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ROLE OF THE GENE IORA IN INDOLE-3-ACETIC ACID DEGRADATION OF BRADYRHIZOBIUM JAPONICUM

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ROLE OF THE GENE *IORA* IN INDOLE-3-ACETIC ACID DEGRADATION OF *BRADYRHIZOBIUM JAPONICUM*

by

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ABSTRACT

ROLE OF THE GENE *IORA* IN INDOLE-3-ACETIC ACID DEGRADATION OF *BRADYRHIZOBIUM JAPONICUM*

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Bradyrhizobium japonicum is a nitrogen-fixing bacterium that forms a symbiotic relationship with legumes such as soybeans. The bacterium plays an important role in providing the soybean plant with usable nitrogen while it receives photosynthetic products in return. *B. japonicum* strain USDA110 can utilize phytohormone indole-3-acetic acid (IAA) as an energy source. It has been found that the *iorA* gene is up-regulated in response to IAA. A mutant strain of the bacterium, *ΔiorA*, had the *iorA* gene removed in a previous experiment. To further investigate the role of IAA and its effects on *B. japonicum*, both the wild-type and mutant strains were exposed to varying concentrations of IAA. The results show that the *iorA* gene allows for growth in IAA treatment conditions, but the growth was reduced in comparison to glycerol treatments. This suggests the *iorA* gene may provide an evolutionary advantage by allowing energy to be obtained from alternate carbon sources.

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CHAPTER 1

INTRODUCTION

Bradyrhizobium japonicum, a nitrogen-fixing bacterium, forms a symbiotic relationship with *Glycine max,* the soybean plant, by infecting the plant creating nodules. Both *B. japonicum* and *G. max* benefit from this symbiotic relationship. Within the nodules, *B. japonicum* plays an important role in providing the plant with usable nitrogen while the bacterium receives photosynthetic products and essential nutrients in return. Atmospheric nitrogen, N_2, is converted by the bacterium into ammonia, NH_3, that the plant can then utilize. Nitrogen is an essential nutrient for all life, as it is the building block for nucleic acids and proteins. Additionally, nitrogen is also one of the essential macronutrients required for plants. Plants deficient in nitrogen display slow or stunted growth.

The soybean plant is an important crop due to its countless number of applications in food as well as industry. Most importantly, soybeans can produce almost twice as much protein per acre as any other vegetable or grain (1). Because of this, it is commonly used as a dairy and meat alternative. Due to the high protein content of soybean, improving its crop productivity could play a large role in feeding a growing world population and reducing malnutrition in third world countries (1). By the year 2050, the world population is estimated to reach nine billion (8). A growing world population will require increased food production despite the diminishing amount of farmable land. Climate change and water availability have also made farming increasingly difficult throughout the world,further decreasing crop yields (8). With this knowledge, scientists are attempting to

find ways to increase agricultural productivity.

It has been found that the presence of nitrogen-fixing bacteria in soil tends to increase crop yields (3). Inoculation of soybean seeds with *B. japonicum* would not only benefit crop yields, but be better for the environment. *B. japonicum* is an organic source of nitrogen for the soybean plant, as it naturally occurs in soil all around the world. Typically, fertilizers are used to introduce essential nutrients, such as nitrogen, that may be lacking in the soil. Nitrogen is a main component of many fertilizers. Much of the nitrates within the fertilized soil are lost in run-off. When agricultural run-off reaches bodies of water, fertilizer creates major environmental problems. Eutrophication is the process by which amounts of excess nutrients in the water promote plant growth and algae blooms. Algal blooms, which are excessive growths of algae, can cause hypoxia, killing animals within the environment due to a lack of oxygen. Fish kills, the mass killing of fish, are most often caused by algal blooms. This causes problems for the fishing industry, especially in communities where fish is a main source of food. The most significant problem is nitrate rich run-off leaching into groundwater, since groundwater is commonly used for public water sources. Ingestion of nitrate rich water can be toxic not only to animals in the environment but to humans as well. (2). By inoculating soybean plants with *B. japonicum*, we can hopefully cut down or eliminate the use of nitrogenous fertilizers for legumes.

