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Characterization of Pinitol Catabolism in Sinorhizobium Meliloti and its Role in Nodule Occupancy

Angela Irene Kennedy-Mendez
Western Michigan University

CHARACTERIZATION OF PINITOL CATABOLISM IN *SINORHIZOBIUM MELILOTI* AND ITS ROLE IN NODULE OCCUPANCY

by
Angela Irene Kennedy-Mendez

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Thesis Committee:

Silvia Rossbach, Ph.D.
Todd Barkman, Ph.D.
Jian Yao, Ph.D.

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Sinorhizobium meliloti, which is the nitrogen fixing symbiont of *Medicago sativa* (alfalfa), was used to test the hypothesis that D-*chiro*-inositol is an intermediate of pinitol catabolism and that pinitol catabolism plays a role in *S. meliloti's* ability to compete for nodule occupancy. An analytical method, involving gas chromatography and mass spectrometry (GC-MS), was developed to detect and separate pinitol and D-*chiro*-inositol. The *S. meliloti* wild type, and three isogenic mutant strains (*idhA*, *mocD*, and *mocE*) were grown in minimal medium with pinitol and pinitol plus glycerol. The *S. meliloti* wild type can utilize pinitol as a sole carbon source, whereas the mutant strains cannot. The *idhA* mutant removed 50% of the detectable pinitol from the medium when grown with pinitol plus glycerol and showed accumulation of D-*chiro*-inositol. Therefore, it was concluded that D-*chiro*-inositol is indeed an intermediate of pinitol catabolism. To test the ability of pinitol catabolism mutants to compete with the wild type for nodule occupancy, alfalfa plants were inoculated with a 1:1 ratio of wild type *S. meliloti* and the *idhA*, *mocD*, and *mocE* mutants. Plants nodules were collected at eight and 16 weeks and the percentage of the nodule inhabitants was determined. Each mutant was found to be outcompeted by the wild type by week 16. Therefore, it was concluded that the ability to catabolize pinitol does indeed play a role in nodule occupancy.

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INTRODUCTION

Throughout the history of agriculture, the main goal has been to increase the yield and quality of crops. One of the main limiting factors of plant yield and quality is the availability of nitrogen that plants need for their metabolic processes. While nitrogen is found abundantly in the atmosphere, it is present in an inert form, two nitrogen atoms triple bonded together (N_2). This atmospheric nitrogen is inaccessible to plants. Therefore, farmers frequently use nitrogen-rich fertilizers on their crops. Between 1960 and 2000 fertilizer use increased by about 800% (Fixen, 2002). Unfortunately, the massive use of fertilizers results in depositions of nitrogen compounds into the environment, which causes a cascade of health and environmental safety issues (Galloway et al., 2008). Two common nitrogen compounds in fertilizers are ammonium and nitrate. Ammonium carries a positive charge while nitrate carries a negative, and because soil usually has a net negative charge, ammonium can associate within soil particles. Nitrate is more mobile and thus is easily washed out from agricultural fields into the ground water and nearby rivers. Contamination of ground water with nitrate, which is the most common contaminant found in ground water, is considered a serious health concern (Solley et al., 1995). The less mobile ammonium does not remain in the top soil very long either, mainly because of the presence of nitrifying bacteria that convert ammonium to nitrite, which then can be further converted to other nitrogenous oxides. For these reasons, it is easy to understand that the efficiency of fertilizers can be below 40% (Canfield et al., 2010), and that the use of excessive amounts of fertilizers is polluting the environment. Currently fertilizer use is essential for the production of food for numerous people around the globe, but perhaps there is a better way to provide plants with the nutrients they need.

Nitrogen fixation

Nitrogen is an essential compound for all life forms because nitrogen is part of the building blocks of nucleotides that are used for DNA and RNA synthesis, as well as in amino acids for protein synthesis. Animals obtain nitrogen by consuming organic matter, while plants rely on their roots to take up ammonium or nitrate from soil. Usable nitrogen can be deposited into the soil by prokaryotic organisms through nitrogen fixation, but currently more often than not, in agriculture, farmers use nitrogen-rich fertilizer to ensure their crop plants have sufficient nitrogen. Without assistance from microorganisms or fertilizers most plants are under nitrogen-limited conditions.

Nitrogen fixed by the Haber-Bosch process is used to create synthetic fertilizers. The Haber-Bosch process uses high temperature and pressure to convert atmospheric nitrogen into ammonium. This requires a lot of energy, which is mainly obtained by the burning of fossil fuels (Canfield et al., 2010). However, some microorganisms that can fix nitrogen via an enzyme called nitrogenase are a sustainable alternative to the use of fertilizers. There are free-living nitrogen-fixing microorganisms, including *Anabaena*, *Azotobacter*, *Clostridium* and *Klebsiella*, but there are also symbiotic nitrogen-fixing microorganisms, which belong mainly to the *Rhizobiaceae* family. *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium, Cupriavidus*, and *Burkholderia* are examples of nitrogen fixing rhizobia (Barea et al., 2005). Most genera in the *Rhizobiaceae* family are within the *alpha*-proteobacterial class, however, within the last decade some bacteria of the *beta*-proteobacteria class such as *Burkholderia* and *Cupriavidus* have been classified as symbiotic nitrogen fixing *beta*-rhizobia (Jz et al., 2017).

Sinorhizobium meliloti, the model organism used in this study, is a gram-negative bacterium in the *alpha*-proteobacteria class. The work in this thesis was completed with the *S. meliloti* strain 2011. The DNA sequence of the complete genome *S. meliloti* strain 1021 was determined in 2001 (Galibert et al., 2001). *Sinorhizobium meliloti* 2011 and 1021 are derived from the same ancestral strain; their genomes should be identical with a few differences due to being maintained in different laboratories. The entire genome contains a 3.65-Mb chromosome and two plasmids, 1.35-Mb pSymA and 1.68-Mb pSymB (Galibert et al., 2001). *Sinorhizobium meliloti* can be found free living in soil or in a symbiotic association with *Medicago sativa* (alfalfa). When *S. meliloti* forms symbiosis with alfalfa it involves the formation of so-called root nodule. Inside the nodules *S. meliloti* differentiates into bacteroids and expresses genes required for nitrogen fixation. The formation of nodules and the differentiation into bacteroids both involve complex reorganization of gene expression in both plants and bacteria (Sallet et al., 2013).

Rhizosphere

The small amount of soil that adheres to the roots of a plant is called the rhizosphere. (One can describe the rhizosphere as the soil that sticks to the roots of a plant that has been pulled out of the ground.) Plants excrete nutrients through their roots on a regular basis, therefore the rhizosphere is different than bulk soil. The high concentration of nutrients in the rhizosphere promotes a dense community of microorganisms leading to a high competition for the nutrients (Berg & Smalla, 2009). In comparison to bulk soil, the rhizosphere can support much more life, but interestingly, the diversity of the microorganisms is not increased (Berg & Smalla, 2009). Since the numbers of cells increase, but diversity does not, there must be pressures acting on the

microbes. The nutrients found in the rhizosphere vary depending on the plant species, therefore different microorganisms are favored based on their ability to utilize compounds produced by the plant roots (Berg & Smalla, 2009).

Infection and nodulation

Via a complex signal exchange system, a specific rhizobial species is able to enter a specific plant species root inner cortex cells. Flavonoids (2-phenyl-1,4-benzopyrone derivatives) are aromatic compounds that are excreted from the roots of legume plants; they act as the first signal to the rhizobia (Jones et al., 2009). Flavonoids are considered secondary plant metabolites and they can differ in their number of residues as well as side groups that allows them to be selective for a particular *Rhizobium* strain by their ability to bind to species-specific bacterial NodD proteins (Jones et al., 2009). Transcription regulation occurs in the rhizobia in response to flavonoids. Activated NodD protein binds to DNA at the "nod-box" promoter and leads to the transcription of *nod* genes that are translated into enzymes that synthesize Nod factors (Capela et al., 2005) The *nod* genes are located on pSymA (Barloy-Hubler et al., 2000). Nod factors are lipo-chito-oligosaccharide compounds that are essential for the ability of rhizobia to invade the plant (Jones et al., 2009). If the plant recognizes the correct Nod-factors in their environment, the plant undergoes various changes that allow for bacterial invasion which includes increasing calcium concentrations in the root cells, reinitiating mitosis of root cortex cells, and alterations to the root hair cytoskeleton (Timmers et al., 1999). After sufficient changes have occurred, the plant root hair grows around the nearby bacteria and traps them. This growth is referred to as a Shepard's crook. These trapped rhizobia are able to progressively travel across the root hair to the inner cortex by producing an infection thread. The infection thread is an important step in the

entire process; the ability for rhizobia to travel through it is influenced by a variety of exopolysaccharides and Nod factors (Gibson et al., 2008). Once the rhizobia reach the inner cortex of the root, they are released into the cortical cells. Several bacteria can be taken up by a single cortex cell at the same time within a plant derived membrane. Because of bacterial and plant factors, the rhizobial cells differentiate into bacteroids which gain the ability to survive in its new environment (Jones et al., 2009). Bacteroids are characterized by being slightly larger and having "Y" shaped cells, which have a higher DNA content (Jones et al., 2009). As plant and bacteria cells grow and divide, and while the rhizobia differentiate, a nodule is formed on the plant roots. Bacteroids within nodules can synthesize the enzyme nitrogenase, the enzyme that catalyzes nitrogen fixation. Like the *nod* genes, the genes required for nitrogen fixation (*nif* genes) are located on pSymA, but a few are also located on pSymB (Finan, et al., 2001).

Nitrogenase is sensitive to oxygen; it's transcription is highly regulated by a cascade of oxygen sensing proteins. Nevertheless, rhizobia are aerobic, and this creates the so-called "oxygen paradox". Plants promote the transcription of nitrogenase by creating and maintaining a low oxygen environment within nodules with the use of a protein called leghemoglobin. Leghemoglobins are oxygen binding proteins, they keep low oxygen partial pressure in the nodules while delivering oxygen to the respiring bacteroids (Jones et al., 2009). Leghaemoglobins contain a heme group, with iron as a co-factor, and this results in nodules having a slight red color. The plants obtain nitrogen from the bacteroids due to nitrogen fixation, in the form of the amino acids glutamine and glutamate (Gibson et al., 2008). Bacteria obtain dicarboxylic acids, such as malate, from the plant as carbon sources through the dicarboxylate transport (DCT) uptake system (Gibson et al., 2008).

Inositol

Inositols, or cyclohexanehexols, are sugar alcohols that can exist in nine different stereoisomers (Fig.1). The *myo*-, *scyllo*-, *neo*-, *muco*-, D- and L-*chiro*- isomers are naturally occurring, whereas the *cis*-, *allo*-, and *epi*- isomers are synthetic inositols. The most common isomer in nature is *myo*-inositol, it plays an important role as a signaling molecule and as a structural compound in many eukaryotic cells (Loewus & Murthy, 2000). While inositols are found abundantly in eukaryotes, they are not found widely in prokaryotes. Also, the ability of some prokaryotes to utilize inositols and their derivatives is not widely distributed (Majumder et al., 2003).

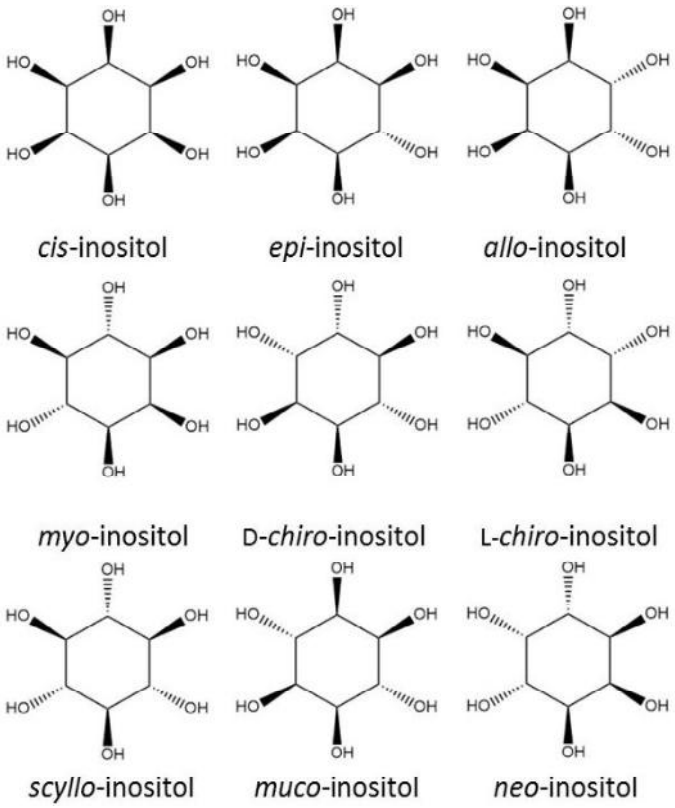

Figure 1. Structures of the nine stereoisomers of inositol.

