversion of allantoin to gyloxylate and ammonia in *Methylobacterium* is shown. The hydrolysis reactions between allantoate/ureidoglycolate and ureidoglycolate/glyoxylate release urea, which is subsequently hydrolyzed by urease.


Allantoin
Allantoinase
Allantoate
Allantoate Amidinohydrolase
Urea
Urease
2 NH3 +CO2
Ureidoglycolate
Ureidoglycolate Urea-Lyase
Urea
Urease
2 NH3 +CO2
Glyoxylate

Our working model states that a nitrogenous compound produced by the plant could be a factor in causing the urease-negative phenocopy of the associated PPFMs. We know that urea accumulates *in vivo* in urease-negative soybean accessory gene mutants and that ureides are transported to the aerial portions of the plant. However, our *in vitro* data does not support a nitrogenous compound (NH_4^+, urea, ureide) as being a factor in the urease status of the associated bacteria. If anything, these nitrogenous compounds increase the urease activity of the PPFMs. And, ureide levels would be expected to vary greatly between fixing plants and those supported by reduced N. Indeed, if an N-signal from the plant were a factor in "shutting down" urease in the associated PPFMs, we are still left with the explanation of why this occurs in *eu2* and *eu3* plants but not *eu4* plants.

So we were obliged, at this point to test the alternative working model: that there was either a Ni^{2+} transport/uptake block in the associated bacteria or that the bacteria somehow co-opt the plant urease activation machinery. To begin to address the model, we determined the urease status of marked PPFMs from recolonized soybean mutants.

4.5 Colonization of soybean with marked *Methylobacterium* spp. strains.

PPFMs associated with urease-negative soybean mutants, which lack functions for insertion of nickel in the plant urease active site, were urease-negative themselves while on the plant [6]. These bacteria were transiently urease-negative in free-living culture and the reacquisition of urease activity was accelerated by nickel supplementation [6]. If Ni^{2+} transport/uptake was blocked between *eu3* plants and their associated PPFMs, then I would predict that PPFMs, upon recolonizing urease-negative plants or callus (*eu3* but

not *eu4*) will become urease-negative while associated with plants or callus. To accomplish recolonization, I utilized a number of marked strains that confer a drug resistance to be able to distinguish them from the resident population of PPFMs associated with the recolonized plants.

Two kanamycin-resistant (Km^r) PPFM isolates, *M*. sp. *At*leaf1-65 (Atleaf65) and *M. extorquens*-OR18 (OR18) (Table 3.1-1), were first tested for their ability to colonize axenic callus cultures of soybean Williams 82 (W82) and *eu3-e1*/*eu3-e1* (eu3-e1). I determined that by coating newly transferred callus with 20 μL of an early stationary-phase culture, the PPFMs remained associated with the callus and did not 'spill out' onto the tissue culture medium or alter the appearance of the callus. The PPFM isolates could be recovered from the callus by grinding the callus in sterile water and plating the macerate onto selective medium (AMS+Km). The PPFM isolates were recovered from the callus of both W82 and eu3-e1 two weeks post-inoculation and urease activity was determined as described [119]. The re-colonized PPFM isolates were urease-positive whether recovered from W82 or eu3-e1 (Figure 4.5-1). Similar results were obtained when PPFMs were isolated from W82 and eu3-e1 one month post-inoculation (Figure 4.5-2). These data did not fit the prediction that urease-positive PPFMs will become urease-negative while associated with urease-negative *eu3-e1*/*eu3-e1* callus. I then proceeded with experiments to test the prediction on *eu3-e1*/*eu3-e1* plants.

Seeds of soybean W82 and eu3-e1 were inoculated with Atleaf65 or OR18 by imbibition of an early-stationary phase PPFM culture at room temperature for 5 h with gentle

Figure 4.5-1 Urease activity of PPFMs from colonized callus two weeks post-inoculation. Axenic callus cultures of soybean Williams 82 (W82) and *eu3-e1*/*eu3-e1* (eu3-e1) were inoculated with *M*. sp. *At*leaf1-65 (Atleaf65) or *M. extorquens*-OR18 (OR18) by coating newly transferred callus with 20 μL of an early stationary-phase culture. The PPFM isolates were recovered from the callus two weeks post-inoculation by grinding the callus in sterile water and plating the macerate out onto selective media. Quantitative urease assays were performed as described [119] based on the release of $^{14}CO_2$ from [^{14}C]urea.