In addition to receiving photosynthetic products, it has been determined that *B. japonicum* can also utilize phytohormone indole-3-acetic acid (IAA), more commonly known as auxin, as a carbon source for energy (5). There have been numerous studies conducted regarding soil bacteria and IAA. DNA fragments of other soil bacteria that can degrade IAA have been annotated, revealing a common *iac* gene cluster in 22 different

species (7). Interestingly, the *B. japonicum* genome does not contain this *iac* gene cluster. These results suggested that alternate genes are responsible for IAA degradation in *B. japonicum* (7). Not only does *B. japonicum* have the ability to catabolize IAA, but it can produce IAA as well. Many more studies have been conducted about the production of IAA, as it is thought to have a role in root nodulation development and/or maintenance (6). Many of the studies investigating IAA catabolism by *B. japonicum* have simply looked to determine the degradation products. Few studies have been conducted regarding the specific gene responsible for IAA degradation in *B. japonicum.*

In a study conducted under my mentor, Dr. Woo-Suk Chang, it was found that the gene *iorA* (locus bll3411), was up-regulated in response to a 1mM treatment with IAA (4). This data suggested that the *iorA* gene likely plays a role in the degradation of IAA by *B. japonicum*. In an additional experiment, a mutant strain, *ΔiorA*, was created by removing the *iorA* gene. With this gene removed, it is possible to further investigate the role of the *iorA* gene in IAA degradation and its effects on *B. japonicum*. Based on previous microarray data, the mutant strain, *ΔiorA*, should show decreased growth in comparison to the wild type strain, USDA110.

CHAPTER 2

METHODS

2.1 Gene Selection

In a previous study, *B. japonicum* strain USDA110 was treated with 1 mM IAA. Through the analysis of DNA microarray data, it was found that 1,323 genes were differentially expressed. Most genes associated with metabolism were repressed (down-regulated) but two subunits of an indolepyruvate ferredoxin oxidoreductase, encoded by gene loci bll3410 and bll3411, were induced (up-regulated). Loci bll3411, expressing the *iorA* gene, was chosen for mutant construction for gene knock-out. Through double-homologous recombination, the wild type USDA110 was mutagenized to replace the *iorA* gene with a kanamycin cassette. This kanamycin cassette confers kanamycin antibiotic resistance in the mutant strain, *ΔiorA*.

2.2 Control Variables

For the experiments conducted in this study, pH, temperature, revolutions per minute, antibiotic usage, and the wavelength used for measuring optical density were constant. Media were adjusted to pH 6.8 and grown at 30°C with shaking at 200 revolutions per minute (RPM) to maintain full aeration. Wild type strain USDA110 and the *ΔiorA* mutant strain were also inoculated with antibiotics chloramphenicol (30 μg/ml) and kanamycin (100 μg/ml), respectively. Growth was measured by recording optical density at 600 nm ($O.D._{600}$) using a UV-Vis spectrophotometer (Genesys 5, Spectronic Instruments).

2.3 Growth Curve in Arabinose-Gluconate Media

Two strains of *B. japonicum*, wild type strain USDA110 and mutant strain *ΔiorA,* were grown in arabinose-gluconate (AG) media. Refer to Appendix A for the contents of the AG media used in this study. Three cultures for each strain were inoculated in 50 ml of AG media and grown in 250 ml flasks. $O.D._{600}$ was recorded every 12h for a total of 96h.

2.4 Growth Curve in Bergersen Minimal Media

The two strains of *B. japonicum* were grown in Bergersen minimal media (BMM). Refer to Appendix B for the contents of the BMM used in this study. Three cultures for each strain were inoculated in 50 ml of BMM and grown in 250 ml flasks. $O.D._{600}$ was recorded every 12h for a total of 132h.

2.5 Growth Curve in 1mM IAA Minimal Media

The two strains of *B. japonicum* were grown in 1mM IAA minimal media. The IAA minimal media contained the same contents as BMM, except the carbon source was changed. Refer to Appendix B for the contents of BMM. Instead of using 4 ml of glycerol, 4 ml of 1mM IAA dissolved in ethanol was used. Three cultures for each strain were inoculated in 50 ml of 1 mM IAA minimal media and grown in 250 ml flasks. $O.D._{600}$ was recorded every 12h for a total of 180h.