There are several inositol derivatives that occur naturally, such as pinitol. Pinitol is a methylated inositol derivative that shares stereochemistry with D-chiro-inositol (Fig. 2).

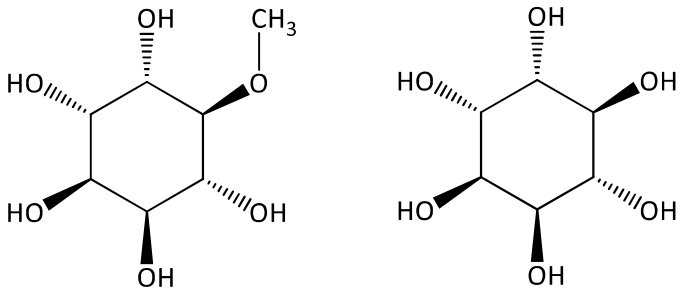

Figure 2. Schematics of pinitol and D-*chiro*-inositol.

It is suggested that pinitol could be a factor that plays a role in *S. meliloti* and alfalfa symbiosis for two reasons. *Sinorhizobium meliloti* can utilize pinitol as a sole carbon source (Thwaites, 2013) and because pinitol is found in high concentrations in alfalfa and other legumes (Klonowska et al., 2018). In fact, pinitol has been shown to function as an osmoregulator in alfalfa because during drought or salt stress pinitol concentrations throughout the plant increased significantly (Aranjuelo et al., 2011; Fougere et al., 1991; Palma et al., 2013).

Inositol catabolism

The catabolism of *myo*-inositol is well understood in the Gram-positive bacterium *Bacillus subtilis*, but not much is known about the catabolism of the other isomers (Yoshida et al., 1997). The genes necessary for the utilization of inositol include *iolABCDEFGHIJ*, which form one operon, and the divergently oriented *iolRS* (Yoshida et al., 1997). The *iolG* gene encodes an inositol dehydrogenase (IdhA) which is the first enzyme in the pathway. It is responsible for the oxidation of *myo*-inositol to generate a ketone (2-keto-*myo*-inositol; 2KMI). Subsequently, a dehydration occurs by the *iolE* gene product which is a 2KMI dehydratase. The dehydrated product is subjected to a ring scission by a hydrolase, the *iolD* gene product. Subsequent action by the gene products, of *iolB*, *iolC*, *iolJ*, and *iolA* results in isomerization,

phosphorylation, and aldol formation, respectively, in order to generate small carbon molecules that can be utilized by the citric acid cycle. The entire catabolic pathway is shown in Fig. 3. Also shown in Fig. 3 is the proposed mechanism of pinitol catabolism.

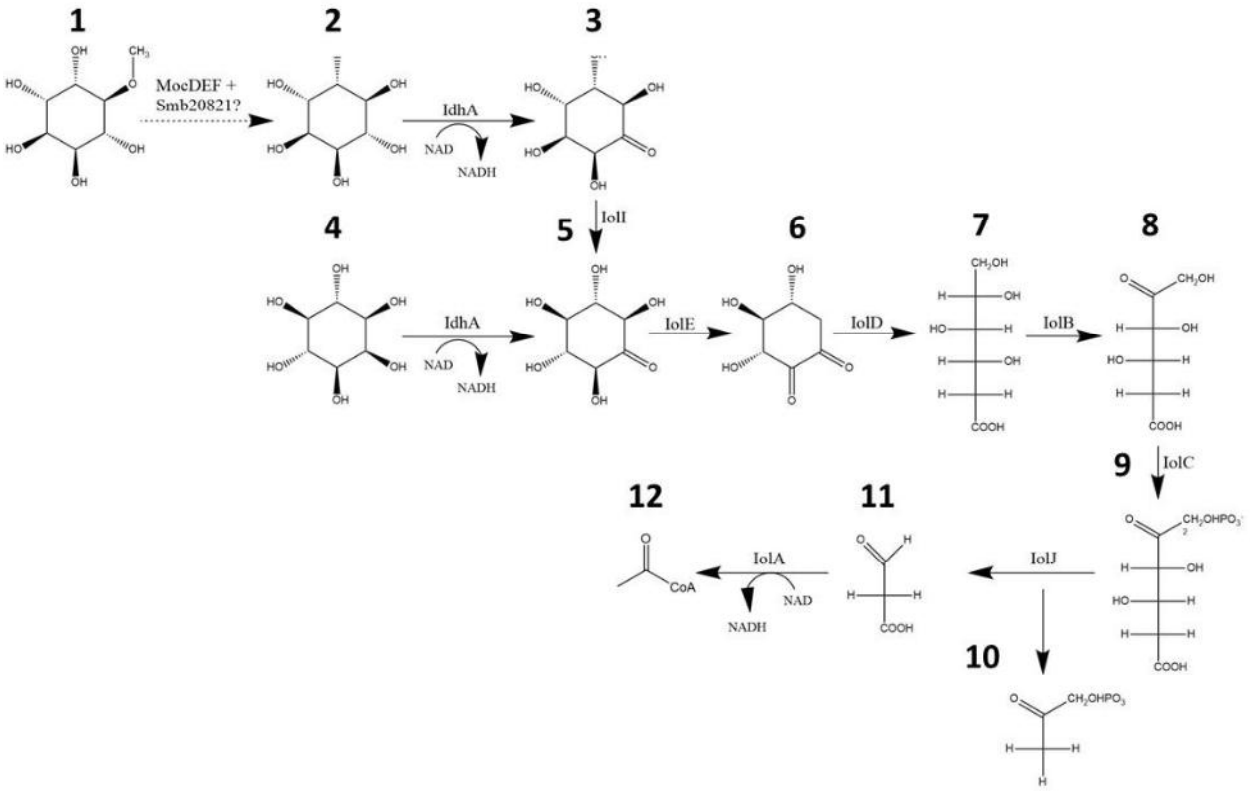

Figure 3. Schematic showing the steps of *myo*-inositol and D-*chiro*-inositol catabolism.

(1) pinitol, (2) D-*chiro*-inositol, (3) 2 keto-D-*chiro*-inositol, (4) *myo*-inositol, (5) 2-keto-*myo*-inositol, (6) 3-D-trihydroxycyclohexane-1,2-dione, (7) 5-deoxy-glucuronic acid, (8) 2-deoxy-5-keto-D-gluconic acid, (9) 2-deoxy-5-keto-D-gluconic acid 6-phosphate, (10) dihydroxyacetonephosphate, (11) malonic semialdehyde, (12) acetyl-CoA. The gene products responsible catalyzing each step in the pathway have been identified in *S. meliloti* and are shown alongside arrows: IdhA (*myo*-inositol dehydrogenase), IolE (2-keto-*myo*-inositol dehydratase), IolD (a THcHDO hydrolase), IolB (5-deoxy glucuronic acid isomerase), and IolA (malonic semialdehyde dehydrogenase). Gene products not found in S. meliloti are also shown alongside arrows: IolI (inosose isomerase) and IolJ (aldolase).

Various Gram-negative *Rhizobiaceae* members, such as *S. meliloti*, have been shown to be able to utilize inositol as a sole carbon source (Fry et al., 2001; Galbraith et al., 1998; Kohler

et al., 2010). However, *S. meliloti* organizes the *iol* genes differently than *B. subtilis* and contains fewer of them. The *idhA* gene is located on the large p*symB*-plasmid and the *iolYRCDEB* genes form one operon on the chromosome, while *iolA* is located much further away on the chromosome (Kohler et al., 2010) The *idhA* gene has been shown to encode the *myo*-inositol dehydrogenase (Galbraith et al., 1998). Knock-out mutagenesis studies using *S. meliloti* suggested the pathway of *myo*-inositol and D-*chiro*-inositol degradation (Kohler et al., 2010; Fig. 2). Kohler et al. (2010) used mutants created with the use of the *sacB*-containing suicide vector, which contained the gene of interest with an insertion of the kanamycin resistance gene. The resulting *iolCDEBA* mutants all lost their ability to utilize *myo*-inositol, *scyllo*-inositol, D-*chiro*-inositol, and 2KMI. The growth studies completed by Kohler et al. (2010) also showed that *iolR* mutants were able to survive on each inositol tested, which supported the claim that *iolR* is a regulatory protein of the inositol catabolism pathway. A mutant with an insertion within an uncharacterized gene at the time, *smc01163*, was able to grow on all tested inositols besides *scyllo*-inositol; thereby providing evidence that this gene encodes a dehydrogenase that acts on *scyllo*-inositol and is now called *iolY*. Lastly, growth studies with the *idhA* mutant provided further evidence for the claim that the *idhA* gene encodes a *myo*-inositol dehydrogenase by not being able to grow with *myo*-inositol as sole C-source but being able to grow with 2KMI which is the product of the IdhA reaction (Fig. 2). Interestingly, it was also shown that the *idhA* mutant could not grow with D-*chiro*-inositol as sole C-source, thus IdhA is not specific only to *myo*-inositol (Thwaites, 2013). Furthermore, with the use of competition assays, Kohler (2010) was able to show that inositol catabolism plays a role in *S. melilots* ability to compete for nodule occupancy; all the mutants were unable to compete for nodule occupancy with the wild-type strain when inoculated in a 1:1 ratio onto alfalfa plants.

Rhizopines

There are methylated inositol derivatives that occur naturally in the environment such as pinitol and rhizopines (Fig. 4). Strain *S. meliloti* L5-30 can synthesize a rhizopine inside nodules that functions much like opines do for *Agrobacterium tumefaciens*. The plant host cells produce compounds called opines because *A. tumefaciens* inserts its DNA into the host's DNA, thus making the plant synthesize opines for the bacteria. *Agrobacterium tumefaciens* can survive on opines as a sole carbon and nitrogen source. The rhizobial version of opines is referred to as rhizopines (Murphy et al., 1988). Two known rhizopines occur naturally: L-3-*O*-methyl-*scyllo*-inosamine (3-O-MSI) and *scyllo*-inosamine (SI; Fig. 4; Murphy et al., 1988).

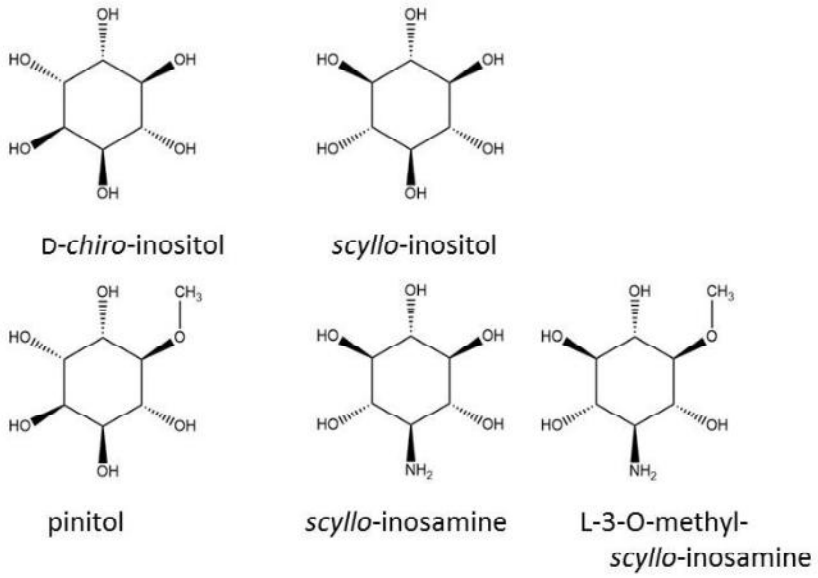

Figure 4. Schematic drawing showing inositols and their related derivatives.

Note how pinitol is a methylated D-*chiro*-inositol. In addition, *scyllo*-inosamine is a *scyllo*-inositol with an amino group, and L-3-O-methyl-*scyllo*-inosamine is a methylated *scyllo*-inosamine.

It has been shown that *S. meliloti* can utilize L-3-*O*-methyl-*scyllo*-inosamine as a sole carbon and nitrogen source (Murphy et al., 1987). It is understood that rhizopines are nutritional mediators; they enhance the formation of symbiotic relationships between legume plants and members of *Rhizobiaceae* family. Much like opines select for *Agrobacterium* in the formation of gall crown tumors, rhizopines select for *Rhizobiaceae* members in the rhizosphere (Gordon et al., 1996). Nevertheless, there is a significant difference between rhizopines and opines. *Agrobacterium* is able to insert it's opine synthesis genes into the host plant, thereby forcing the plant to produce a metabolite that promotes the bacteria's growth; basically, they establish their own ecological niche. In contrast, rhizopines are produced by the invading species of rhizobia during endosymbiosis within the plant nodules.

