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Isolation and characterization of bacterial symbionts from Crotalaria spectabilis grown on trichloroethene contaminated soil

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ISOLATION AND CHARACTERIZATION OF BACTERIAL SYMBIONTS FROM *Crotalaria spectabilis* GROWN ON TRICHLOROETHENE CONTAMINATED SOIL

by

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ABSTRACT

The discovery of several members of the Leguminosae family growing on a TCE contaminated site demonstrated that they have the capabilities to tolerate the TCE contamination. This led to an investigation of the plants' ability to metabolize TCE. Research by Dr. Lee Newman showed that nodules from *Crotalaria spectabilis* grown on the site had TCE metabolites present while nodules from other legumes at the site did not. The presence of TCE metabolites within the *C. spectabilis* nodules suggests that the bacterial symbiont may be responsible for TCE metabolism. This led us to investigate the symbiont of *C. spectabilis* and its ability to metabolize TCE.

Nodules from *C. spectabilis* grown on TCE contaminated and non-contaminated soils were used to isolate the bacterial symbiont. Some species of *Crotalaria* are known to host *Methylobacterium nodulans* and this unique symbiont may explain why only the *C. spectabilis* nodules metabolize TCE. However, methane and methanol enrichments from the nodules were not successful and led to other approaches of isolating and identifying the symbiont. Sequencing of the 16S rRNA gene amplified from crushed nodules identified the symbionts as members of the *Mesorhizobium* genus. Symbiotic bacteria were isolated from control and dosed nodules and isolates of potential symbionts were verified by comparing PCR products from nodules and isolates using DGGE analysis. Sequencing of the 16S rRNA gene from the isolate confirmed that the isolates are members of the genus *Mesorhizobium*. This bacterium is different from symbionts isolated from other *Crotalaria* species and represent a new bacterium capable of nodulating *Crotalaria* sp. Attempts to demonstrate TCE metabolism by the isolated symbionts were unsuccessful suggesting that TCE metabolism may depend on a partnership with the host plant.

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1. INTRODUCTION

1.1. BACKGROUND

The *Crotalaria* genera can be found throughout the world and some *Crotalaria* species have been used as a green manure (6). The few *Crotalaria* species that have been studied have been nodulated with *Methylobacterium nodulans*. *M. nodulans* would have the potential to use single carbon compounds such as methane or methanol. It may also have the ability to use other simple carbon compounds such as trichloroethylene (TCE) which would be beneficial in bioremediation. The growth of *Crotalaria spectabilis* on a TCE contaminated suggests that *M. nodulans* may be the symbiont to *C. spectabilis* and have the ability to metabolize TCE.

Trichloroethylene is a colorless liquid that was commonly used as an industrial solvent from the 1930s until the 1970s. Because of the frequency of its use, many people were exposed before the health consequences were realized. Since that time, TCE has been shown to be a known carcinogen and has many health implications depending on exposure. Ingestion and inhalation of TCE can pose serious health issues with consequences as serious as death for the patient exposed to TCE (1). Cardiovascular problems, as well as death, have been reported due to inhalation of high levels of TCE. Renal, hepatic, along with other body parts, can be impacted as well from TCE exposure. Mice exposed to TCE via ingestion had an increased number of tumors in renal and hepatic tissues compared to the control mice (22). Voluntary and unintentional exposures have occurred and led to unfavorable health implications. Occupational exposure to TCE is the most common manner in which people are exposed to TCE and are more likely to have the serious health consequences associated with TCE exposure.

1.2. GOALS AND OBJECTIVES

The goals of this research were to isolate and identify the bacterial symbionts of *Crotolaria spectabilis* from TCE contaminated sites and to test the symbiont's ability to metabolize TCE as a pure culture. The isolation and identification of the symbiont will provide a better understanding of *C. spectabilis* in a non-native environment and its potential use in TCE bioremediation. Specific objectives to reach these goals are as follows.

Objective 1: Isolation of symbiont in pure culture.