2.6 Growth Curve in Arabinose-Gluconate Media in Response to IAA

The two strains of *B. japonicum* were grown in arabinose-gluconate (AG) media. Refer to Appendix A for the contents of the AG media used in this study. For each strain one culture was grown in a 2-liter flask containing 1 L of AG media. Cultures were grown until mid-log phase ($O.D._{600}$ ~0.8 to 1.0) was reached. Mid-log phase was reached at 108h of growth. The mid-log phase is characterized by exponential growth of the bacteria as

cells are in a metabolically active state. For each strain the AG culture was separated into eighteen 50 ml cultures in 250 ml flasks. Therefore, for each strain there were three biological replicates for each treatment condition. Cultures were treated with either 0.25, 0.5, 1, 2, or 5 mM of IAA. A control group was treated with only ethanol, as ethanol was used as a solvent to dissolve IAA in the experimental treatment groups. $O.D._{600}$ was recorded every 12h for a total of 192h.

CHAPTER 3

RESULTS

3.1 Growth Curve in Arabinose-Gluconate Media

There was a slight difference in growth between USDA110 and the *ΔiorA* mutant in AG media. A statistically significant difference ($P < 0.05$ by t-test) was seen at 84h and 96h. Data shown are the mean ± standard error of the mean of three biological replicates.

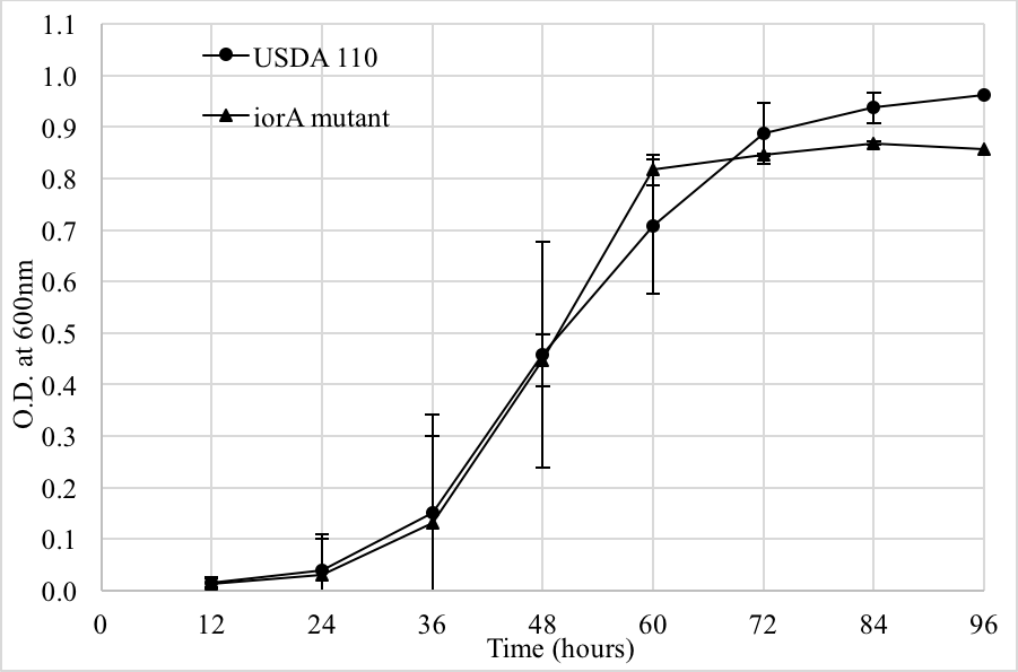

Figure 3.1: Growth Curve in AG Media

3.2 Growth Curve in Bergersen Minimal Media

There was a slight difference in growth between USDA110 and the *ΔiorA* mutant in BMM. A statistically significant difference ($P < 0.05$ by t-test) was seen at 84h and onwards. Data shown are the mean ± standard error of the mean of three biological replicates.