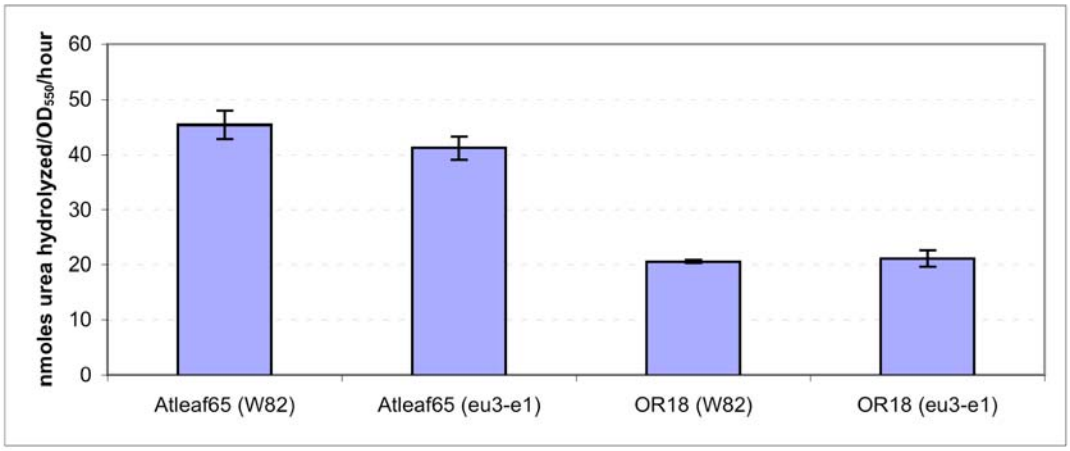
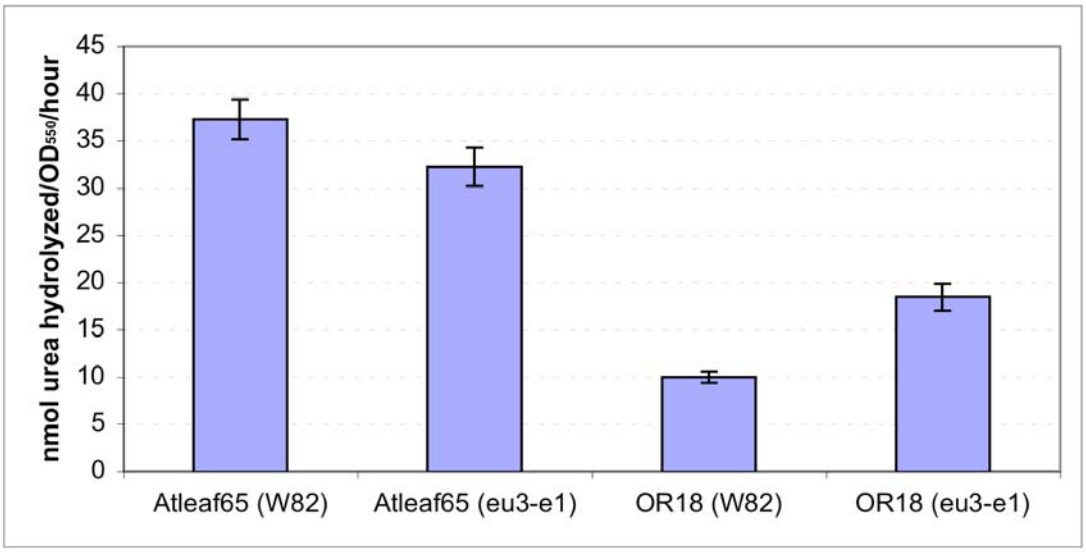

nmoles urea hydrolyzed/OD550/hour
60
50
40
30
20
10
0
Atleaf65 (W82)
Atleaf65 (eu3-e1)
OR18 (W82)
OR18 (eu3-e1)

Figure 4.5-2 Urease activity of PPFMs from colonized callus one month post-inoculation. Axenic callus cultures of soybean Williams 82 (W82) and *eu3-e1*/*eu3-e1* (eu3-e1) were inoculated with *M*. sp. *At*leaf1-65 (Atleaf65) or *M. extorquens*-OR18 (OR18) by coating newly transferred callus with 20 μL of an early stationary-phase culture. The PPFM isolates were recovered from the callus two weeks post-inoculation by grinding the callus in sterile water and plating the macerate out onto selective media. Quantitative urease assays were performed as described [119] based on the release of $^{14}CO_2$ from [^{14}C]urea.


nmol urea hydrolyzed/OD550/hour
45
40
35
30
25
20
15
10
5
0
Atleaf65 (W82)
Atleaf65 (eu3-e1)
OR18 (W82)
OR18 (eu3-e1)

shaking as described [8]. The PPFM isolates were recovered from the unifoliate leaves of greenhouse grown plants by grinding the leaves in sterile water and plating the macerate onto selective medium (AMS+Km). The PPFM isolates recovered from the leaves of eu3-e1 were urease-positive (Figure 4.5-3). This result also did not fit the prediction that urease-positive PPFMs will become urease-negative while associated with urease-negative *eu3-e1*/*eu3-e1* plants.

Strains Atleaf65 and OR18 (Table 3.1-1) were not originally isolated from soybean. Therefore, the results obtained above could be explained by a potential difference in host specificity even though the isolates do colonize the plant for at least one generation. To address possible host-specificity, I generated a Km^r isolate of *M.* sp. soyleaf2 by performing a bi-parental mating of the recipient soyleaf2 strain and an *E. coli* strain (S17-1) carrying the plasmid pSUP5011 containing transposon Tn*5*-mob [115]. *M.* sp. soyleaf2-140 (soyleaf2-140) was isolated (Table 3.1-1) and resembled the parent strain (soyleaf2) in growth conditions and was also urease-positive (data not shown) in free-living culture indicting that the transposon did not insert in a gene required for active urease.

Seeds of soybean W82 and eu3-e1 were inoculated with soyleaf2-140 by imbibition of an early-stationary phase PPFM culture at room temperature for 5 h with gentle shaking as described [8]. The Km^r PPFM isolates were recovered from the first trifoliate as described above. The soyleaf2-140 strain recovered from both W82 and eu3-e1 remained urease- positive (Figure 4.5-4). The urease status of the resident PPFMs from eu3-e1 was

Figure 4.5-3 Urease activity of PPFMs from colonized soybean plants. Seeds of soybean Williams 82 (W82) and *eu3-e1*/*eu3-e1* (eu3-e1) were inoculated with *M*. sp. *At*leaf1-65 (Atleaf65) or *M. extorquens*-OR18 (OR18) by imbibition of an early-stationary phase PPFM culture at room temperature for 5 hours with gentle shaking as described [8]. The PPFM isolates were recovered from the unifoliate leaves by grinding the leaves in sterile water and plating the macerate out onto selective media. Quantitative urease assays were performed as described [119] based on the release of $^{14}CO_2$ from [^{14}C]urea.