Rhizopine metabolism

The rhizopine synthesis (*mos*) genes have been identified and sequenced from *S. meliloti* strain L5-30 (Murphy et al., 1987). The results indicated that there are four open reading frames; ORF1 and *mosABC*. Rhizopine catabolism (*moc*) genes also have been characterized in *S. meliloti* L5-30 (Rossbach et al., 1994). The *mocABC* and *mocR* are the genes required for rhizopine utilization. Tn5-mutagenesis resulted in the delineation of the *moc* genes. Studies indicated that the *mocABC* genes are key for the transport of rhizopines into the cell and subsequent catabolism, while *mocR* is important for regulating the process (Rossbach et al., 1994). MocR showed homology to the GntR family of regulatory proteins, as well as to aminotransferases. A helix-turn-helix in the N-terminal domain which allows it to bind to DNA, as well as an effector binding site on the C-terminus are usually conserved in this type of protein (Haydon & Guest, 1991). Rossbach et al. (1994) was able to deduce that the *mocA* gene encodes a rhizopine dehydrogenase based on significant similarity with other genes known to encode dehydrogenases and possessing a motif that is key for NAD(H) binding. The *mocB* gene product is suggested to be a periplasmic rhizopine-binding protein necessary for the uptake of rhizopine (Rossbach et al., 1994). The function of *mocC* was left unknown by Rossbach et al. (1994) due to lack of similarity with other proteins in the database, however, it was later learned that it encodes a dehydratase similar to the IolE protein, which is necessary for inositol catabolism. The *mocD* and *mocE* genes were found to be required for the degradation of the 3-O-MSI rhizopine, but interestingly, not for SI (Bahar, de Majnik, Saint, & Murphy, 2000). The significant difference between 3-O-MSI and SI is a methyl-group, therefore these genes seemed to be essential for the demethylation of 3-O-MSI. In the proposed mechanism by Bahar et al., (2000) MocD, an oxygenase, converts the methyl group of rhizopine into a hydroxyl group. Similarly,

MocE, a ferredoxin, is proposed to accept electrons during this metabolic step, cooperating with the product of the adjacent MocF, which works as a ferredoxin reductase. It is suggested that the *mocDEF* (Fig.4) gene products work together to convert 3-OMSI to SI for further breakdown by other Moc proteins (Bahar et al., 2000). In addition, *mocDEF* are transcribed together (Thwaites, 2013; Fig. 5). Interestingly there is a fourth open reading frame, probably on the same transcript, *smb20821* (Thwaites, 2013; Fig. 5).

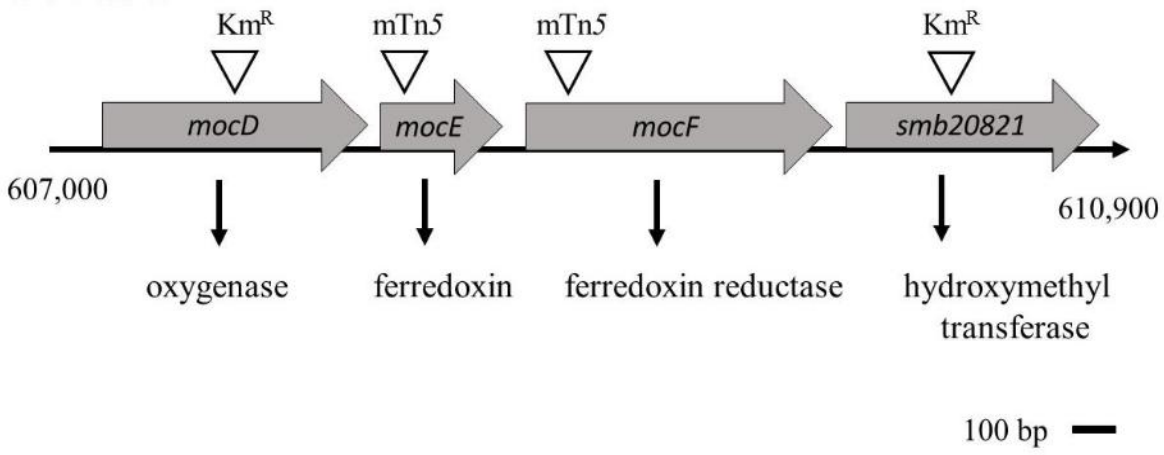

Figure 5. Physical map of the *S. meliloti mocDEF* and *smb20821*genes.

Specific aims of this thesis

Rhizopine degradation (*moc*) genes have been characterized in *S. meliloti* strain L5-30 (Rossbach et al., 1994). Nevertheless, the *mocDEF* are also present in *S. meliloti* strain 1021, which is not able to synthesize or degrade rhizopines. However, it is able to use pinitol as sole C source. Since pinitol and rhizopines are similar in structure, it was hypothesized that the *mocDEF* genes are essential for pinitol catabolism (Thwaites, 2013). Because pinitol is a methylated inositol compound, it was then further shown that pinitol catabolism also required the *myo*-inositol catabolism genes (Thwaites, 2013).

The work for my thesis was designed to study the relationship between pinitol catabolism and inositol catabolism, as well as to study how pinitol catabolism effects *S. meliloti* ability to compete for nodule occupancy. My first hypothesis was that D-*chiro*-inositol is an intermediate of pinitol catabolism. (Fig. 6).

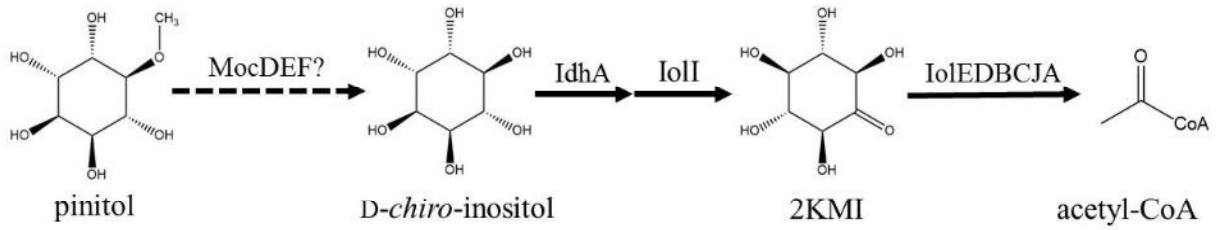

Figure 6. Schematic drawing of proposed pinitol catabolism.
2KMI is short for 2-keto-*myo*-inositol.

Our approach to test this hypothesis entails the use of gas chromatography in tandem with mass spectrometry to analyze *S. meliloti* cultures for the presence of pinitol and D-*chiro*-inositol. The *mocD*, *mocE*, and *idhA* mutants were compared to wild type and an uninoculated media control. We hypothesized that the *idhA* mutant produces D-*chiro*-inositol concentrations that are higher from the ones produced by the wild type, in addition to the accumulation of D-*chiro*-inositol. If our proposed model of pinitol catabolism is correct, the *idhA* mutant should degrade pinitol and accumulate D-*chiro*-inositol since it is unable to grow on it as a sole carbon source. We also hypothesized that the *mocD* and *mocE* mutants will exhibit pinitol and D-*chiro*-inositol concentrations that are the same as in the uninoculated media control, because it has been shown that these mutants cannot survive on pinitol as their sole carbon source.

In addition, we hypothesized that pinitol catabolism is a favorable trait for nodule occupancy. Using nodule competition essays, wild-type strain and each mutant (*mocD, mocE,*

and *idhA*) were inoculated on germinated plants in a 1:1 ratio. After certain time periods, the wild-type and mutant strains were extracted from the nodules and quantified. We hypothesized that the wild-type strain will outcompete each mutant.

MATERIALS AND METHODS

Bacterial strains

S. meliloti strains used in this study are shown in Table 1.

Table 1. *S. meliloti* strains used in this study

WT 2011	wild type, Sm^R derivative of SU47	(Meade & Signer, 1977)
WIDHA	2011mTn*5*STM.5.11.A04 *idhA::gus*, Sm^R, Km^R	(Pobigaylo et al., 2006)
WMOCD	2011*ΔmocD* Km^R, Sm^R	(Thwaites, 2013)
WMOCE	2011mTn*5*STM.1.11.D07 *mocE*::*gus*, Sm^R, Km^R	(Pobigaylo et al., 2006)
GLYA2	2011mTn*5*STM4.01.D11Tn5STM *glyA2*::*gus*, Sm^R, Km^R	(Pobigaylo et al., 2006)

Full medium

Full medium used for all *S. meliloti* strains was tryptone-yeast (TY) medium (Beringer et al., 1974), consisting of yeast extract (5 g/L), tryptone (5 g/L), and calcium chloride dihydrate (0.5 g/L). Solid medium required the addition of 15 g/L agar. When antibiotics were used, streptomycin (Sm) was added to a final concentration of 250 μg/mL and kanamycin (Km) was added to a final concentration of 200 μg/mL.

Minimal medium

Minimal medium (MM) for *S. meliloti* contained K_2HPO_4 (1.4 g/L), KH_2PO_4 (0.8 g/L), $CaCl_2$ x 2 H_2O (0.02 g/L), $MgSO_4$ x 7 H_2O (0.25 g/L), NaCl (0.2 g/L), $Fe(C_6H_5O_7)$ x H_2O (4 mg/L), Na_2MoO x 7 H_2O (0.2 mg/L), $MnSO_4$ x $4H_2O$ (0.2 mg/L), H_2BO_2 (0.25 mg/L), $CoCl_2$ x $4H_2O$ (0.001 mg/L), thiamine HCl (1 mg/L), $CuSO_4$ x $5H_2O$ (0.02 mg/L), Ca-panthothenate (2 mg/L), $ZnSO_4$ x $7H_2O$ (0.16 mg/L), biotin (0.001 mg/L), and EDTA (0.15 mg/mL) with 0.1% pinitol or 0.1% glycerol or combined as the sole carbon source and 0.1% KNO_3 as the sole nitrogen source (Rossbach et al. 1994).

Broughton and Dilworth (B&D) plant growth medium

Broughton and Dilworth (B&D; Broughton and Dilworth, 1971) plant growth medium for *Medicago sativa* contained KH_2PO_4 (0.136 g/l), K_2HPO_4 (0.1228 g/l), K_2SO_4 (0.087 g/l), $CaCl_2$ x H_2O (0.294 g/l), $Fe(C_6H_5O_7)$ x H_2O (0.676 mg/l), $MgSO_4$ x $7H_2O$ (0.123 g/l), $MnSO_4$ (0.338 mg/l), $ZnSO_4$ x $7H_2O$ (0.288 mg/l), $CuSO_4$ x $5H_2O$ (0.100 mg/l), $CoSO_4$ x $7H_2O$ (0.056 mg/l), Na_2Mo_4 x $2H_2O$ (0.048 mg/l), and H_3BO_3 (0.247 mg/l).

Culture conditions

Agar plates with *S. meliloti* strains were placed in an incubator set at 28 ºC for 72 hours for growth. Broth cultures were grown in 10 ml medium in a rotary shaker (C25 Incubator Shaker, New Brunswick Scientific, Edison, NJ) at 200 rpm set at 28 ºC for 72 hours for growth.

Growth studies

Precultures of *S. meliloti* wild-type and mutant strains were obtained by inoculating the strains in 10 ml of full media with appropriate antibiotics. Three-day old precultures were used to inoculate minimal medium in 1:100 ratio. Minimal media contained a 0.1% concentration of a defined carbon source: glycerol, D-*chiro*-inositol, pinitol, or glycerol together with pinitol. Growth was measured spectrophotometrically at 600 nm (OD_{600}) with a DU-640 spectrophotometer, (Beckman Coulter, Brea, CA). Growth studies with pinitol and pinitol plus glycerol were carried out in triplicate, whereas the growth studies with D-*chiro*-inositol and glycerol were carried out in duplicate.

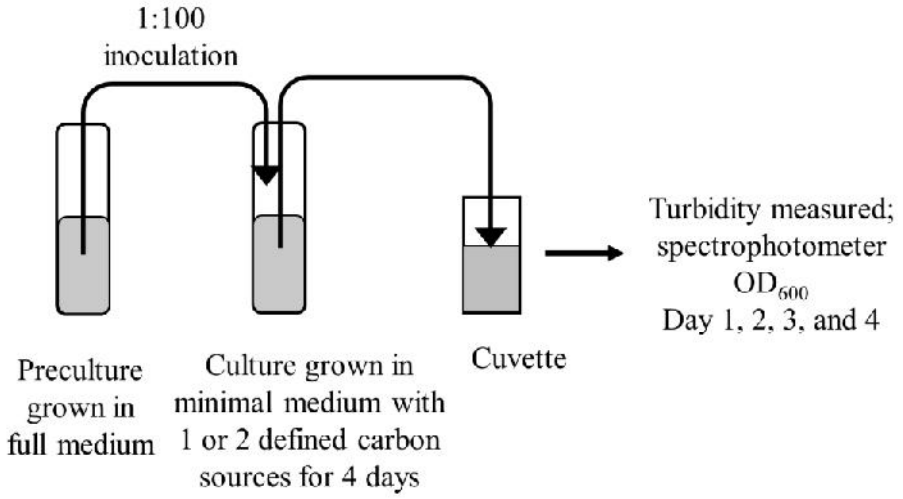

Figure 7. Illustration demonstrating *S. meliloti* growth studies.

