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).

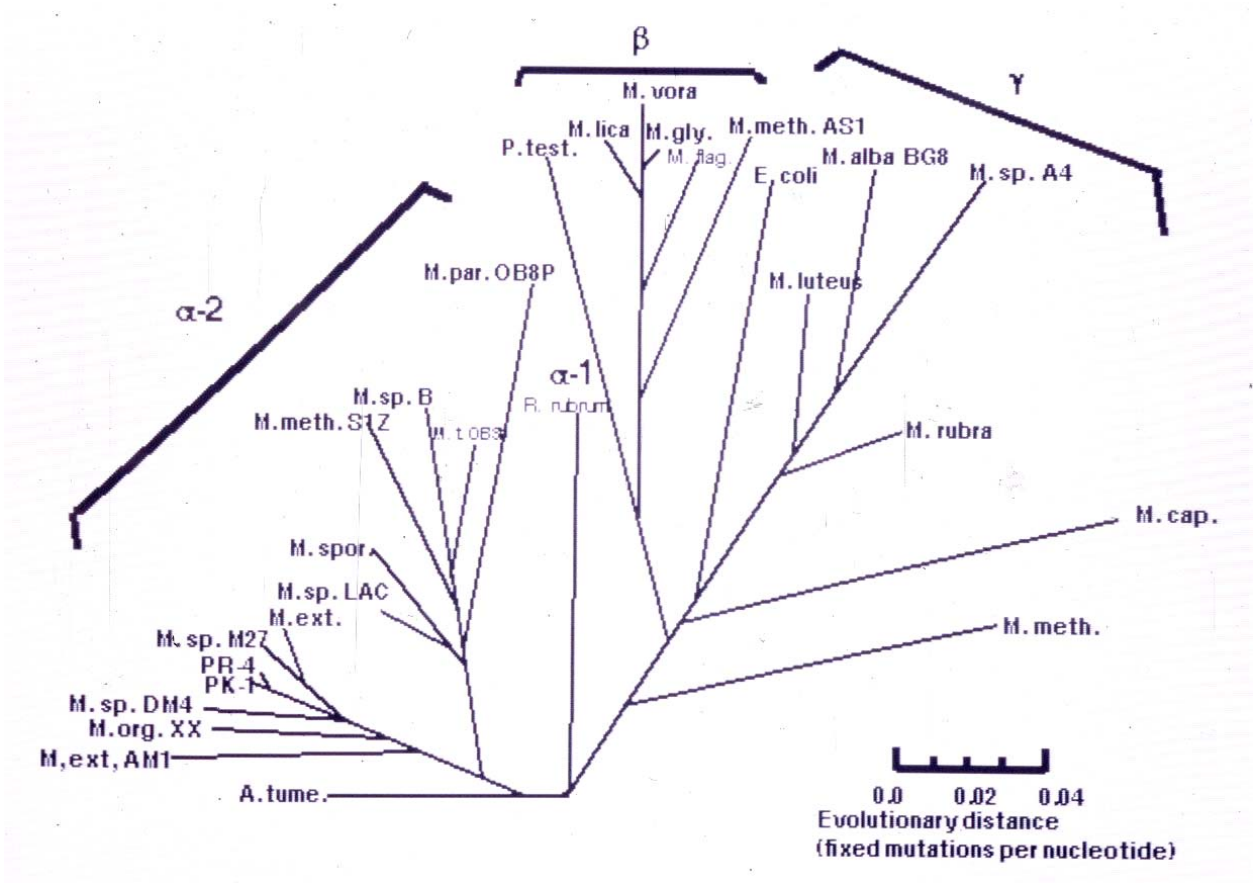
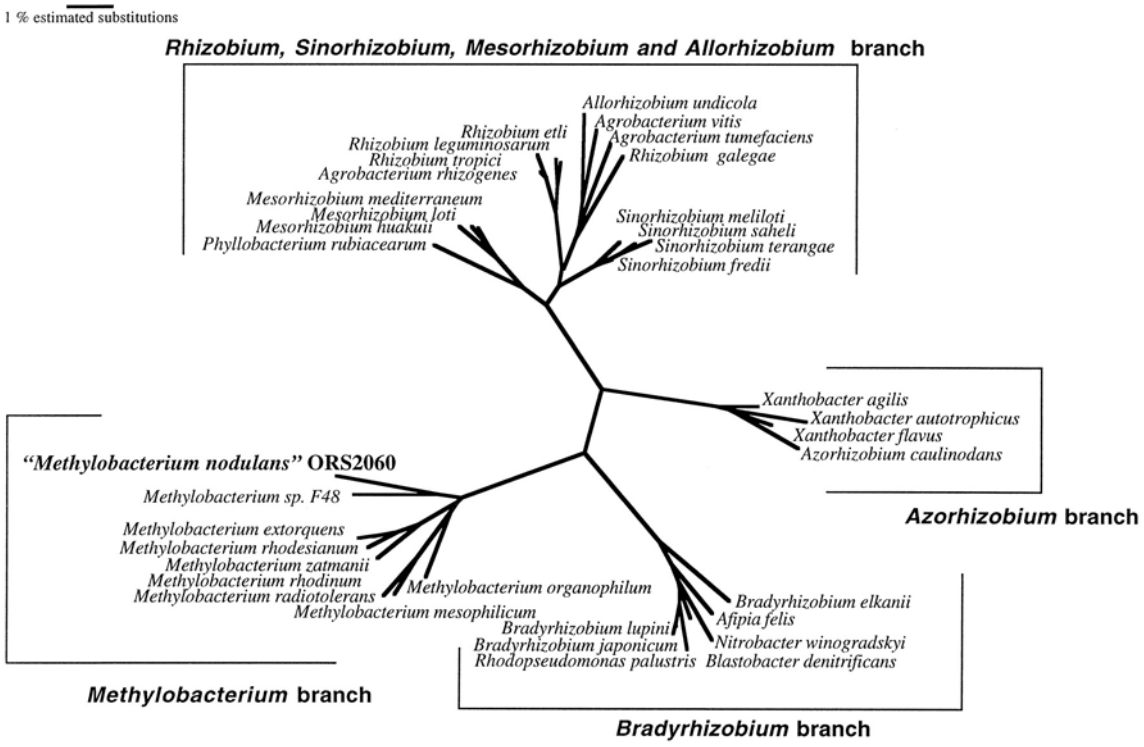