Hypothesis: Nodules will contain one bacterial species.

Significance: To date, *Crotalaria* species from several locations have been shown to be infected with various rhizobium and non-rhizobium genera. None of the nodule symbionts have been isolated from *Crotalaria* grown in North America.

Objective 2: Re-inoculation of *C. spectabilis* seedlings with isolated symbionts.

Hypothesis: The isolated pure culture will be capable of infecting *C. spectabilis*..

Significance: The symbiont isolated from *C. spectabilis* nodules should be able to nodulate the same plant species. Re-infection will verify the bacteria isolated from the original nodules are the correct symbiont.

Objective 3: Analysis of the ability of nodule isolates to metabolize TCE.

Hypothesis: The bacterial symbiont has the potential to metabolize TCE.

Significance: The TCE degradation products found within this plant at the original site is hypothesized to be due to the symbiont's ability to degrade TCE.

Completion of these three objectives will help in identification of the symbiont of a non-native plant and the symbiont's characteristics. It will also provide insight into the

possible use of *C. spectabilis* and its symbiont in the TCE bioremediation processes. This has the potential for being an inexpensive remediation process that can be implemented at TCE contaminated sites, both NPL and sites that are not severe enough to be on the NPL.

2. REVIEW OF LITERATURE

2.1. *Crotalaria* species BACKGROUND

2.1.1. *Crotalaria* species. There are over 600 *Crotalaria* species worldwide and they are predominately native to Africa and Asia (4). A few of the Crotalaria species are used as green manure in Japan (6). *C. spectabilis* is native to the Indo-Malaysia area but found worldwide, including the southeastern United States (4). The plant contains alkaloids which are of concern to many farmers and have prevented it from being commonly used as a green manure.

2.1.2. Known symbionts of *Crotalaria* sp.. The symbionts of *C. spectabilis* have not been well documented and reported in literature. Symbionts from a handful of *Crotalaria* species have been well documented in numerous literature publications. Most literature has shown that *Crotalaria* species are frequently nodulated with *Methylobacterium nodulans* (11, 16, 17, 27, 28, 35). *Crotalaria* species have also been shown to be nodulated by *Bradyrhizobium* sp. (35) along with *Rhizobium leguminosarum*, *B. japonicum*, and *Burkholderi*a sp. (16). The non-native location of *C. spectabilis* in the southeastern United States may encourage a symbiotic relationship with a bacterial species that is not typically associated with *Crotalaria* species.

2.1.3 Nodulation. *Crotalaria* sp. form determinate nodules in response to their bacterial symbiont. The process of nodulation involves chemical signals between leguminous plants and bacteria. Flavonoids are released by the plant as an attractant for potential symbiotic partners. The bacteria recognize these flavonoids through their NodD proteins. Upon this recognition, additional Nod genes are activated and Nod factors are released by the bacteria. This stimulates proliferation of cells within the root cortex which

will eventually differentiate into root nodules. This stimulation of nodulation of the root by the soil rhizobia only occurs if the plant recognizes the Nod factors (20). The interaction between the plant and rhizobia are based on specific signal recognition between the two. Plants are usually nodulated by specific bacteria which infers that bacteria are rather specific in which plants they nodulate. Although, some rhizobia have been shown to nodulate other plant species.

After chemical signals are recognized, bacteria associate with the tip of the root hair and stimulate root hair curling leading to formation of an infection thread. The infection thread which is the diameter of a single bacterium extends toward the root cortex as the bacteria divide within the infection thread. As a result of this process, an infection thread contains a pure culture of a single strain of bacteria which will infect the root hair of the plant. Once the bacteria have entered the differentiating cells of the root cortex, they differentiate and multiply within the root. The differentiated bacteria are referred to as bacteroids. The plant cell where the 'bacterial infection' has occurred becomes differentiated as well such that the nodule has the structure of a new tissue. The increase in bacteroids (the differentiated bacterial cells) causes the plant root cell to grow, which initiates the formation of the nodule. The plant provides a source of carbohydrates in the form of dicarboxylic acids, malate, sucinate, and fumarate to the bacteria and the bacteria fix nitrogen into usable form for the plant (20).