BiOLOG ecoplate characterization of carbohydrate utilization

Precultures of *S. meliloti* were used to inoculate a microbial community analysis EcoPlate™ (BiOLOG, Hayward, CA). The cultures were centrifuged at 7,000 rpm (4,930 x g) for 15 minutes (Eppendorf Centrifuge 5415D, Brinkman Instruments, Westbury, NY, USA) to pellet the cells. Minimal medium with no added carbon source was added and the cells were

resuspended. The resuspended cells were pipetted into the right-most four wells of an EcoPlate™. A figure showing the carbon sources in those wells is provided in results section. The EcoPlate™ was left for 6 days and the OD_{590} was taken at day 2, day 3, and day 6 with an Epoch microplate reader (Biotek, Winooski, VT).

Harvesting of *S. meliloti* cells for GC analysis

Rhizobial cultures were sonicated in a water bath for 15 minutes (Mettler Electronics Corporation, Anaheim, CA). Sonicated samples were then centrifuged at 7,000 rpm (4,930 x g) for 15 minutes (Eppendorf Centrifuge 5415D, Brinkman Instruments, Westbury, NY) to pellet the cells. The supernatant was pipetted (200 µl) into gas chromatography vials (Thermoscientific, Rockwood, TN, 60180-723) and dried completely in a vacuum pressurized heating oven (VWR, Sheldon manufacturing, model 1430, Cornelius, OR) and subsequently capped (Themoscientific, Rockwood, Tennessee, 60180-729).

Trimethylsilylation derivatization

One milliliter containing 950 microliters hexane and 50 microliters of N-trimethylsilylimidazole (Sigma, 33068-U) was added to the dried samples. Biphenol was used as an external standard and was dissolved in hexane prior derivatization. Derivatized samples were left sitting for 30 min at room temperature before injection into a gas chromatograph (GC 2010, Shimadzu, Japan). Samples were obtained prior to inoculation (day 0) as a control, and then once a day on day 1 through day 4. Each sample from each day was completed in triplicate. Pinitol and D-*chiro*-inositol concentrations were calculated using a calibration curve generated from pinitol (Sigma, 441252) and D-*chiro*-inositol (Sigma, 468045) standards.

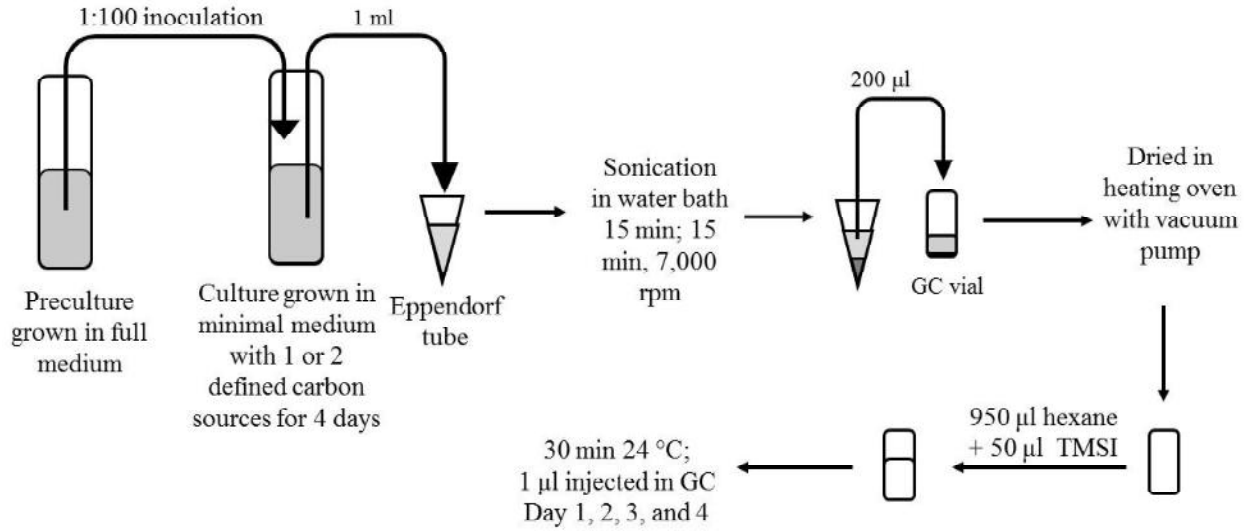

Figure 8. Illustration demonstrating *S. meliloti* cell harvesting and sample derivatization.

GC-MS

The gas chromatograph (GC 2010, Shimadzu, Japan) was equipped with a Shimadzu Shr5xlb capillary column (30 m by 0.25 µm) and a mass spectrometer (GCMS - Qp2010S, Shimadzu). Helium was used as a carrier gas with a velocity of 36.8 cm/sec. The column was subjected to 80 °C for 1 minute and adjusted to 300 °C at a rate of 20 °C/min with the final temperature being held for 5 minutes. The injector port was operated in a split-less mode (1:10) at 320 °C. Before and after each sample the needle was rinsed several times with hexane to prevent sample carry over and plunger errors.

Statistical analysis of GC data

Analysis of data was completed using the student *t*-test of the IBM® SPSS® Statistics version 24 (2016). Using the Bonferroni correction it was decided that P values less then 0.005 would be considered significant (Bonferroni 1935).

Plant competition assay

Seeds of *Medicago sativa* (alfalfa; vernal) were weighed and placed in a sterile petri dish. Seeds were imbibed using 70% ethanol for 30 minutes while shaking gently on an orbital shaker (BT30-GM low speed orbital shaker, VWR, Taiwan), followed by three washes with water. Surface-sterilization was carried out by the addition of full strength bleach (8.25% sodium hypochlorite) for 30 minutes, while shaking gently followed by three washes with sterile water. Whatman 3MM filter paper was cut into 7 cm x 5 cm pieces. These pieces were folded twice and placed into glass tubes (2 mm diameter and 20 mm long). To each tube 20 ml of B&D medium was added, followed by sterilization in the autoclave. Surface-sterilized seeds were placed on the top of the filter paper and allowed to germinate for one week at room temperature with 16 hours of fluorescent light and 8 hours of darkness. Cultures of *S. meliloti* wild type and mutant were centrifuged at 7,000 rpm (4,930 x g) for 15 minutes (Eppendorf Centrifuge 5415D, Brinkman Instruments, Westbury, NY). The pelleted cells were resuspended into sterile dH_2O. The turbidity was measured (OD_{600}) with a DU-640 Spectrophotometer (Beckman Coulter, Brea, CA). Germinated plants were inoculated with a 1:1 ratio of wild type *S. meliloti* and each mutant. The *S. meliloti idhA, mocD* and *mocE* mutants were tested. As a control, a *S. meliloti glyA2* mutant was used. Sixteen plants were inoculated for each experiment. In addition, four plants were inoculated with each strain by itself as a control. After eight and 16 weeks, nodules were

removed from the plants with forceps. These nodules were surface-sterilized in 95% ethanol for one minute, followed by three washes with sterile H_2O. Surface-sterilized nodules were crushed in 100 µl sterile H_2O followed by addition of 900 µl of sterile H_2O. Crushed nodules were serial diluted in sterile H_2O and 100 µl of each dilution was plated on either TY Sm (for selection of wild type and mutant) or TY Km (for selection of mutant only). Plates were incubated at 28°C for three days. Colonies were counted, the CFU/mL were calculated and the ratios of wild-type to mutant strains was calculated. This was completed in duplicate.

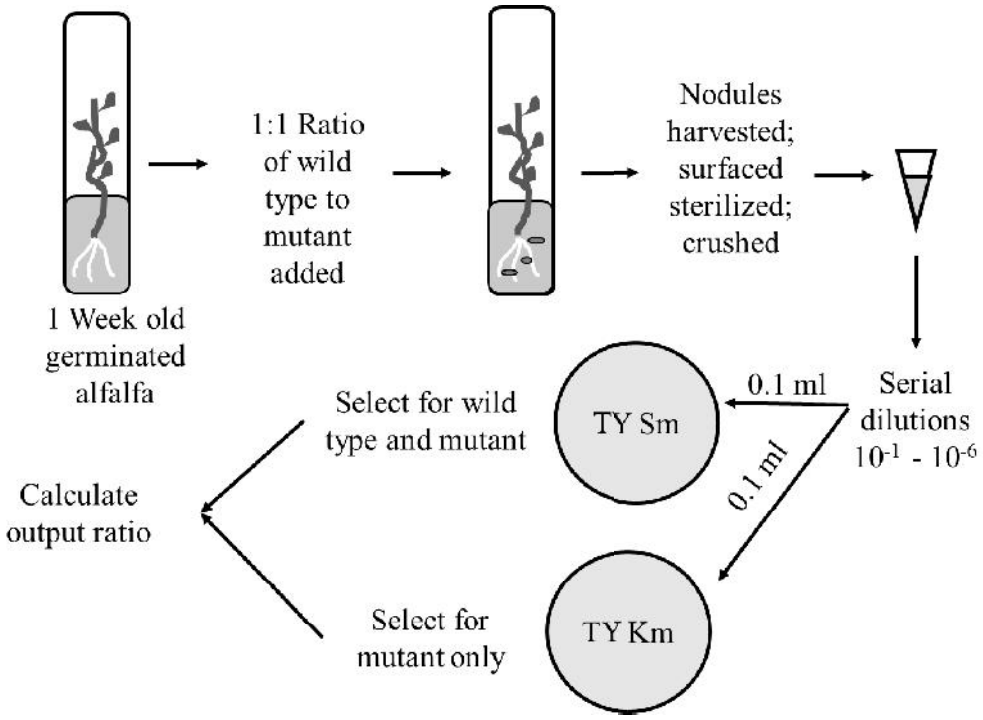

Figure 9. Illustration demonstrating plant competition assay.

RESULTS

Method development

To test the hypothesis that pinitol is degraded to D-*chiro*-inositol by the *mocDEF* gene products, time was invested into the development of an analytical chemical method to detect pinitol and D-*chiro*-inositol. At the beginning the goal was to utilize high pressure liquid chromatography (HPLC) because the sugar alcohols are water soluble. After some testing, it was determined that the detectors available on accessible HPLC's at Western Michigan University were not able to detect pinitol or D-*chiro*-inositol; the UV wavelength needed was out of range of the detectors. The only other option was gas chromatography (GC). First it needed to be addressed how to make the sugars soluble in an inorganic solvent. After some research it was decided that Trimethylsilylation (TMSI) derivatization would be the best option for sugar alcohols (Harvey, 2011; Ruiz-Matute et al., 2011). A solution containing 100 ppm pinitol and 100 ppm D-*chiro*-inositol was derivatized and injected; and after several attempts with different GC MS parameters, we were able to detect and separate both sugars.

After we had an analytical method developed that could detect both sugars and separate them, we wanted to test the stability of pinitol. A 100 ppm solution of pinitol was prepared and left shaking at 28°C for 4 days. A sample was taken each day and injected into the GC. Pinitol peak size was consistent for all four days and no D-*chiro*-inositol could be detected (data not shown). We also tested the derivatization time to know if complete derivatization was occurring. A 100 ppm solution of pinitol was prepared and dried as described in the method section. TMSI was added and incubated for 10, 15, 30, 45, and 60 minutes. Pinitol peak size reached a maximum at 30 minutes and the peak size was stable for 45 and 60 minutes (data not shown). Thus, the derivatization time chosen for the experiment was 30 minutes. Next, we attempted to

identify a carbohydrate that *S. meliloti* could not degrade for use as an internal standard. With the use of an BiOLOG EcoPlate™, it was determined that *S. meliloti* could not use pyruvic acid methyl ester, D-galacturonic acid, 2-hydroxyl-benzoic acid (salicylic acid), L-phenylalanine, 4-hydroxy benzoic acid, D-glucosaminic acid, itaconic acid, glucose-1-phosphate, phenylethyl-amine, D-L-α-glycerol-phosphate, and putrescine (Table 2).

TMSI derivatization involves the replacement of an active hydrogen by a trimethylsilyl (TMS), therefore it was decided to evaluate the use of 2-hydroxyl benzoic acid (salicylic acid), 4-hydroxy benzoic acid or itaconic acid as internal standards. Unfortunately, upon completing growth studies with each carbohydrate, it was determined that *S. meliloti* could indeed use all of them as sole C-sources. Since *S. meliloti* can degrade so many different carbohydrates it was decided it would be difficult to identify an internal standard. Nevertheless, an external standard could be used that would be added to the samples after growth and it was decided to use biphenyl. An example chromatogram is provided that shows the separation of the external standard biphenyl, pinitol, and D-*chiro*-inositol (Fig. 10).