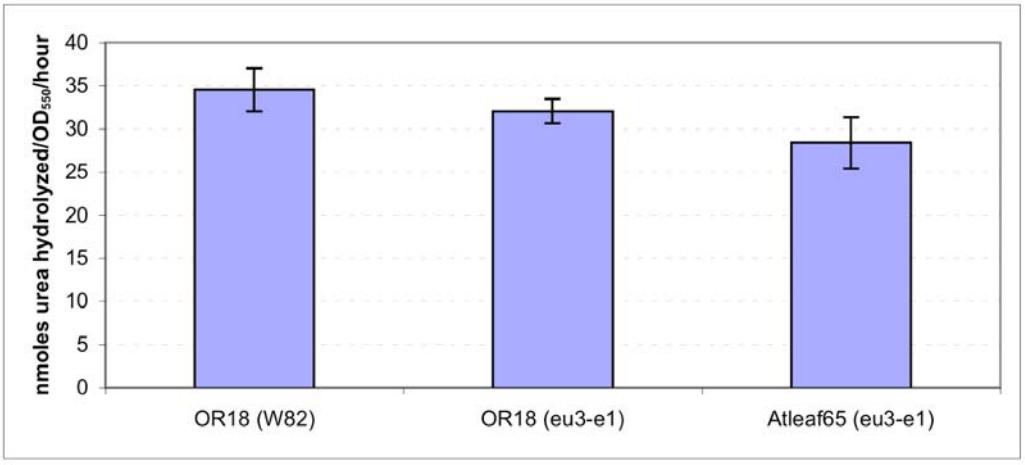
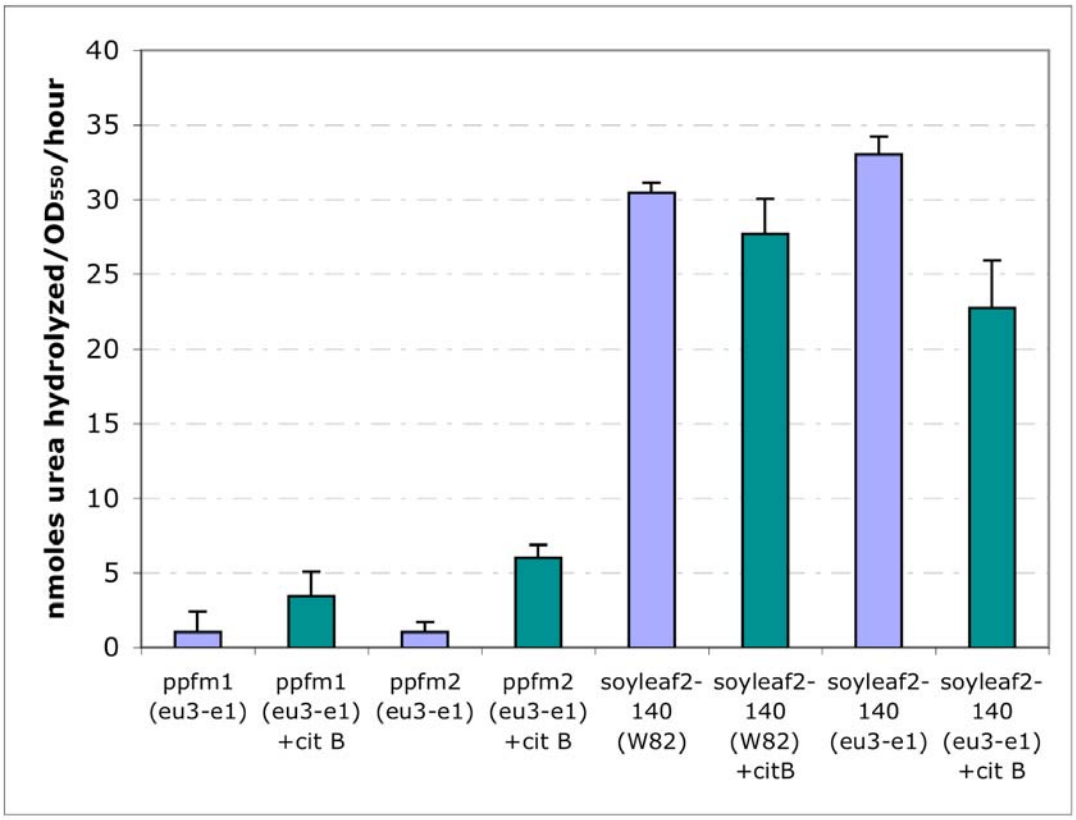

nmoles urea hydrolyzed/OD550/hour
40
35
30
25
20
15
10
5
0
OR18 (W82)
OR18 (eu3-e1)
Atleaf65 (eu3-e1)

Figure 4.5-4 Urease activity of PPFMs from soybean plants. Seeds of soybean Williams 82 (W82) and *eu3-e1*/*eu3-e1* (eu3-e1) were inoculated with *M*. sp. soyleaf2-140 by imbibition of an early-stationary phase PPFM culture at room temperature for 5 hours with gentle shaking as described [8]. The PPFM isolates were recovered from the first trifoliate leaves by grinding the leaves in sterile water and plating the macerate out onto selective media. Quantitative urease assays were performed as described [119] based on the release of $^{14}CO_2$ from [^{14}C]urea.