2.2. SAVANNAH RIVER SITE BACKGROUND

2.2.1. Purpose of the Savannah River Site. The site is located along the Savannah River in South Carolina. DuPont was initially contracted to design and build a

facility for nuclear weapon enrichment in the early 1950's. The site has been expanded over the years to continue with its primary goal of nuclear weapon enrichment and became a disposal site for nuclear waste, both on-site and off-site, and other wastes produced by nuclear enrichment activity (18). It continues to function as a Department of Energy site, although managed and operated by a subcontractor, and is projected to be the site of continued energy research (39).

Figure 2-1. Location of Savannah River Site (40)

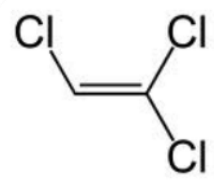
2.2.2. Contaminants at the Savannah River Site. The nuclear activity occurring at the site led to the use of many chemicals and radioactive material. Previously there were no regulations on how to properly dispose of these compounds, which led to significant site contamination (41). The primary environmental concern at the site is the radioactive waste, but there are other contaminants on site having varying levels of toxicity. The other contaminants, such as TCE, have the potential of contaminating ground water and spreading to nearby communities. Milson and Bledsoe have reported

that over 13 million pounds of TCE and PCE have been released at the Savannah River Site settling basin or in the A-14 Outfall (19).

2.3. TRICHLOROETHYLENE BACKGROUND

2.3.1. Discovery and Use of Trichloroethylene. TCE was first synthesized in 1864 by Fisher and patented in 1906 by Konsortium für Elektrochemische Industrie (44). After the capabilities of TCE were discovered, manufacturing of TCE on a large scale began. TCE was primarily used as an industrial solvent but was present in many household cleaners and solvent; it was also used in the medical field as an analgesic and anesthetic. Of the TCE manufactured in the United States up until the early 1970s, 90-95% was used for degreasing operations (31).

Figure 2-2. Structure of Trichloroethylene



2.3.2. TCE as a Common Contaminant. The incredible degreasing capabilities of TCE led to its broad use in many industries and this wide-spread use led to numerous contaminated sites. As of February 2011, 427 NPL sites contained TCE. This is not all sites that have TCE; these are just the sites meeting the NPL requirements (38). There are three primary ways in which a contaminated site can be placed as a NPL site. The first of these is that the site has been scored on an EPA hazard scale and its score has placed the

site as hazardous and in need of remediation. Another way in which a site can be placed on the NPL is that each state or territory is allowed to have one site that is a priority, regardless of its hazard score. The third method in which a site becomes placed on the NPL is that the site is determined to be a public health risk by the Agency for Toxic Substances and Disease Registry (ATSDR) and the EPA believes that they can remediate the site for less than what it would cost to relocate the population. The Toxic Release Inventory (TRI) showed between 1988 and 2009, 1,989 facilities released TCE into the environment (38).

2.3.3. TCE as a Health Concern. TCE has many health implications depending on type and duration of exposure. TCE vapors can be present when showering with TCE contaminated water or from using products containing TCE (ie. Spot removers and typewriter correction fluid). Inhalation of small amounts of TCE vapors has the potential to cause headaches, lung irritation, dizziness, poor coordination, and difficulty concentrating. Large amounts of TCE vapor inhalation can impair heart function, cause unconsciousness, and lead to death. Chronic TCE vapor exposure has the potential to cause nerve, kidney, and liver damage. Ingesting large amounts of TCE can cause nausea, liver damage, unconsciousness, impaired heart function, and eventually death. Chronic ingestion of small amounts of TCE can cause liver and kidney damage, impaired immune system function, and impaired fetal development in pregnant women (38). According to the National Toxicology Program in the 12th Report on Carcinogens, TCE is "reasonably anticipated to be a human carcinogen" (22).