β
γ
α-2
α-1
M. vora
M. lica
M.gly.
M.meth. AS1
P.test.
M. flag.
E. coli
M. alba BG8
M. sp. A4
M.par. OB8P
M. luteus
M.sp. B
M.meth. S1Z
M. t OB3
R. rubrum
M. rubra
M. cap.
M. spor.
M.sp. LAC
M.ext.
M. meth.
M. sp. M2Z
PR-4
PK-1
M. sp. DM4
M.org. XX
M,ext, AM1
A.tume.
0.0
0.02
0.04
Evolutionary distance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

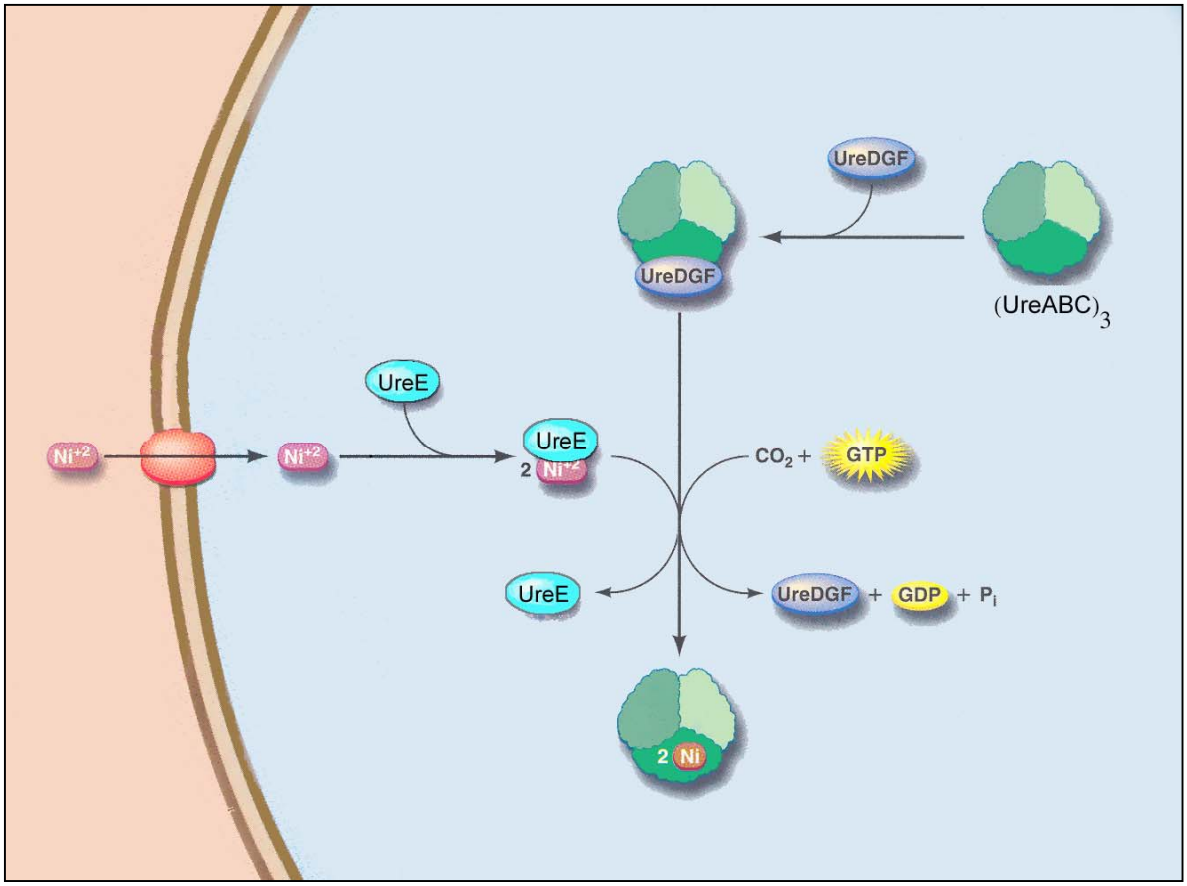
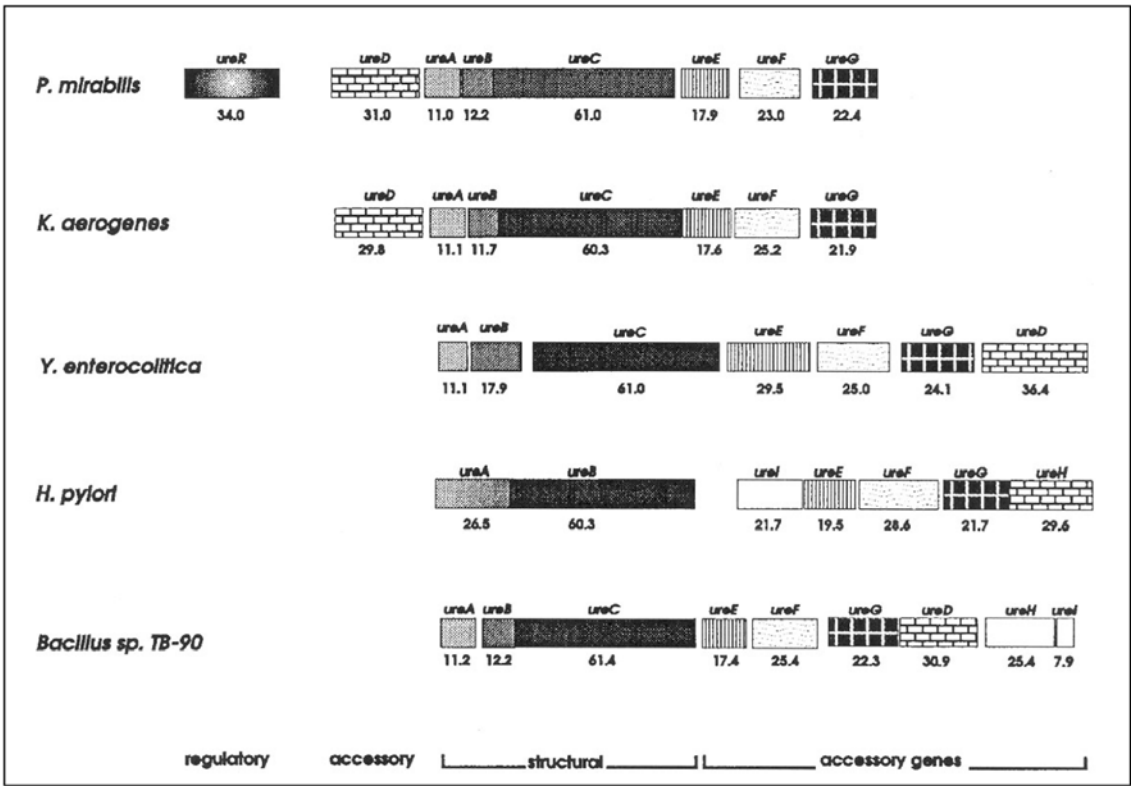

UreDGF
UreDGF
(UreABC)3
UreE
Ni+2
Ni+2
UreE
2 Ni+2
CO2 +
GTP
UreE
UreDGF + GDP + Pi
2 Ni

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-

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:

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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].

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

6. REFERENCES

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