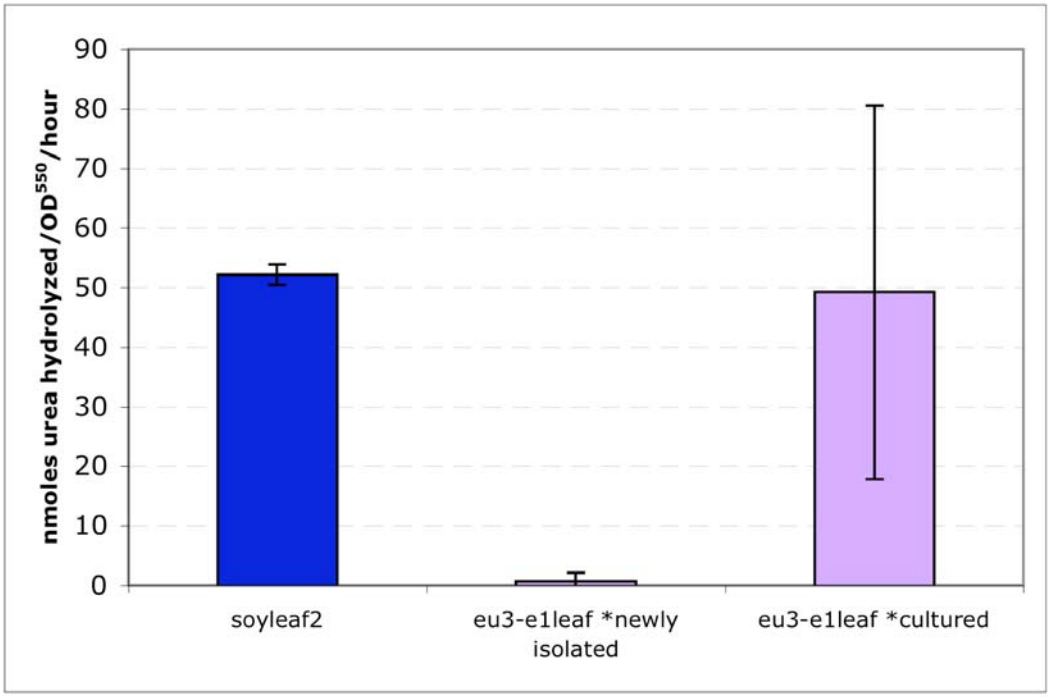

nmoles urea hydrolyzed/OD550/hour
40
35
30
25
20
15
10
5
0
ppfm1 (eu3-e1)
ppfm1 (eu3-e1) +cit B
ppfm2 (eu3-e1)
ppfm2 (eu3-e1) +cit B
soyleaf2-140 (W82)
soyleaf2-140 (W82) +citB
soyleaf2-140 (eu3-e1)
soyleaf2-140 (eu3-e1) +cit B

assayed from two fresh isolates, ppfm1 and ppfm2. The urease status of these fresh isolates was urease-negative and some activity was restored with the addition of a nickel chelate (citB) as described [6] (Figure 4.5-4).

Although the soyleaf2-140 strain is a derivative of a strain isolated from soybean, it was not a strain that was originally urease-negative (it came from a urease-positive plant) so that the possibility that this strain does not behave as ppfm1 and ppfm2, above (i.e. it is not urease-negative while associated with *eu3-e1/eu3-e1*) cannot be excluded. To address this possibility I generated a Km^r strain of an *eu3-e1/eu3-e1* isolate that was transiently urease-negative while associated with the plant. Unfortunately, the isolates described by Holland and Polacco [6] were not saved, so I was obliged to recover and characterize new *eu3-e1/eu3-e1* isolates. The *M*. sp. *eu3-e1*leaf isolate (eu3-e1leaf) was recovered from a trifoliate leaf of *eu3-e1/eu3-e1* and was transiently urease-negative, becoming fully urease-positive in culture (Figure 4.5-5). Km^r *eu3-e1*leaf isolates, *M*. sp. *eu3-e1*leaf-C4 and *eu3-e1*leaf-C5 (eu3-e1leafC4 and eu3-e1leafC5 respectively) were then generated as described above (Table 3.1-1).

Seeds of soybean W82 and *eu3-e1* were inoculated with eu3-e1leafC4 or eu3-e1leafC5 by imbibition of an early-stationary phase PPFM culture at room temperature for 5 h with gentle shaking as described [8]. The Km^r PPFM isolates were recovered from the first trifoliate leaves as described above. Kanamycin-sensitive (Kan-S) PPFMs were recovered from AMS imbibed controls as well as from the PPFM-inoculated *eu3-e1/eu3-e1* plants. The Km^r strains eu3-e1leafC4 and eu3-e1leafC5 were both urease-positive