2.3.4. Current Methods for TCE Clean Up. There are many methods used by the EPA to clean up TCE contaminated sites. The specific process is dependent on the

site. The most common method used to clean up TCE is a pump and treat system. This method involves the pumping of ground water to the surface, treating it to meet the safe drinking water act requirements, and then the water is usually returned to the ground or it can be kept at the surface (42). Some of the other methods used by the EPA include: monitored natural attenuation, phytoremediation, thermal processes, volatilization technologies and treatment walls (38). A few of these methods can be used in the pump and treat system as the process for removing the TCE from the ground water; for example, two of these methods would be a thermal process and volatilization. They can also be used at the site without the pump and treat system. Some of these methods are expensive and are no more efficient than less expensive methods depending on the TCE contaminated site.

2.3.4.1. Pump and Treat TCE removal. Removal of TCE at a contaminated site is the primary concern of nearby residents and the organized group at the site. The most common technique used by the EPA at TCE contaminated sites is the pump and treat method. Surfactants may also be used to make the TCE more available within the ground water for easier removal. The pump and treat method requires a continued energy source and monitoring of the TCE plume to ensure that the TCE is being removed with minimal spreading in the groundwater. This method does not guarantee that all contaminants will be removed from the site, as some compounds may adhere to soil surfaces. There is also potential for damage with the pump and treat system such as over pumping the aquifer, increased contaminants at the surface, and if not returning the water to the aquifer, dealing with the large volume of water needing to be discharged after treatment (34). The required energy and manpower to maintain a pump and treat facility can increase the

costs of bioremediating the site. Alternative remediation efforts may be able to significantly decrease expenses from by eliminating energy requirements and reducing manpower required to maintain the clean-up.

2.3.4.2. Phytoremediation. One of the more attractive methods for cleaning up contaminated sites is the use of plants for phytoremediation. Plant research has shown the incredible uptake and degradation potential of many plants. In addition to plants degrading the contaminants in the soil themselves, they create an environment that is conducive for microorganisms with diverse metabolic capabilities. Some of these microorganisms are able to degrade contaminants and have shown this potential since the early 1990s (43). By providing the right environment in the soil, some plants host microorganisms that are able to degrade TCE. The use of both plants and their associated rhizosphere microorganisms for the clean up of contaminated sites is referred to as rhizoremediation. A particularly interesting combination of plants and their root associated microorganisms for rhizoremediation is the legume/rhizobium symbiosis. The intimate association of rhizobia and their host plants provides a controlled environment for the bacterial symbiont and exchange of nutrients (or contaminants) between the plant and the bacterium. The utilization of plants and their root associated microorganisms to remove contaminants in the environment may be an inexpensive solution to some waste sites that have vegetation.

2.3.5. Microorganisms known to Degrade TCE. Early observations into microbial TCE degradation led many to believe that it was an anaerobic process. Up until recently, microbial TCE degradation was believed to only occur anaerobically (24). Many of the early experiments used environmental samples and looked for TCE

degradation (12). The consortia of bacteria in the sample were able to degrade TCE but no individual organisms were isolated or identified. Mixtures are still being analyzed for their role in TCE degradation and its composition of organisms is still largely unknown (7). Genetic analysis is being done to determine what microorganisms are present, but it appears that it is the combination that makes degradation possible. Table 2-1 shows organisms documented to degrade TCE and if they do so aerobically or anaerobically. The chart does not include all organisms, but those organisms that are specifically identified in literature as being capable of degrading TCE. Many mixtures or environmental samples are able to degrade TCE but the individual organisms responsible have not been individually identified as degrading TCE. According to Pant and Pant, there are three mechanisms in which chlorinated ethenes are degraded (26). One of these mechanisms is reductive dechlorination which is an anaerobic process in which the TCE would act as an electron acceptor. A second method is direct oxidation; this is TCE acting as an electron donor. A third method is co-metabolism in which the organism degrades the TCE biochemically but does not benefit. There are various mechanisms in which TCE could be degraded aerobically and a chart showing these potential pathways is shown in Figure A-1 (33). In one series of experiments, TCE degradation has been shown to occur through co-metabolism of methane in methanotrophs (32). The research showed that the methanotrophic communities not only survived in the presence of TCE, the bacteria were able to grow and degrade the TCE. Of particular interest for phytoremediation, *Ralstonia taiwanensis*, a bacteria isolated from *Mimosa sp*., has been shown to degrade TCE (5). In the presence of plant carbohydrates, *R. taiwanensis* had increased TCE removal from the