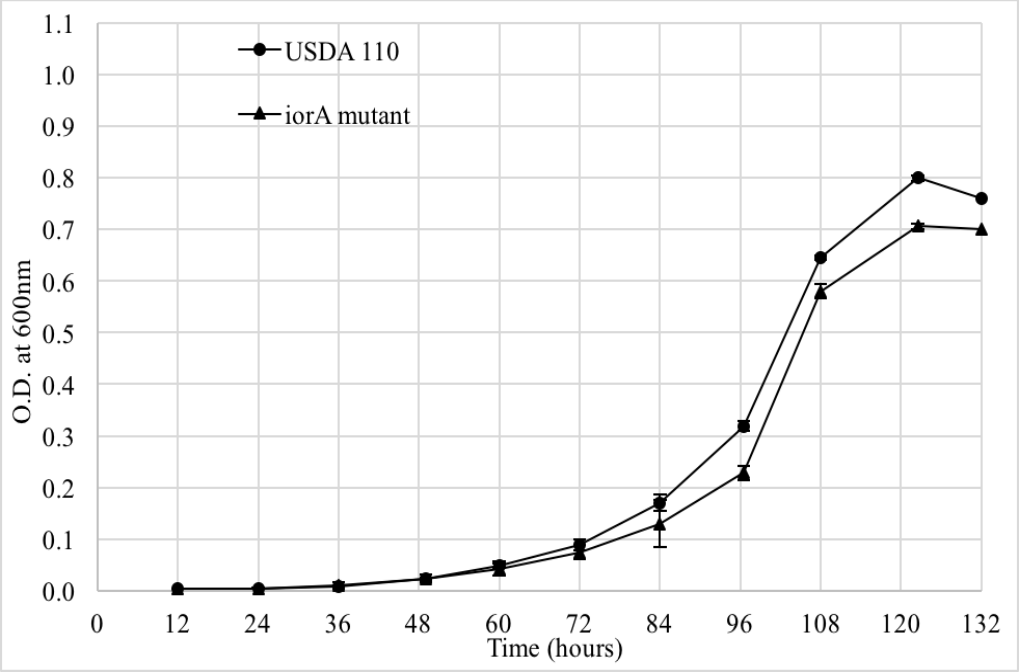

Figure 3.2: Growth Curve in BMM

3.3 Growth Curve in 1mM IAA Minimal Media

There was a statistically significant difference ($P < 0.05$ by t-test) in growth between USDA110 and *ΔiorA* mutant in 1 mM IAA minimal media beginning at 60h and onwards. Data shown are the mean ± standard error of the mean of three biological replicates.

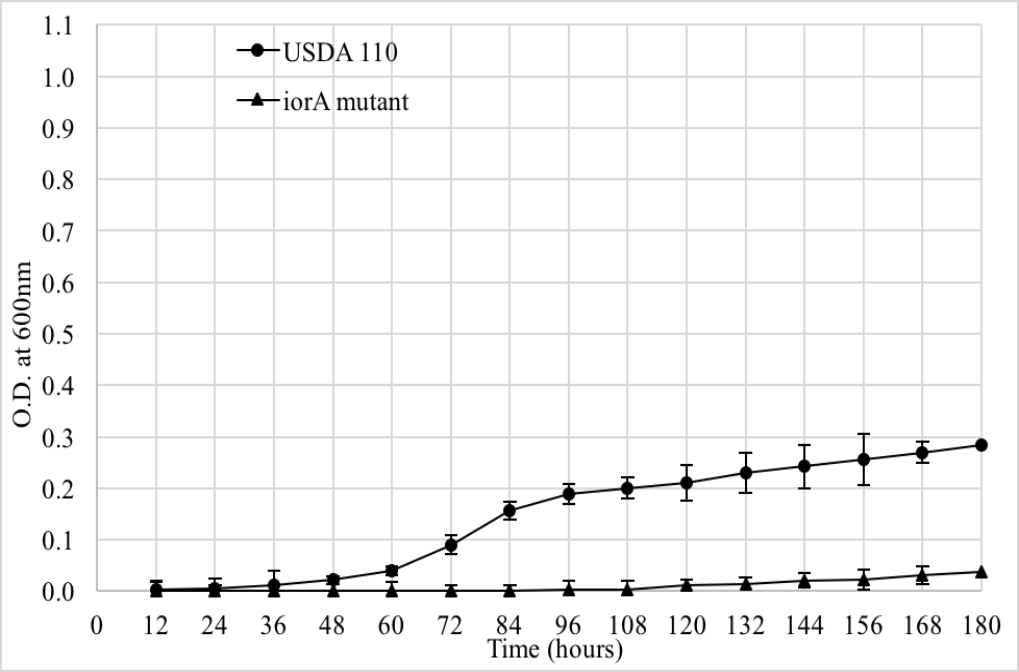

Figure 3.3: Growth Curve in 1mM IAA Minimal Media

3.4 Growth Curve in Arabinose-Gluconate Media in Response to IAA

There was a statistically significant difference ($P < 0.05$ by t-test) in growth between USDA110 and *ΔiorA* mutant in AG media treated at mid-log phase with various concentrations of IAA. There were also statistically significant differences in growth at the different concentrations of IAA among each strain. No error bars are shown due to too small variances.