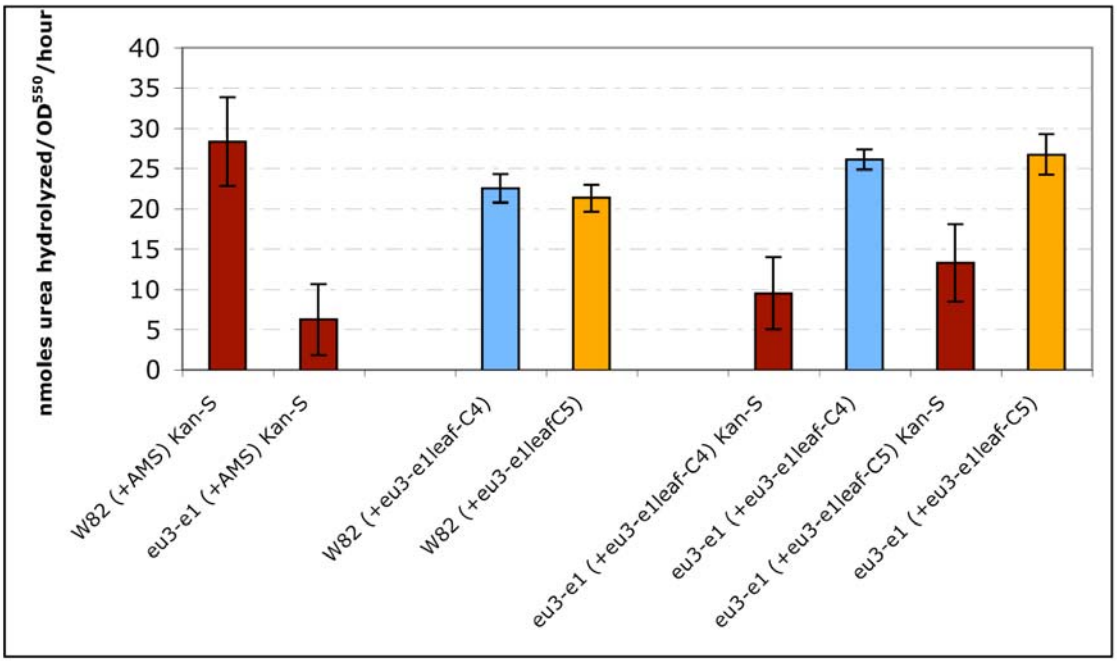
Figure 4.5-5 A PPFM isolate from *eu3-e1/eu3-e1* is transiently urease-negative. The *M*. sp. *eu3-e1*leaf isolate (eu3-e1leaf) recovered from a trifoliate leaf of *eu3-e1/eu3-e1* was transiently urease-negative and becomes fully urease-positive in culture. The *M*. sp. soyleaf2 isolate (soyleaf2) was included in the assay as a control. Quantitative urease assays were performed as described [119] based on the release of $^{14}CO_2$ from [^{14}C]urea.


90
80
70
60
50
40
30
20
10
0
nmoles urea hydrolyzed/OD550/hour
soyleaf2
eu3-e1leaf *newly isolated
eu3-e1leaf *cultured

when recovered from both W82 and *eu3-e1/eu3-e1* inoculated plants (Figure 4.5-6). The activity of the Kan-S isolates from the AMS imbibed control plants are shown in Figure 4.5-6. The Kan-S resident isolates from the inoculated plants were not completely urease-negative but had 34% to 48% of the activity of the Km^r isolates from the same plants. Since it is documented that the isolates from *eu3-e1/eu3-e1* plants are transiently urease-negative ([6] and Figure 4.5-5), this activity can be explained by the isolates already gaining activity in culture before the assay. Thus, our prediction that PPFMs recolonized on urease-negative plants will become urease-negative while associated with plants but will regain urease activity when cultured away from the tissue was refuted. It is worth mentioning that the recolonized strains were all marked with kanamycin resistance and this could affect the results. However, efforts to reduce this possibility included using multiple marked strains all expected to contain the insertion in different genomic regions. This result led us to alter our model (Figure 2.1-1) to explain (1) why the PPFMs from soybean urease-negative accessory gene mutants were urease-negative and (2) why this trait was not seen in PPFMs re-introduced to urease-negative accessory gene mutants.

Our working model suggests that there is some signal from the plant that either inhibits the production of the urease gene products in the associated bacteria or inhibits the function or transport of Ni^{2+} from the plant to the bacteria. Our altered model suggests that a "special" or "stable" PPFM-plant association results in the urease-negative phenocopy of the resident PPFM population on *eu3-e1* plants. And, that this association

Figure 4.5-6 Urease activity of Kan-resistant PPFMs recovered from inoculated Williams 82 and *eu3-e1/eu3-e1* plants compared to the Kan-sensitive resident population. Seeds of soybean Williams 82 (W82) and *eu3-e1*/*eu3-e1* (eu3-e1) were inoculated with *M*. sp. *eu3-e1*leaf-C4 (eu3-e1leafC4) or *M*. sp. *eu3-e1*leaf-C5 (eu3-e1leafC5) as in Figure 4.5-4. These isolates are derivatives of the isolate that was originally urease-negative when recovered from *eu3-e1*/*eu3-e1* (Figure 4.5-5). The Kan-resistant PPFM isolates were recovered from the first trifoliate leaves by grinding the leaves in sterile water and plating the macerate onto selective media. Kan-sensitive (Kan-S) PPFMs were recovered from AMS imbibed controls as well as from the PPFM-inoculated *eu3-e1/eu3-e1* plants. Quantitative urease assays were performed as described [119] based on the release of $^{14}CO_2$ from [^{14}C]urea.


nmoles urea hydrolyzed/OD550/hour
40
35
30
25
20
15
10
5
0
W82 (+AMS) Kan-S
eu3-e1 (+AMS) Kan-S
W82 (+eu3-e1leaf-C4)
W82 (+eu3-e1leafC5)
eu3-e1 (+eu3-e1leaf-C4) Kan-S
eu3-e1 (+eu3-e1leaf-C4)
eu3-e1 (+eu3-e1leaf-C5) Kan-S
eu3-e1 (+eu3-e1leaf-C5)

is different in recolonized inhabitants. To address this, we attempted to recover the re-introduced isolates from the next plant generation.