Table 2. BiOLOG Ecoplate results

Growth on each carbohydrate or amino acid indicated by optical density (OD_{590}); values less than 1.5 are bolded (A). Supplied nutrient in each well provide (B); bolded compounds were considered unable to support growth of *S. meliloti*.

A

	Day 6 BiOLOG EcoPlate results			
	1	**2**	**3**	**4**
A	**1.31**	3.30	1.79	3.34
B	2.93	3.44	1.35	3.34
C	2.77	3.27	**1.30**	**1.33**
D	2.36	3.05	**1.33**	3.23
E	**1.36**	3.12	3.25	2.01
F	**1.35**	**1.48**	**1.27**	3.27
G	2.99	**1.24**	3.07	**1.28**
H	2.77	**1.23**	2.94	**1.25**

B

	1	**2**	**3**	**4**
A	**Water**	β-methyl-D-glucoside	D-galactonic acid γ-lactone	L-arginine
B	pyruvic acid metyl ester	D-xylose	D-galacturonic acid	L-asparagine
C	tween 40	i-erythritol	**2-hydroxy benzoic acid**	**L-phenylalanine**
D	tween 80	D-mannitol	**4-hydroxy benzoic acid**	L-serine
E	**α-cyclodextrin**	N-acetyl-D-glycosamine	**γ-hydroxylbutyric acid**	L-threonine
F	**Glycogen**	**D-glycosaminic acid**	**itaconic acid**	glycyl-L-glutamic acid
G	D-cellobiose	**Glucose-1-phosphate**	α-ketobutric acid	**phenylethyl-amine**
H	α-D-lactose	**D,L-α-glycerol phosphate**	D-malic acid	**putrescine**

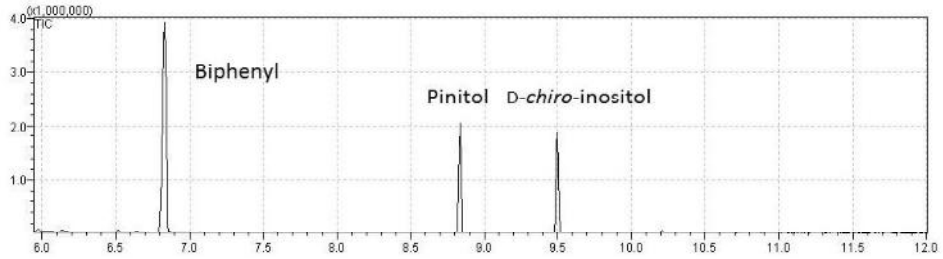

Figure 10. Example chromatogram.

Shown are 200 ppm biphenyl (retention time 6.8), 100 ppm pinitol (retention time 8.8), and D-*chiro*-inositol (retention time 9.5).

With the use of the external standard biphenyl, calibration curves were created for both pinitol and D-*chiro*-inositol so that the data could be presented in ppm instead of peak area size. After several months of refining the method, it was found that for best results, the column should be baked before and after use, the first and last two injections should be a blank, and a blank should be injected between every two samples.

The connection between pinitol catabolism and D-*chiro*-inositol catabolism

Since the *mocDEF* operon and the inositol catabolism genes were found to be essential for the catabolism of pinitol (Thwaites, 2013), the goal was to determine the link between pinitol catabolism and inositol catabolism. Mutants with insertions in the *idhA*, *mocD*, and *mocE* genes were available from the collection of Pobigaylo et al. (2006) and Thwaites (2013). To verify the phenotype of the wild type and each mutant strain, growth studies on glycerol, D-*chiro*-inositol, pinitol, and pinitol and glycerol were conducted (Fig. 7). When grown with 0.1% glycerol as sole carbon source, the growth of each mutant was comparable with the wild type (Fig. 11A). As a control, supernatant from cultures grown with 0.1% glycerol as the sole carbon source were injected into GC and it was confirmed that each strain of *S. meliloti* used in this study did not

produce pinitol or D-*chiro*-inositol from glycerol (data not shown). When grown with 0.1% D-*chiro*-inositol, the *mocD* and *mocE* mutants exhibited growth that was comparable with wild-type, but the *idhA* mutant was unable to grow (Fig. 11B). Each mutant showed an inability to grow when provided with 0.1% pinitol as the sole carbon source, whereas the wild type yielded an OD_{600} of 0.75 by day 4 (Fig. 11C). When grown with 0.1% pinitol plus 0.1% glycerol as carbon sources, the wild-type strain achieved an OD_{600} of 1.9 by day 4, whereas the *idhA* mutant reached an OD_{600} of 1.6, and the *mocD* and *mocE* mutant reached an OD_{600} of only 1.4 (Fig. 11D).

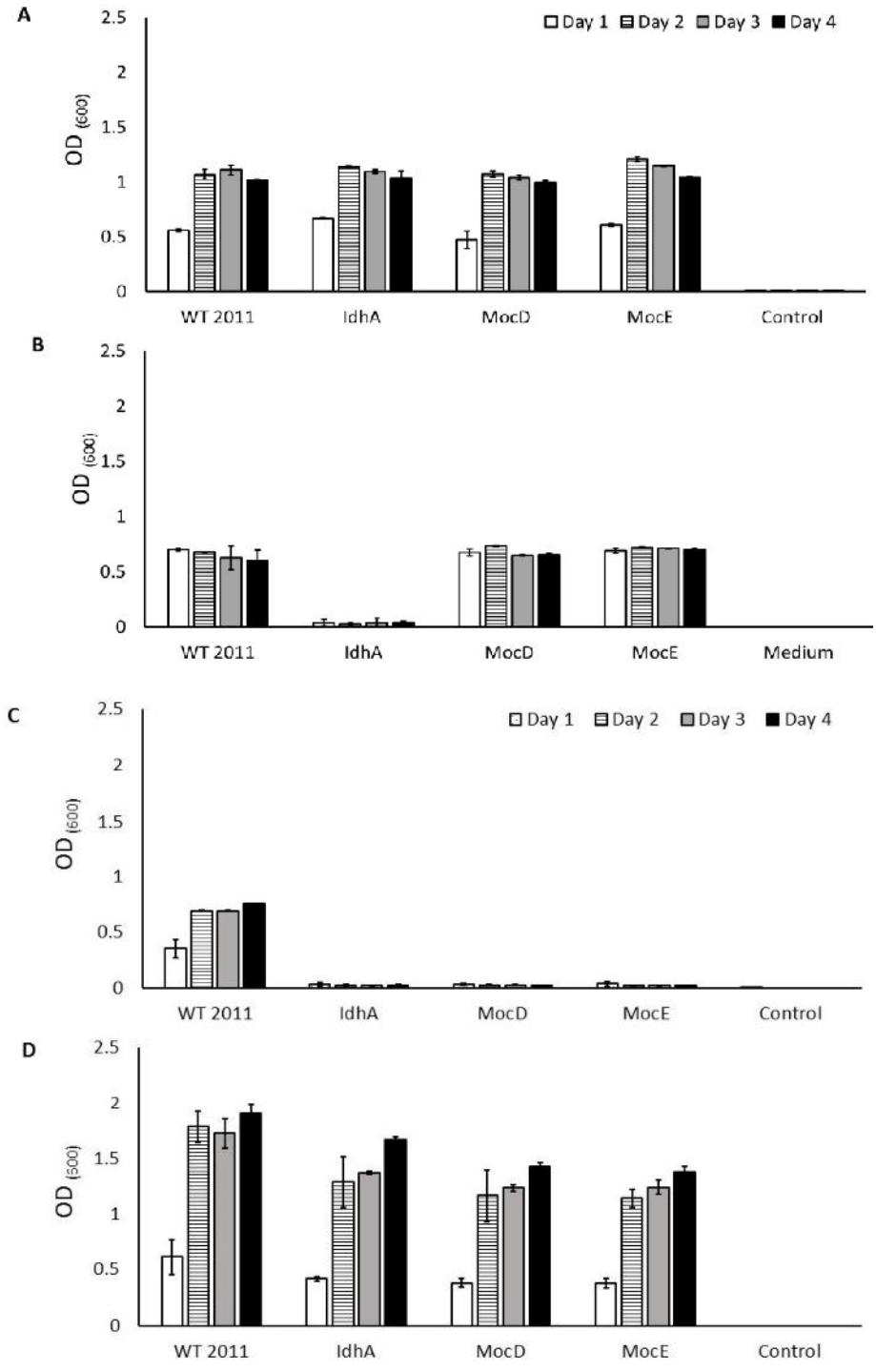

Figure 11. Growth study with wild type *S. meliloti* and *idhA*, *mocD*, and *mocE* mutants.

Strains were grown in minimal media with glycerol (A), D-*chiro*-inositol (B), pinitol (C) or pinitol plus glycerol (D). Experiments with glycerol as the sole carbon source was performed with n=2, D-*chiro*-inositol n=2, pinitol n=3, and pinitol plus glycerol n=3. Growth were measured spectrophotometrically at 600 nanometers for 4 sequential days. Error bars denote the standard deviation.

To test cultures for metabolic intermediates, the method outlined in Fig. 8 was followed. Supernatants from cultures grown with 0.1% pinitol as a sole C-source were injected into the GC. Pinitol concentrations and D-*chiro*-inositol concentrations are shown in Fig. 12A and Fig. 13A, respectively. Supernatant from cultures grown on 0.1% pinitol plus 0.1% glycerol as sole carbon sources were injected into the GC and resulting pinitol concentrations and D-*chiro*-inositol concentrations data are shown in Fig. 12B and Fig. 13B, respectively.

Since the wild-type strain can utilize pinitol as a sole carbon source it would be expected that the wild type would remove all detectable pinitol. Wild type *S. meliloti* was able to remove all detectable pinitol from its medium by day 2, regardless of being grown with pinitol as the sole carbon source or being grown with pinitol plus glycerol (Fig. 12A; Fig. 12B).

The *idhA* mutant cannot utilize pinitol as a sole carbon source, because, it has the gene mutated that encodes the enzyme that is responsible for oxidizing D-*chiro*-inositol. If D-*chiro*-inositol is indeed an intermediate of pinitol catabolism, it would be expected that the *idhA* mutant would remove at least some of the detectable pinitol. Because of feedback mechanisms (buildup of end product often results in repression of continued degradation of substrate), it was not expected that all the pinitol would be removed from the medium by the *idhA* mutant. When grown on pinitol as a sole carbon source the *idhA* removed approximately 60% of the detectable pinitol from the medium by day 1 and maintained these pinitol concentrations through day 4 (Fig. 12A). The *idhA* mutant grown with pinitol and glycerol was able to remove 53% of the detectable pinitol from the medium by day 2 and maintained the same pinitol concentration through to the day 4 (Fig. 12B).

The *mocD* and *mocE* mutants cannot utilize pinitol as a sole carbon source and have mutations in the enzymes expected to be responsible for the conversion of pinitol into D-*chiro*-inositol. If MocD and MocE are indeed the enzymes that convert pinitol into D-*chiro*-inositol, it would be expected to observe no change in pinitol concentration. When grown on pinitol as a sole carbon source the *mocD and mocE* mutants both removed approximately 60% of the detectable pinitol from the medium by day 1 and maintained these pinitol concentrations through day 4 (Fig. 12A). The *mocD* mutant grown with pinitol plus glycerol was able to remove 53% of the detectable pinitol from its medium by day 2, 68% by day 3, and 87% by day 4 (Fig. 12B). The *mocE* mutant grown with pinitol plus glycerol was able to remove 53% of the detectable pinitol from the medium by day 2, 72% by day 3, and 83% by day 4 (Fig. 12B).

Pinitol concentrations found in uninoculated medium control were expected; pinitol concentrations were consistent for all four days regardless of having 0.1% pinitol or 0.1% pinitol plus 0.1% glycerol (Fig. 12A; Fig. 12B).