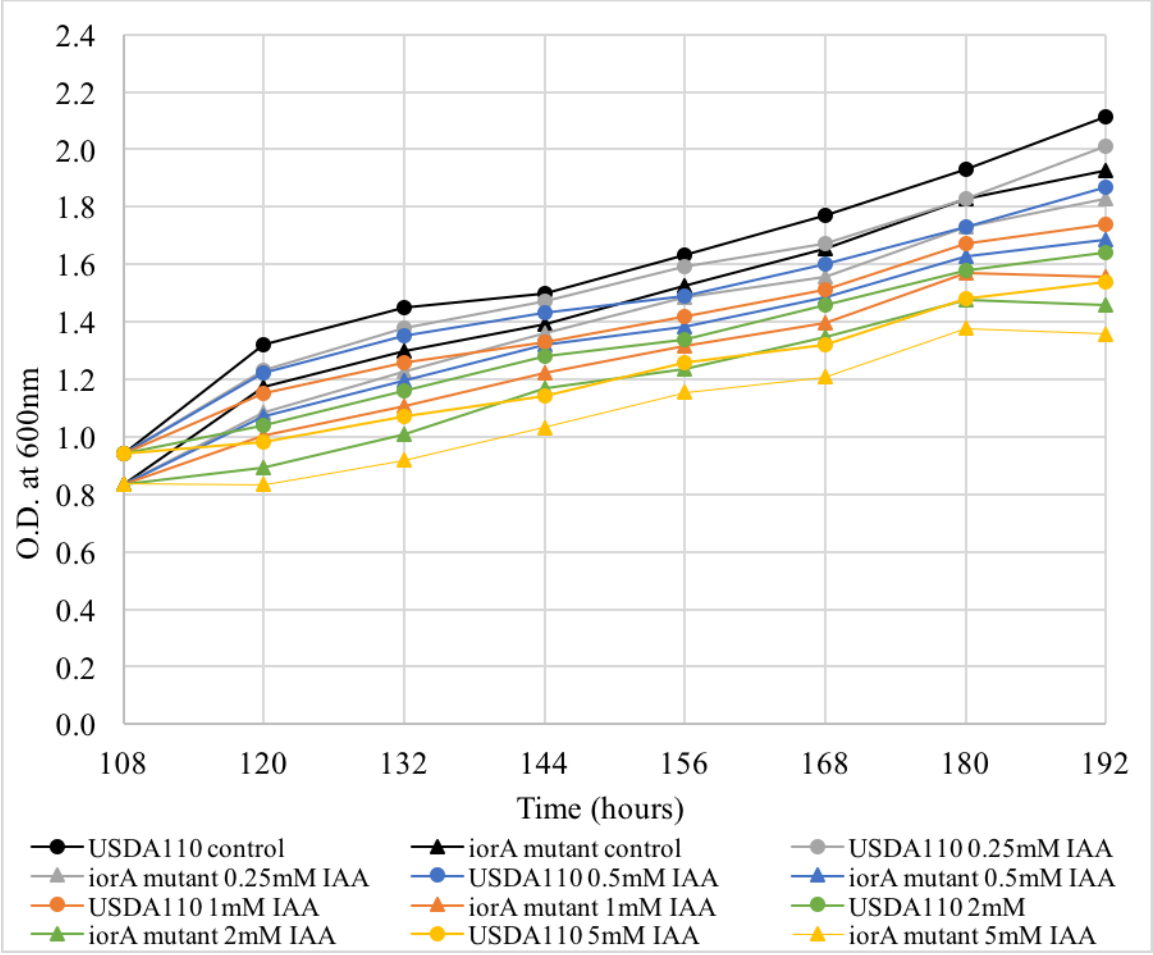

Figure 3.4: Growth Curve in AG Media in Response to Various IAA Concentrations

CHAPTER 4

DISCUSSION

Studying *B. japonicum* has important implications in agriculture, food production, and water pollution. Not only can the inoculation of *B. japonicum* increase crop yields for a growing world population, but may also cut down on the use of potentially toxic nitrogenous based chemical fertilizers. The relationship between *B. japonicum* and IAA is an important topic of research, as IAA is thought to play a role in the formation and/or maintenance of the root nodules where the bacteria lives. Because *B. japonicum* does not contain the *iac* gene cluster that is believed to be responsible for IAA degradation in other rhizobia (nitrogen-fixing soil bacteria) species, it has been of interest to find the gene responsible for IAA degradation in *B. japonicum*.