PPFMs are seed transmitted in soybean [3] and surface sterilization treatments are not effective for removal suggesting that bacteria on the leaf surface are descendents of seed-borne bacteria [6]. In the recolonization experiments described above, I was not able to recover any of the marked strains from the next generation, i.e. neither from seeds set on the recolonized plants nor from the leaves of those seeds sowed in greenhouse conditions. This result was not expected and may suggest a competitive advantage for the endogenous PPFM population passing on to the next plant generation. Another unexpected result was the inability to recover PPFMs from *eu3-e1/eu3-e1* segregant plants after 10 generations of single seed descent of heterozygous *eu3-e1*/Eu3. It appears that these *eu3-e1/eu3-e1* seeds have been rid of their PPFMs.

The alternative hypothesis states that a "special" or "stable" PPFM-plant association results in the urease-negative phenocopy on *eu3-e1* plants. If altered host Ni metabolism results in the bacterial urease-negative state, sub-hypotheses can posit: 1) lack of Ni in plant tissues; 2) a block in Ni transport; or 3) co-opting of bacterial urease activation by the plant activation machinery. The latter would predict that the host plant could correct the urease-negative phenotype of an associated PPFM-*ureG* mutant. However, this experiment was not tested because we could not duplicate the urease-negative trait in wild type PPFMs (Figure 4.5-5 and 4.5-6). So the PPFM-ureG disruption mutant was not made.

Sub-hypotheses 1 and 2 predict that PPFMs from *eu3-e1* and from *eu2* plants will have less internal Ni^{2+}. The first posits that Ni^{2+} pools are virtually zero in the *eu3-e1* and *eu2* plant hosts. However, Ni^{2+} uptake and translocation appear normal in *eu2/eu2* and *eu3-e1/eu3-e1* plants [6], so Ni^{2+} should be available to PPFMs.

To test sub-hypothesis 2 I am in the process of pooling sufficient culture from urease-negative PPFMs isolated from *eu3-e1* and wild type plants to be able to determine their Ni^{2+} content and the ability to take up ^{63}Ni.

Why PPFMs are urease-positive on *eu4* plants and urease-negative on *eu3* plants lies in the difference between these two plant genotypes. Eu4 is the structural gene for the ubiquitous urease, whereas Eu3 is an accessory gene required to activate urease. In *eu4* plants the activation machinery required to deliver nickel to the urease active site is functional. This could be a signal to the bacteria associated with those plants to activate their urease. In *eu3* plants the activation machinery is not active. Those plants produce the urease holoenzyme but it is not activated. Nickel is not utilized by the urease activation machinery and does not get into the urease active site. In this scenario, the nickel could be sequestered off to some unknown function in the plant cell or could be bound by a nickel delivery protein within the plant (i.e. UreE, some other nickel-binding protein?) in transit for the activation machinery unavailable to the associated bacteria.

4.6 Urease activity of *Methylobacterium* spp. from urease-negative *Arabidopsis.*

PPFMs associated with urease-negative soybean mutants, which lack functions for insertion of nickel in the plant urease active site, were urease-negative themselves while on the plant [6]. My goal was to determine the urease status of PPFMs associated with urease-negative *Arabidopsis* mutants. The prediction is that PPFMs associated with *Arabidopsis* urease-negative accessory gene mutants (*At-ureD,F,G*), but not the structural gene mutant (*At-ure-1*), will be urease-negative while associated with plant, but regain urease activity in culture.

I first wanted determine conditions required to remove (or reduce) and recover the PPFM population from *Arabidopsis.* The prediction was that reduced populations of PPFMs in the seeds of *Arabidopsis* would reduce the germination percentage of those seeds. These experiments were important to determine the conditions needed to recover the PPFM isolates from *Arabidopsis* for the urease assay. Seedlings from seeds exposed to several standard treatments (including dry heat, chemical treatment and microwaves) growing on ½ MS were ground in sterile water and the macerate plated on selective medium (AMS). Surprisingly, no PPFMs were recovered from any of the plants, including the surface sterilization treatments involving ethanol/bleach or Cl_2 gas (data not shown). However, if *Arabidopsis* seeds were planted onto PPFM selective AMS (solidified with tissue culture grade agar) without surface sterilization, PPFM colonies become visible around some, but not all, of the seedlings about 5 d after planting and began to form a ring or halo around the seedling. It appears the PPFMs associated with *Arabidopsis* seeds are not as protected as those associated with soybean where surface sterilization techniques to

remove PPFMs have proven not to be 100% effective [7]. I used this method to recover PPFM isolates from *Arabidopsis* seeds for the urease assay.

Wildtype *Arabidopsis thaliana* (WT) and urease-negative mutants *At-ure-1, At-ureD-1, At-ureF-1* and *At-ureG-2* were surface sterilized and germinated on ½ MS, then transplanted to sterilized pro-mix and grown in a growth chamber. The PPFM isolates were recovered by grinding the leaves in sterile water and plating the macerate onto AMS. PPFMs recovered from the plants were all urease-positive (Figure 4.6-1). Given the results above that PPFMs were not recovered from surface sterilized *Arabidopsis* seeds in tissue culture, these results could be explained by PPFMs already present in the growth chamber which became associated with those plants, i.e. that the resident PPFM population on the *Arabidopsis* mutants was not in fact that assayed. However, in this case if the PPFMs were 'covert contaminants', the results support the data shown for recolonized soybean in Chapter 4.5 (Figure 4.5-6).