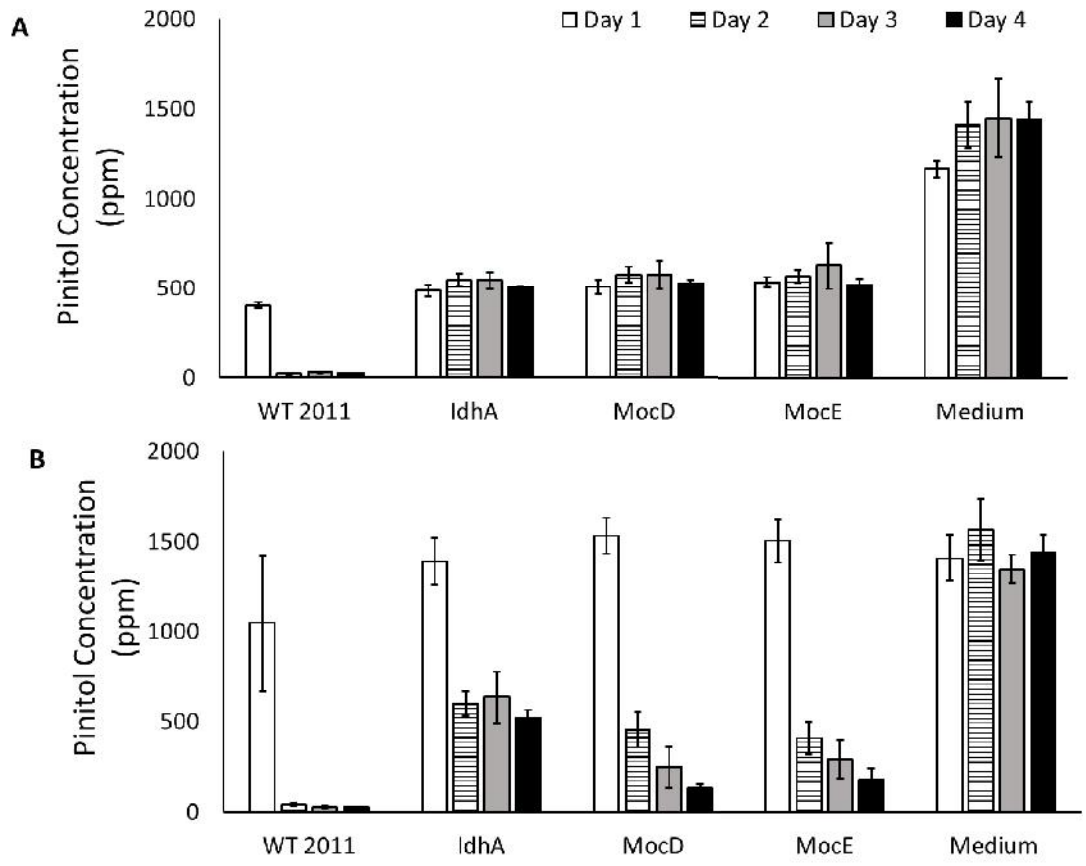

Figure 12. Pinitol concentrations.

Analysis of supernatants for pinitol concentrations from *S. meliloti* cultures grown in minimal media with pinitol as a sole carbon source (A) and with pinitol plus glycerol (B) as carbon sources (n =3). Error bars denote the standard deviation.

All *P* values for pinitol concentrations are shown in Table 3. The student's *t*-test showed that there were significant differences between the pinitol concentrations detected in the wild type and all mutant cultures and the uninoculated control medium for day 2, day 3, and day 4 when grown with pinitol alone. When provided with glycerol plus pinitol, the *t*-test showed that there was no difference between the pinitol concentration detected in all strains at day 1. The wild-type strain was shown to be significantly different from all strains and the medium for day 2. In addition, each strain was statistically different from the medium. On day 3 each strain was

different from the medium control. Each strain was different from all other strains except wild type and *mocE* mutant and *mocD* and *mocE* mutants on day 4.

Table 3. *P* values for pinitol concentrations for cultures

		Grown on pinitol				Grown on pinitol and glycerol			
Day		Idha	MocD	MocE	Control	Idha	MocD	MocE	Control
1	WT	0.029	0.027	**0.005**	**0.000**	0.295	0.155	0.178	0.271
	IdhA		0.660	0.207	**0.001**		0.279	0.396	0.880
	MocD			0.444	**0.001**			0.827	0.350
	MocE				**0.001**				0.481
2	WT	**0.000**	**0.000**	**0.000**	**0.000**	**0.003**	0.012	0.008	**0.003**
	IdhA		0.488	0.593	**0.001**		0.622	0.836	**0.002**
	MocD			0.804	**0.001**			0.783	**0.002**
	MocE				**0.001**				**0.002**
3	WT	**0.000**	**0.000**	**0.003**	**0.000**	0.042	0.060	0.075	**0.001**
	IdhA		0.659	0.443	**0.005**		0.332	0.214	**0.003**
	MocD			0.670	0.006			0.708	**0.001**
	MocE				0.010				**0.001**
4	WT	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**	0.011	0.069	**0.001**
	IdhA		0.140	0.587	**0.000**		**0.000**	**0.003**	**0.000**
	MocD			0.769	**0.000**			0.414	**0.000**
	MocE				**0.000**				**0.000**

**P* values smaller than 0.005 are shown in bold.

Since the wild type can utilize pinitol as a sole carbon source, it would be expected that if wild type would produce detectable D-*chiro*-inositol it would only be seen in the early growth phases. In the culture inoculated with the wild-type strain, an appearance of D-*chiro*-inositol was seen on day 1 followed by its disappearance by day 2 and the low concentration is maintained through day 4 regardless of being grown with pinitol as the sole carbon source or being grown with pinitol plus glycerol (Fig. 13A; Fig. 13B).

The *idhA* mutant cannot utilize pinitol as a sole carbon source, however, it contains a mutation in the enzyme responsible for oxidizing D-*chiro*-inositol. If D-*chiro*-inositol is indeed an intermediate of pinitol catabolism, it would be expected that the *idhA* mutant would accumulate detectable D-*chiro*-inositol. When grown on pinitol as a sole carbon source, very little D-*chiro*-inositol is observed (Fig. 13A). When grown with pinitol plus glycerol, the *idhA* mutant showed an appearance of D-*chiro*-inositol on day 1 with the mean value of 450 ppm, followed by a decrease to 200 ppm and this concentration was maintained through day 4 (Fig. 13B).

The *mocD* and *mocE* mutants cannot utilize pinitol as a sole carbon source and contain mutations in the genes encoding enzymes presumably responsible for the conversion of pinitol into D-*chiro*-inositol. If MocD and MocE are indeed the enzymes that convert pinitol into D-*chiro*-inositol, it would be expected to see no production of detectable D-*chiro*-inositol. When grown with pinitol as a sole carbon source, very little D-*chiro*-inositol is observed (13A). The *mocD* and *mocE* mutants showed an appearance of D-*chiro*-inositol on day 1 with the mean value of 250 ppm, followed by a decrease to 50 ppm and the low concentration was maintained through day 4 (Fig. 13B)

D-*chiro*-inositol concentrations found in uninoculated medium control were not consistent with expectations; there should have been no D-*chiro*-inositol detection in the control. Furthermore, medium containing 0.1% glycerol plus 0.1% pinitol produced higher concentrations of D-*chiro*-inositol detected than medium containing 0.1% glycerol alone (Fig. 13).

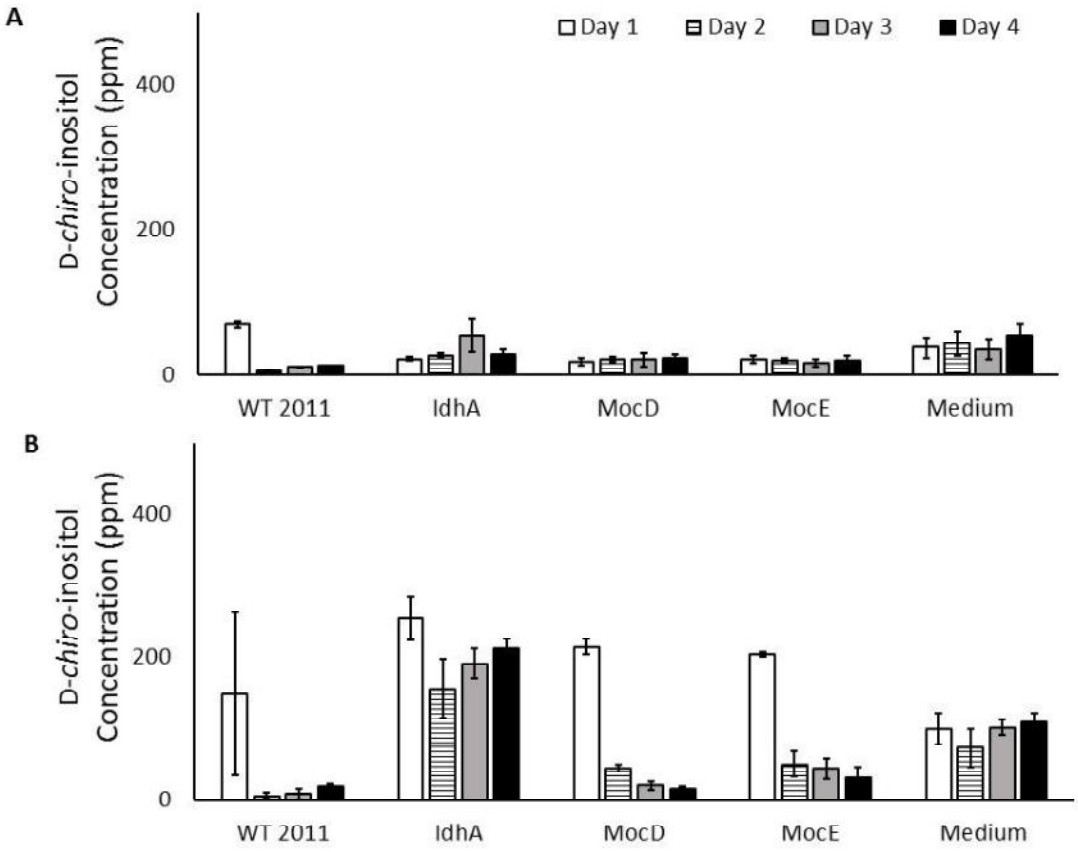

Figure 13. D-*chiro*-inositol concentrations.

Analysis of supernatants for D-*chiro*-inositol concentrations from *S. meliloti* cultures grown in minimal media with pinitol as a sole carbon source (A) and with pinitol plus glycerol (B) as carbon sources (n =3). Error bars denote the standard deviation.

All *P* values for D-*chiro*-inositol concentrations are shown in Table 4. For cultures grown with pinitol only, in regard to D-*chiro*-inositol concentrations, the student's *t*-test showed that there were significant differences between wild type and all strains on day 1, day 2, and day 4. More differences were seen in cultures grown with pinitol and pinitol plus glycerol. On day 1, the *idhA*, *mocD*, and *mocE* mutants showed significant differences in D-*chiro*-inositol concentrations in comparison to the concentrations in the control medium. On day 2, the wild type differed from the *mocD* mutant and the *idhA* mutant differed from the *mocE* mutant significantly. On day 3, the wild type differed from the *idhA* mutant and the control medium,

while the *idhA* mutant differed from the *mocD* and *mocE* mutants. Lastly for day 3, the *mocD* mutant different from the control medium. On day 4 the wild type differed from the *idhA* mutant and the control medium, while the *idhA* mutant differed from all other strains and the control medium. Also the *mocD* and *mocE* mutant differed from the control medium significantly.

Table 4. *P* values for D-*chiro*-inositol concentrations for cultures

		Grown on pinitol				Grown on pinitol and glycerol			
Day		Idha	MocD	MocE	Control	Idha	MocD	MocE	Control
1	WT	**0.000**	**0.000**	**0.000**	0.045	0.321	0.493	0.329	0.620
	IdhA		0.382	0.998	0.221		0.162	0.083	**0.004**
	MocD			0.473	0.156			0.241	**0.003**
	MocE				0.234				**0.003**
2	WT	**0.001**	**0.003**	**0.004**	0.032	0.030	**0.005**	0.031	0.083
	IdhA		0.140	0.081	0.257		0.019	**0.029**	0.077
	MocD			0.696	0.145			0.674	0.211
	MocE				0.125				0.379
3	WT	0.125	0.353	0.272	0.164	**0.003**	0.305	0.035	**0.003**
	IdhA		0.125	0.081	0.373		**0.000**	**0.001**	0.006
	MocD			0.625	0.307			0.103	**0.001**
	MocE				0.161				0.010
4	WT	**0.000**	**0.000**	**0.000**	**0.000**	**0.001**	0.308	0.479	**0.004**
	IdhA		0.140	0.231	**0.000**		**0.000**	**0.000**	**0.001**
	MocD			0.769	**0.000**			0.257	**0.000**
	MocE				**0.000**				**0.004**

		Grown on pinitol				Grown on pinitol and glycerol			
Day		Idha	MocD	MocE	Control	Idha	MocD	MocE	Control
1	WT	0.000	0.000	0.000	0.045	0.321	0.493	0.329	0.620
	IdhA		0.382	0.998	0.221		0.162	0.083	0.004
	MocD			0.473	0.156			0.241	0.003
	MocE				0.234				0.003
2	WT	0.001	0.003	0.004	0.032	0.030	0.005	0.031	0.083
	IdhA		0.140	0.081	0.257		0.019	0.029	0.077
	MocD			0.696	0.145			0.674	0.211
	MocE				0.125				0.379
3	WT	0.125	0.353	0.272	0.164	0.003	0.305	0.035	0.003

	IdhA		0.125	0.081	0.373		0.000	0.001	0.006
	MocD			0.625	0.307			0.103	0.001
	MocE				0.161				0.010
4	WT	0.000	0.000	0.000	0.000	0.001	0.308	0.479	0.004
	IdhA		0.140	0.231	0.000		0.000	0.000	0.001
	MocD			0.769	0.000			0.257	0.000
	MocE				0.000				0.004

**P* values less than 0.005 are shown in bold

Examples of chromatograms obtained in this study are provided in Figure 14. The control contained no D-*chiro*-inositol (Fig. 14A), the wild-type strain showed less pinitol in the medium than the control and no D-*chiro*-inositol (Fig. 14B), while the *idhA* mutant showed less pinitol in the medium than the control and D-*chiro*-inositol (Fig. 14C).