Because the *iorA* gene is knocked out in the *ΔiorA* mutant, it was unable to utilize IAA as a carbon source, resulting in less growth. This can be seen in the growth curve experiment in 1mM IAA minimal media and in AG media in response to varying IAA concentrations. IAA decreased growth in both strains in a concentration-dependent manner. Higher concentrations of IAA more negatively affected growth. USDA110 did grow slightly better in AG media and BMM compared to the *ΔiorA* mutant. It may be possible that the *iorA* gene could play a role in other energy metabolism processes besides IAA degradation. From previous microarray data, it has been shown that IAA down-regulates most energy metabolism processes. The exception of up-regulated energy metabolism in relation to IAA degradation was evident, as USDA110 showed slow but

continued growth in high IAA concentration environments. The *iorA* gene therefore may provide an evolutionary advantage as the bacteria is able to continue to grow in what would otherwise be unfavorable environmental conditions.

It may be possible through genetic modification to introduce the *iorA* gene in other soil bacteria that can currently not utilize IAA as a carbon source. Introducing the *iorA* gene in other soil bacteria that form symbiotic relationships with other plants may help increase crop yields in many important crops besides the soybean plant. Many more studies will need to be conducted regarding the *iorA* gene and its role in IAA degradation in *B. japonicum*, but this study proves that the *iorA* gene does indeed confer the bacterium's ability to catabolize IAA.

APPENDIX A

ARABINOSE-GLUCONATE (AG) MEDIA

Per 1L of deionized water (DI water)

125 mg	Disodium phosphate
250 mg	Sodium sulfate
320 mg	Ammonium chloride
180 mg	Magnesium sulfate heptahydrate
10 mg	Calcium chloride
4 mg	Iron(III) chloride
1.3 g	HEPES (hydroxyethylpiperazin ethane sulfonic acid)
1.1 g	MES (morpholineethanesulfonic acid)
1.0 g	Yeast extract
1.0 g	L-Arabinose
1.0 g	Sodium D-gluconate

APPENDIX B

BERGERSEN MINIMAL MEDIA (BMM)

Per 1L of DI water

300 mg	Dipotassium phosphate
300 mg	Monopotassium phosphate
500 mg	Ammonium nitrate
100 mg	Magnesium sulfate heptahydrate
50 mg	Sodium chloride
4 ml	Glycerol
1 ml	Trace Elements*
1 ml	Vitamin Stock**

*Trace Elements (1000X)

Per 1L of DI water

5 g	Calcium chloride
145 mg	Calcium chloride
125 mg	Iron(II) sulfate heptahydrate
70 mg	Cobalt(II) sulfate heptahydrate
5 mg	Copper(II) sulfate heptahydrate
4.3 mg	Manganese(II) chloride tetrahydrate
108 mg	Zinc sulfate heptahydrate
125 mg	Sodium molybdate
7 g	Nitrilotriacetate

**Vitamin Stock

Per 1L of DI water

20 mg	Riboflavin
20 mg	p-Amino benzoic acid
20 mg	Nicotinic acid
20 mg	Biotin
20 mg	Thiamine hydrochloride
20 mg	Pyridoxine hydrochloride
20 mg	Calcium pantothenate
120 mg	Inositol

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BIOGRAPHICAL INFORMATION

Amanda Plein graduated with an Honors Bachelor of Science in Biology at UT Arlington, and has been a part of the Honors College since joining the university her freshman year. Although she is mostly interested in the health sciences, researching within the Microbiology Department exposed her to many different topics she may have never been interested in otherwise. Many of the projects she conducted in Dr. Woo-Suk Chang's laboratory involved *B. japonicum* strain USDA110 and studying its ability to utilize IAA as an energy source. Her undergraduate research experience has interested her in participating in additional research opportunities in her post-graduate studies. She will be studying medicine starting in July of 2017 at the University of North Texas, Texas College of Osteopathic Medicine. As of now she is interested in becoming a forensic pathologist, but is keeping her options open to the many different medical specialties.