To be certain that endogenous PPFMs were recovered from the *Arabidopsis* urease-negative mutants, I planted the seeds directly on PPFM selective medium (AMS, solidified with tissue culture grade agar) without surface sterilization. PPFMs were recovered from all of the treatments by selecting colonies as soon as they became visible around the seedlings. The urease activity of the PPFMs in the *Arabidopsis* structural gene mutant and the accessory gene mutants were all urease-positive (Figure 4.6-2). This result did not agree with the soybean situation ([6] and Figure 4.5-6). PPFMs associated with *Arabidopsis* urease-negative accessory gene mutants (*At-ureD,F,G*), as well as the

Figure 4.6-1 Urease activity of PPFMs from urease-negative mutants of *Arabidopsis* grown in sterile soil. Wildtype *Arabidopsis thaliana* (WT) and urease-negative mutants *At-ure-1, At-ureD-1, At-ureF-1* and *At-ureG-2* were surface sterilized and germinated on ½ MS media, then transplanted to sterilized pro-mix and grown in a growth chamber. The PPFM isolates were recovered by grinding the leaves in sterile water and plating the macerate out onto selective media. Quantitative urease assays were performed as described [119] based on the release of $^{14}CO_2$ from [^{14}C]urea.

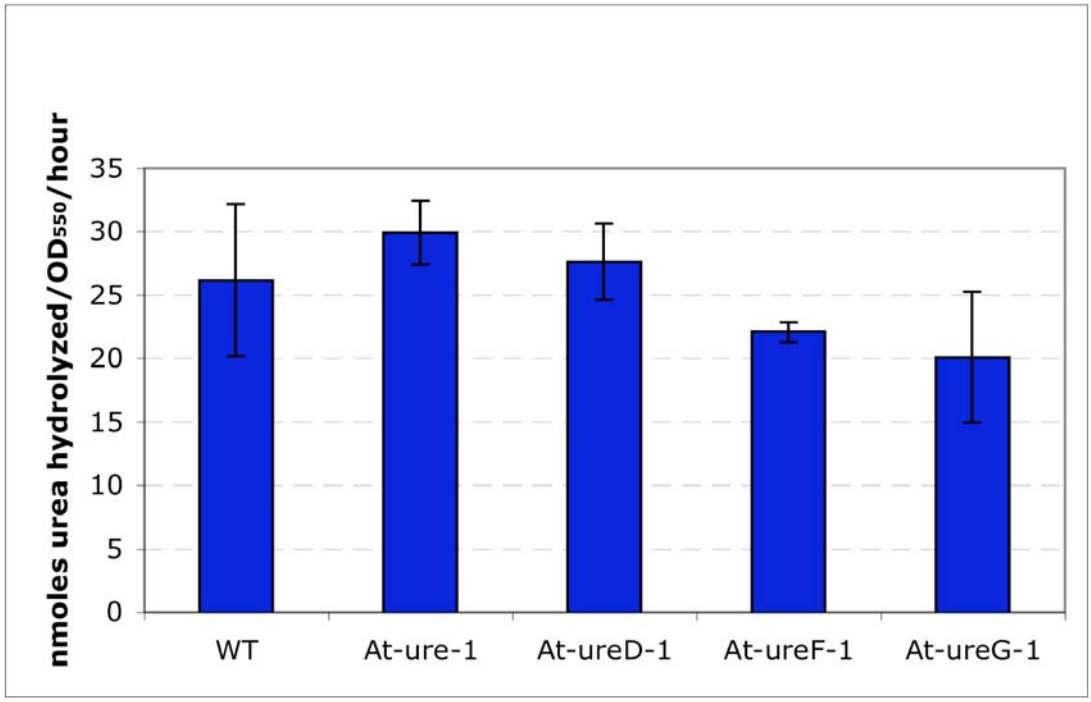
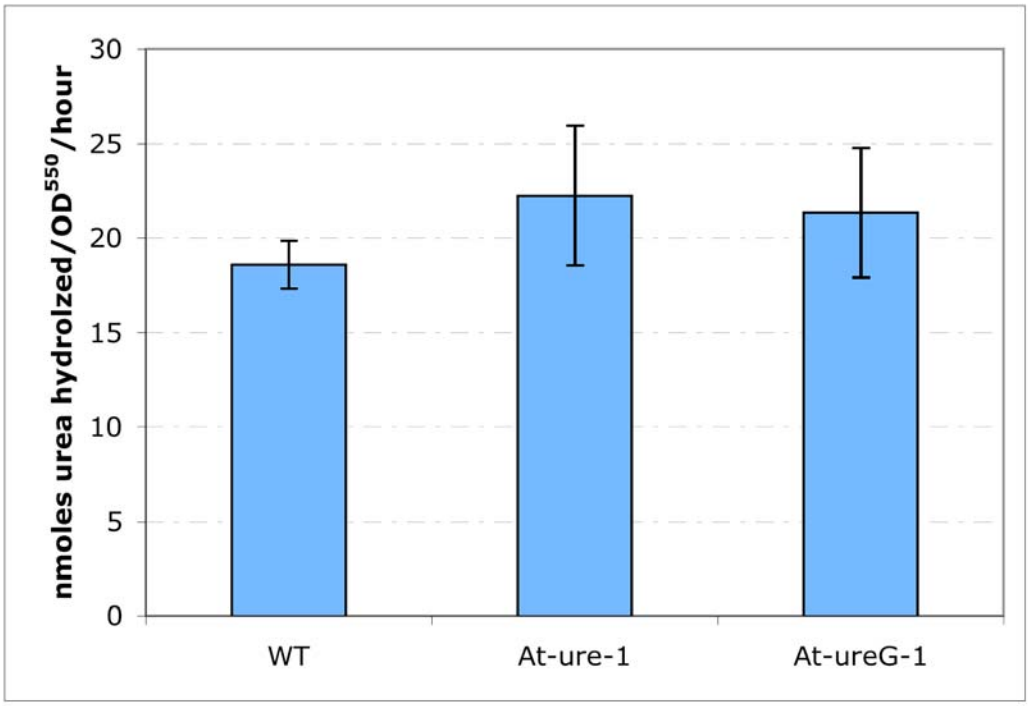

nmoles urea hydrolyzed/OD550/hour
35
30
25
20
15
10
5
0
WT
At-ure-1
At-ureD-1
At-ureF-1
At-ureG-1