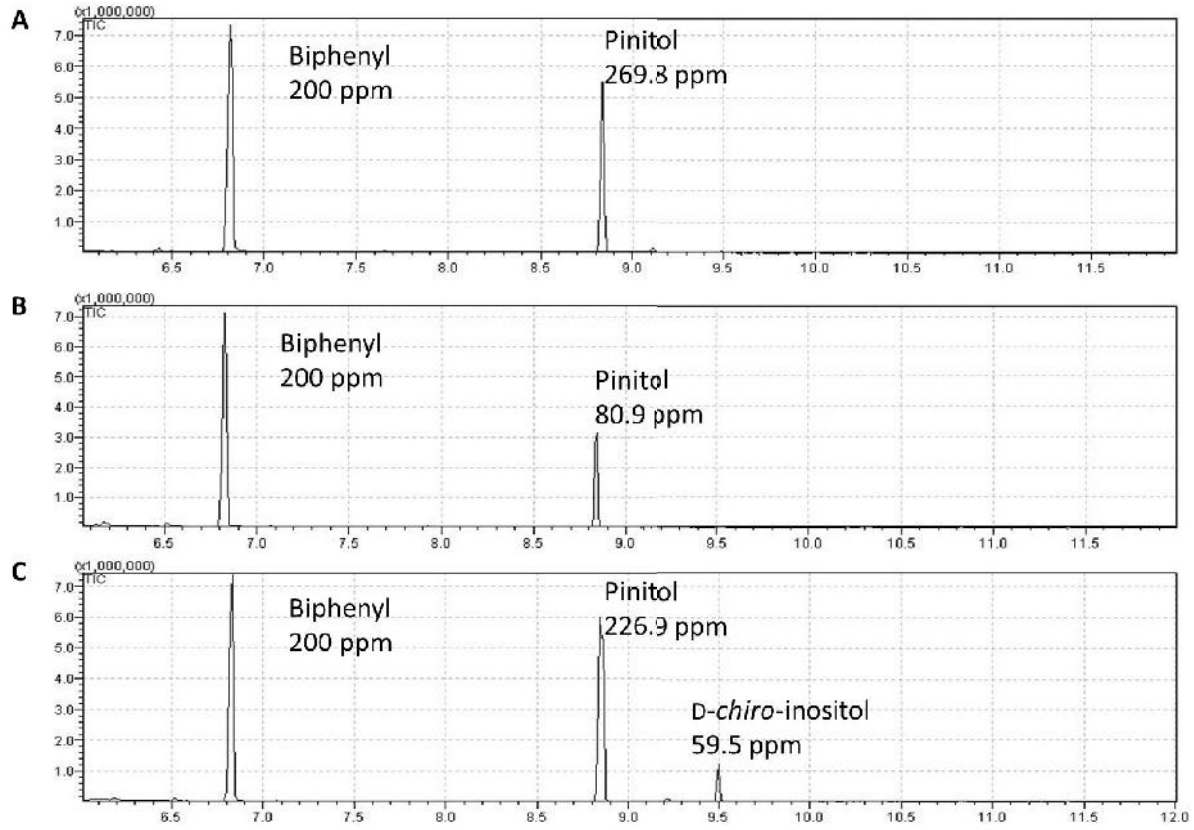

Figure 14. Example GC chromatograms obtained in this study.

Shown are the results from the control (uninoculated medium) on day 2 (A), wild-type strain on day 2 (B), and the *idhA* mutant on day 2 (C).

Role of pinitol catabolism in *S. meliloti's* ability to compete for nodule occupancy

The ability of the *idhA*, *mocD*, and *mocE* mutants to compete with the wild type for nodule occupancy was tested according to the schematic shown in Fig. 9. This was done to evaluate whether the ability to catabolize pinitol plays a role in the efficiency of plant-bacteria interactions. Plants were inoculated with a 1:1 ratio of wild type to mutant strains to give equal opportunity for infection of alfalfa. Nodules were harvested, surface sterilized, and crushed after 8 and 16 weeks. Percentages of each strain isolated from the nodules after 8 weeks are shown in Fig. 15A and after 16 weeks are shown in Fig. 15B. If pinitol catabolism plays no role in nodulation formation, a 50:50 output ratio would be expected at each collection time point. The *glyA2* mutant was used as a control since the gene mutated in it is not involved in pinitol catabolism. After 8 weeks the *idhA* mutant was found to be in a 50% ratio with the wild type (Fig. 15A). The *mocD* and *mocE* mutants were outcompeted to almost 100% by week 8 (Fig. 15A). The *glyA2* mutant was found in a 20% ratio with wild type (Fig. 15A). After 16 weeks, it was found that the *idhA* and *mocD* mutants could not compete with the wild type and the *mocE* and *glyA2* mutants were found in a 20% ratio with the wild type (Fig. 15B). As a control, plants inoculated with only one strain were also studied. At week 16 nodules from these plants were harvested and treated like the nodules created from tubes with wild type and mutant strains. Each strain was shown to be able to form nodules and to contain correct antibiotic resistance (Data not shown)

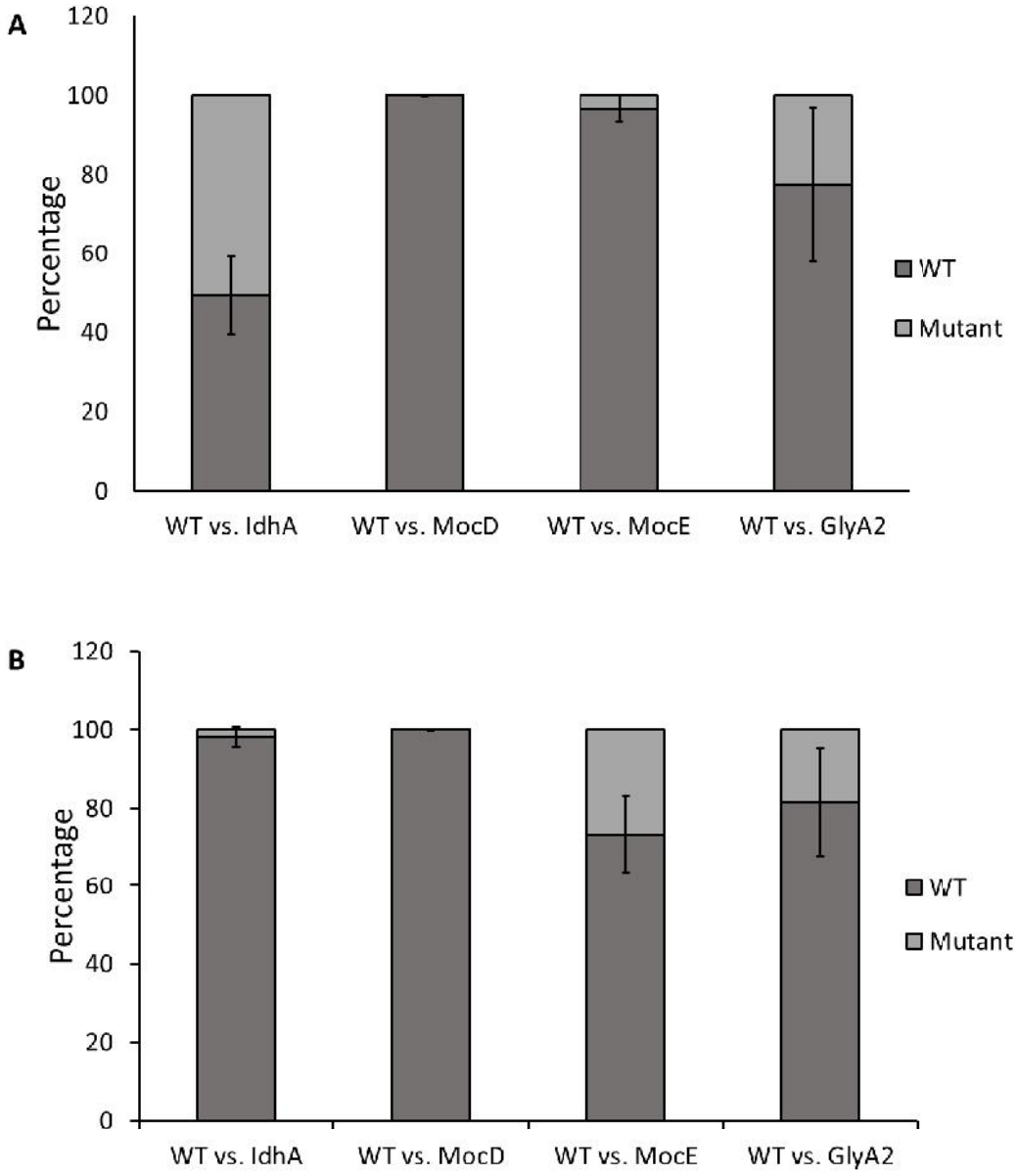

Figure 15. Results of the competition assay.

Percentages of bacteria occupying *Medicago sativa* nodules resulting from a 50:50 inoculation between the *S. meliloti* wild type and the *idhA*, *mocD*, *mocE*, and *glyA* mutants. Nodules were collected after 8 weeks (A) and after 16 weeks (B). The bacteria were re-isolated from surface-sterilized nodules on full

medium with streptomycin to select for both wild type and mutants in addition to kanamycin to select for mutants only. Error bars denote the standard deviation (n=3).

DISCUSSION

This thesis describes experiments designed to test two hypotheses, both of which are related to the metabolism of pinitol by *S. meliloti*. The first hypothesis was that D-*chiro*-inositol is an intermediate of pinitol catabolism. The second hypothesis was that the wild-type strain will outcompete each mutant for nodule occupancy. In fact, the evidence collected in the work described in this thesis supports both hypotheses.

First, we will discuss the catabolism of pinitol via D-*chiro*-inositol. The *mocDE* genes of *S. meliloti* L5-30 were shown to be essential for the demethylation of the rhizopine 3-*O*-MSI (Bahar et al., 2000). The DNA sequence data suggested that MocD is a hydrocarbon oxygenase and MocE a ferredoxin. Co-transcribed, but not essential for the demethylation of the rhizopine 3-O-MSI, is *mocF*; the MocF protein is suggested to be a ferredoxin reductase. The *mocDEF* genes were found to be present in *S. meliloti* strain 1021, which is a strain unable to degrade rhizopines. It was thought the genes might be essential for the demethylation of general inositol derivatives such as pinitol, not only for the demethylation of rhizopines. *Sinorhizobium meliloti* 2011 *mocD*, *mocE,* and *mocF* mutant strains were obtained and both *mocD* and *mocE* mutants were unable to grow with pinitol as a sole carbon source, while the *mocF* mutant was able to do so (Thwaites, 2013). Thus, *mocD* and *mocE* are essential for pinitol catabolism. Inositol catabolism genes were also shown to be essential for pinitol catabolism (Thwaites, 2013). This led us to our first hypothesis: pinitol is degraded by MocDE into D-*chiro*-inositol. In an *idhA*

mutant, which is unable to grow on D-*chiro*-inositol, D-*chiro*-inositol would accumulate when the mutant is grown on pinitol as a sole carbon source.

It was thought that mutant cultures provided with pinitol as a sole C source would not produce much change in pinitol or D-*chiro*-inositol concentrations, because the mutants cannot utilize pinitol as a sole C source. No cell growth occurs in the mutant strains when provided with pinitol as the sole C source. Therefore, cultures were grown also on 0.1% pinitol plus 0.1% glycerol. Indeed, it is interesting to note that there were differences observed between cultures grown with only pinitol and with pinitol plus glycerol. Those differences will be discussed further below.

The *S. meliloti* wild-type strain can use pinitol as a sole carbon source. It was expected that if pinitol or D-*chiro*-inositol were detected, it would only be so during early growth phases. Data collected were consistent with this expectation. When grown with pinitol plus glycerol, day 1 pinitol concentrations were higher than when grown with only pinitol. This suggests that the rhizobia prefer glycerol and focus on degrading glycerol prior to acting on pinitol; a typical example of catabolite repression. When grown with pinitol plus glycerol, D-*chiro*-inositol concentrations were higher on day 1 than when grown with only pinitol. This could be because of the delayed consumption of the pinitol or because there was a higher cell count in the cultures grown with both pinitol plus glycerol.