Figure 4.6-2 Urease activity of PPFMs from urease-negative mutants of *Arabidopsis* grown in sterile culture. Wildtype *Arabidopsis thaliana* (WT) and urease-negative mutants *At-ure-1* and *At-ureG-2* were germinated without surface sterilization on PPFM selective media (AMS) solidified with plant tissue culture agar. The PPFM isolates were recovered by tooth-picking colonies that grew on the medium surrounding the germinated plants. Quantitative urease assays were performed as described [119] based on the release of $^{14}CO_2$ from [^{14}C]urea.


nmoles urea hydrolzed/OD550/hour
30
25
20
15
10
5
0
WT
At-ure-1
At-ureG-1

structural gene mutant (*At-ure-1*), were all urease-positive. It appears that the condition causing the bacteria associated with soybean to remain urease-negative while on the plant is not present in *Arabidopsis*. Given this result, this relationship provides an excellent tool for comparison to determine the factors in some urease-negative soybean that render their associated bacteria urease-negative. In a side-by-side comparison with the PPFMs isolated from soybean mutants, it would of interest to measure nickel levels in the PPFMs isolated from *Arabidopsis*. PPFM isolates from urease-negative *Arabidopsis* are urease-positive. What is different between soybean and *Arabidopsis* that could explain these results? It is possible that nickel is delivered to the activation machinery differently in each of these plants. This difference could be the signal that accounts for the associated bacteria to alter the expression of their own urease.

5. SUMMARY

This research focused on furthering our understanding of the interactions between Pink-Pigmented Facultative Methylotrophic bacteria (PPFMs) and plants. PPFMs (*Methylobacterium* spp.) have been found to be the most abundant microorganisms among phylloplane microflora, and have been recovered from all plants examined. I focused on *Arabidopsis thaliana* and *Glycine max* (soybean), two dicots with which our lab works and which provide valuable mutants. While the genetics, and genome, of each plant is increasingly known, little was known about the identity of the PPFMs. To this end I established phylogenetic relationships of various PPFMs employed in the Polacco lab and elsewhere. The *Arabidopsis* and soybean leaf isolates clustered with *M. extorquens* AM1 while *M. extorquens* clustered with *zatmanii* and sp. CM4 suggesting that all these strains are closely related and may all be best fit into the species '*extorquens*' with strain designations. The barley1, maize1 and broccoli1 sequences all clustered with *mesophilicum, radiotolerans* and *fujisawaense* suggesting that all of these isolates may be best fit into one species, for example '*mesophilicum*'.

One point of interaction between PPFMs and plants was in nitrogen metabolism. Especially intriguing to us was the ability of resident PPFMs to mimic the urease-negative phenotype of two urease-negative soybean host plants. I developed a working model that suggests that there is some signal from the plant that either inhibits the production of the urease gene products in the associated bacteria or inhibits the function or transport of Ni^{2+} from the plant to the bacteria. This signal could be a nitrogenous signal (ureides, urea, ammonia) or simply a block in a bacterial transporter required to

take up Ni^{2+} from the plant cell periphery or apoplast. I attempted the recolonization of plants with PPFMs to determine how this affects urease activity in recovered isolates. PPFM isolates, used to colonize the seed, can be recovered from the aerial portions of the plant, though the urease-activity of those from urease-negative soybean mutants was urease-positive. These recolonized isolates do not mimic the phenotype of the host plant (urease activation-negative), and in this we showed that they differ from the resident population which have little urease activity. In the recolonization experiments, I was not able to recover any of the marked strains from the next generation, i.e. neither from seeds set on the recolonized plants nor from the leaves of those seeds sowed in the greenhouse. This result was not expected and may suggest a competitive advantage for the endogenous PPFM population to be passed on to the next plant generation.

Examination of urease expression *in planta* or in culture requires knowledge of the urease genes and the regulation of urease in the PPFMs. Urease expression was directly related to the role of urease in nitrogen assimilation. My studies led to the overall conclusion that urease was essential for assimilation of urea and of ureides, that urease has a constitutive basal level of expression and was "induced" by the ureide allantoin and "repressed" by the preferential nitrogen source, ammonium, but that these nitrogenous signals were not responsible for the urease-negative status of the plant-associated PPFMs. Our working model thus has shifted to investigate nickel metabolism. We posit that nickel was not taken up by the bacteria in activation-negative hosts, or that if taken up, the activation machinery was inoperative. Determining whether nickel is transported into

the bacterial cell will be a big step forward in understanding how the soybean genotype affects the expression of urease in the associated PPFMs.

We found that the urease activity of all PPFM isolates from urease activation and structural gene mutants of *Arabidopsis* were urease-positive. It appears that the condition causing the bacteria associated with soybean to remain urease-negative while on the plant was not operative in *Arabidopsis*. Given this result, this relationship provides an excellent tool for comparison to determine the factors in urease-negative soybean that render their associated bacteria urease-negative.

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