In our experiment we expected that D-*chiro*-inositol is an intermediate of pinitol catabolism. It was expected that the *idhA* mutant would remove at least some of the detectable pinitol and show an accumulation of D-*chiro*-inositol. The *idhA* mutant removed about 50% of the detectable pinitol from the medium when grown with pinitol and when grown with pinitol plus glycerol. Very little D-*chiro*-inositol was detected when grown with only pinitol. The *idhA*

mutant showed an appearance of D-*chiro*-inositol on day 1 when grown with pinitol plus glycerol which was maintained. The difference in D-*chiro*-inositol concentration is probably due to the fact that the mutant is unable to produce a high cell count when grown with only pinitol as a sole carbon source. The data collected met the expected results, thus provided evidence that D-*chiro*-inositol is indeed an intermediate of pinitol catabolism (Fig. 16.)

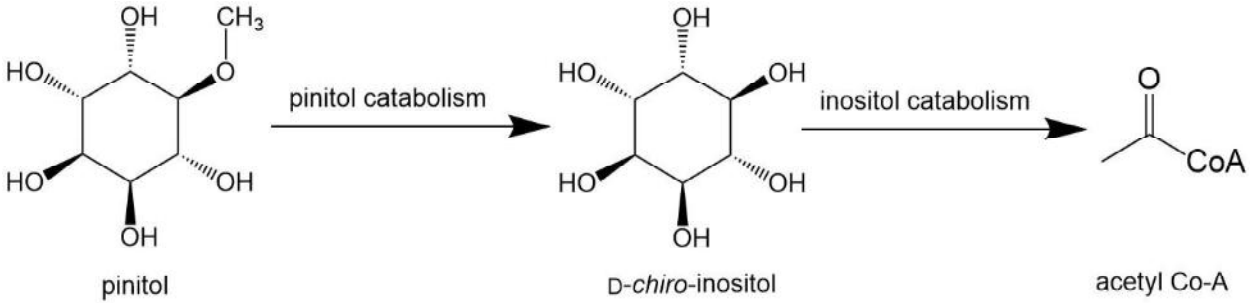

Figure 16. D-*chiro*-inositol is an intermediate of pinitol catabolism.

Some results obtained were unexpected. The *mocD* and *mocE* mutants were not expected to show any removal of pinitol from the medium, regardless of being provided with pinitol or pinitol plus glycerol, because each mutant is unable to grow when provided with pinitol as sole carbon source. Each mutant was able to remove about 50% of the detectable pinitol when grown with only pinitol. In addition, the *mocD* and *mocE* mutants were able to remove about 80% of detectable pinitol from the medium when grown with pinitol plus glycerol by day 4. It is hypothesized that the pinitol is being hoarded by the cells, perhaps in a polymer which would not be detectable by the methods used in this experiment (Fig. 17).

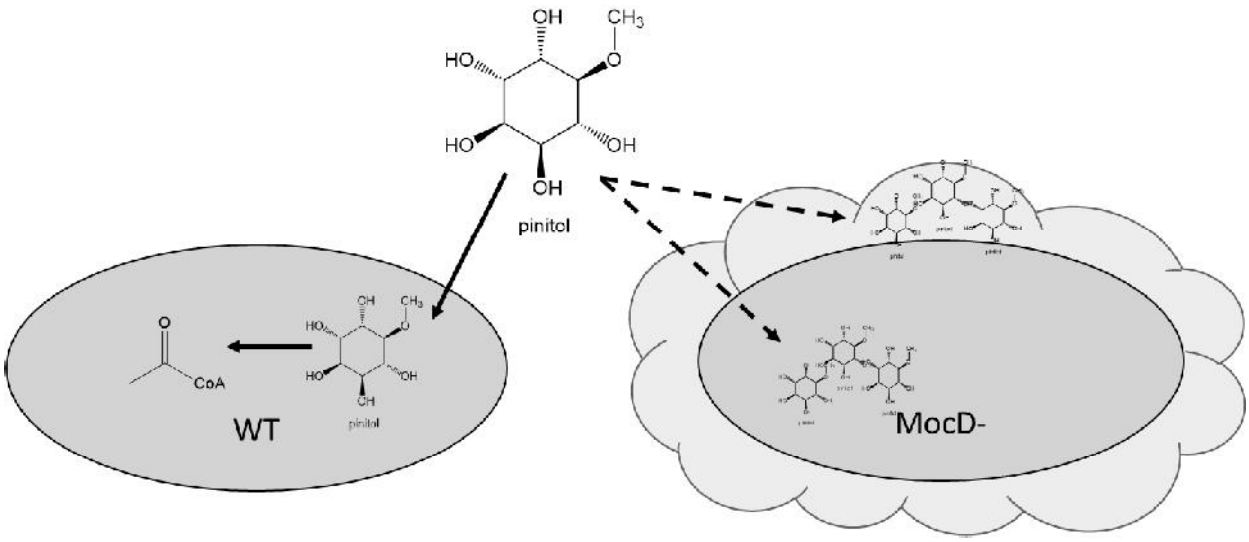

Figure 17. Schematic of pinitol use by *S. meliloti* wt and by the MocD mutant.

To support this notion, a *chiro*-inositol polymer has been found in *Bifidobacterium bifidum* (Ju et al., 2017). The polysaccharide was found to consist of 26.4% *chiro*-inositol, 3.9% rhamnose, 31.5% glucose, 11.0% galactose, and 23.8% ribose (Ju et al., 2017). Furthermore, it was observed that the *mocD* mutant produces more exopolysaccharides than the wild type (Fig. 18).

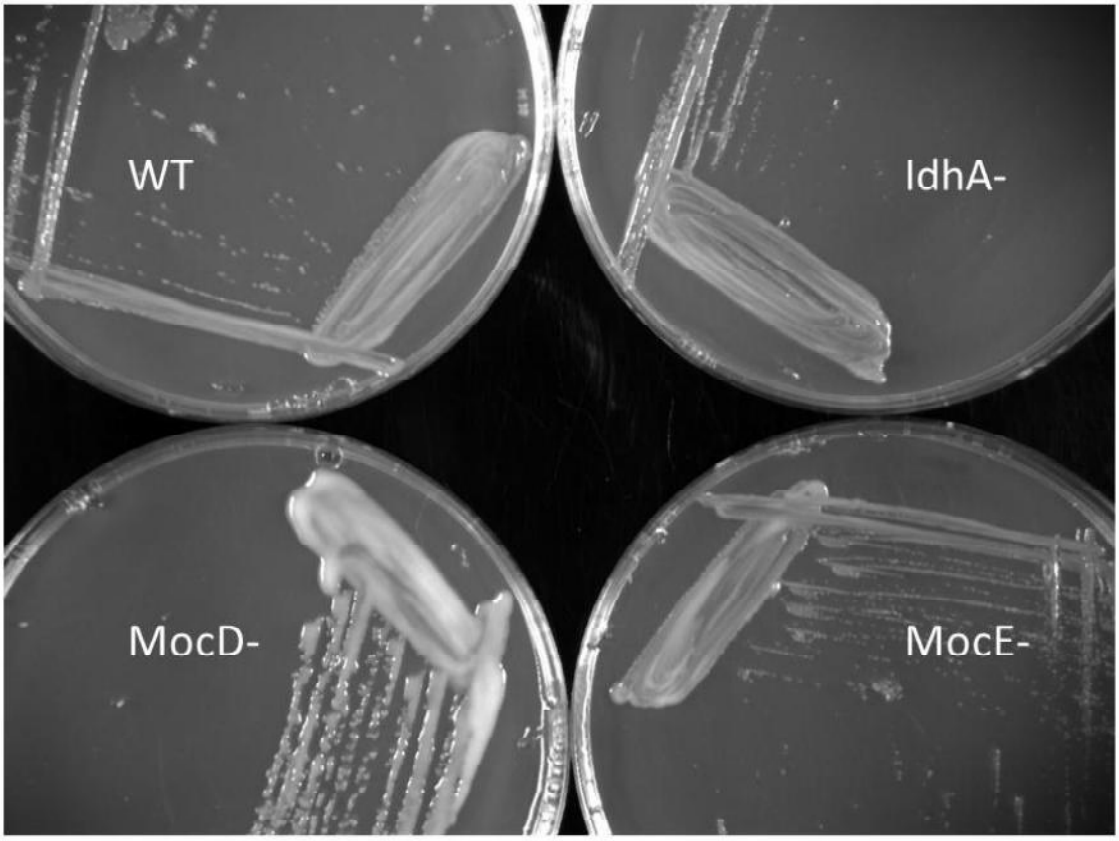

Figure 18. Colony morphology of *S. meliloti* cultures grown on TY plates for 72 hours.
Wild type *S. meliloti* is abbreviated as WT; also shown are the *idhA*, *mocD*, and *mocE* mutants.

The *mocD* and *mocE* mutants were expected to show no production of D-*chiro*-inositol. Just like the *idhA* mutant, very little D-*chiro*-inositol was detected when cultures were grown with only pinitol, but when grown with pinitol plus glycerol both *mocD* and *mocE* showed detectable D-*chiro*-inositol followed by its removal, which was maintained through day 4. It is hypothesized that there may be another enzyme that can act on pinitol when pinitol is present at high concentrations, because this enzyme may not be highly specific for its own substrate. Nevertheless, no enzyme has been found in the literature to support this idea.

Since some of the results obtained were not expected, the efficiency of the water bath sonication method to lyse cells was tested. It was thought that sonicating cultures for 15 minutes in a water bath sonicator would result in completely lysed cells, but it did not (A. Kennedy-Mendez, unpublished observation). Therefore, this experiment should be repeated with a method that will lyse the cells completely. Early in method development, however, I used a known method to lyse gram negative cells with a sonicator tip. A trial was conducted which involved separating the supernatant and resuspending the cells. Very little detectable pinitol was observed within resuspended lysed cells. The lysing efficiency of that method was never tested and should be tested in future, as part of identifying a successful lysing method for a future repeat of this study.

In addition, finding D-chiro-inositol in the uninoculated control was also unexpected. During method development it was shown that 100 ppm pinitol was stable in water. No testing was completed to show that pinitol was also stable in minimal medium. In the future, it should be shown that pinitol is stable in minimal medium with and without glycerol.

Other future work could involve working with another gram-negative bacterium, such as *Escherichia coli*. *Escherichia coli* cannot utilize pinitol as a sole carbon source, so it would serve as a control for pinitol "hoarding". If a culture of *E. coli* would also reduce pinitol concentrations regardless of being able to utilize it, it would support the hypothesis that the cells are hoarding the sugar or that it is being used to make a polysaccharide. Past work identified the *ibpA* gene, which is involved in inositol and pinitol transport in the *S. meliloti* (Thwaites 2013). Using the *ibpA* mutant would also be beneficial as a control for the GC-MS assay, because pinitol concentrations should not decrease unless an extracellular protein is adding it to the exopolysaccharides.

Concerning the second hypothesis, we evaluated the ability of the *idhA*, *mocD*, and *mocE* mutants to compete with the wild type for nodule occupancy when inoculated in a 50:50 ratio with the wild type (Fig. 10). Pinitol catabolism in regard to symbiosis is of interest because pinitol is found in high concentration in plants If pinitol catabolism plays no role in nodulation occupancy, a 50:50 output ratio would be expected. Plants inoculated with wild type and the *idhA* mutant resulted in nodules that contained a 50:50 ratio by week 8, but by week 16 the *idhA* mutant was outcompeted by the wild type. The *mocD* mutant was completely outcompeted by week 8 also by week 16. The *mocE* mutant was almost completely outcompeted by week 8 and and a 25:75 ratio by week 16. The *glyA2* mutant was used as a control, because it contains a mutation that does not interfere with pinitol catabolism. However, by week 8 the *glyA2* mutant was found to be at a 25:75 ratio with wild type, which was similarly maintained through week 16. The results were indeed consistent with what was expected if pinitol catabolism does play a role in nodulation occupancy. This experiment should be repeated with the use of more plants. Another improvement suggested is the identification of a better control mutant. Since pinitol is a secondary metabolite that plants use to handle osmotic stress, this experiment could also be repeated with different concentrations of salt and sugar stress. This would show if the wild type still outcompetes the mutants when the plant is under osmotic stresses. Lastly, it is suggested that plant growth studies could be conducted with and without salt stresses. This would show how the plant is affected by being inoculated with rhizobia that can or cannot degrade pinitol under osmotic stress. It would be interesting if the plant would benefit from one particular strain.

In summary, the results presented in this thesis have helped to refine our understanding of pinitol catabolism in *S. meliloti* by demonstrating that D-*chiro*-inositol is an intermediate of pinitol catabolism and that pinitol catabolism is advantageous for nodule occupancy.

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