sample. It was unable to degrade TCE without another carbon source suggesting co-metabolism of the TCE.

Table 2-1. Microbial Organisms Shown to Degrade TCE

Organism	Oxygen tolerance	Source
Bacillus sp.	Aerobic	(13)
Dehalococcoides ethenogenes	Anaerobic	(21)
Mesorhizobium loti	Anaerboic	(30)
Methylosinus trichosporium	Aerobic	(25)
Pseudomonas putida	Aerobic	(10)
Ralstonia taiwanensis	Aerobic	(5)

2.4. SUMMARY

The ability of a non-native legume to survive on a TCE contaminated site and the presence of TCE metabolites in their root nodules suggests the potential use of legumes and symbionts for phytoremediation of TCE contaminated sites. Root nodule bacteria in association with their host plant are potentially responsible for the metabolism of TCE in *C. spectabilis*. The symbiont of *C. spectabilis* has not been identified in previous literature. Based on symbionts isolated from other *Crotalaria* sp. one might expect to isolate a *Methylobacterium* sp. or *Bradyrhizobium* sp. However, as a non-native species, the typical symbionts of *Crotalaria spectabilis* may not be at the Savannah River site and an atypical symbiont may be responsible for nodule formation.

The identification of the symbiont of *C. spectabilis* from TCE contaminated soils may identify a new candidate for TCE metabolism. This symbiotic relationship may show not only a newly discovered relationship between bacteria and a host but also show promising new TCE phytoremediation possibilities.

3. MATERIALS AND METHODS

3.1. SAMPLING METHODS

Nodule samples were obtained from Dr. Lee Newman, currently at State University of New York College of Environmental Science and Forestry. Dr. Newman worked on this project in South Carolina at the Savannah River Site National Laboratory. The samples were harvested from twelve different *C. spectabilis* plants. Six of the plants were used as controls and not exposed to TCE (identified as NC for nodule control); the remaining six plans were exposed to TCE, (identified as ND for nodule dosed). Nodules for each plant were quick frozen in liquid nitrogen and placed in a glass vial. The glass vials were and shipped overnight on dry ice. The nodules were stored at -30°C until cleaned and sterilized.

3.2. NODULE STERILIZATION AND CRUSHING

All nodules were first cleaned of any external debris (ie. Soil) by rinsing with water. Three different sterilization methods were attempted on the nodules and are shown in the following table.

Table 3-1. Various Nodule Sterilization Methods

Method 1	Method 2	Method 3
Rinse with 70% ethanol.	Vortexed in washing solution for 1 minute.	Rocked 15 minutes in 6%NaClO solution.
Soak in H_2O_2 for 5 minutes.	Rocked for 5 minutes. Vortexed for 1 minute. (2 times)	6%NaClO solution discarded. Rocked in 70% ethanol for 1 minute.
Sterile H_2O rinse (2 times).	Washing solution removed and sterile water was added.	Ethanol solution discarded.
	Vortexed for 1 minute. (3 times).	Rocked in sterile water for 1 minute (4 times).
	Sterile water sample plated and used as a wash control.	
	Water removed.	

Sterile nodules were transferred to a sterile microcentrifuge tube. The nodule was crushed with a sterile micro pestle in 200 µL sterile water. The nodule suspension was stored at -30°C for further analysis.

3.3. MEDIA USED

3.3.1. Media for Bacterial Isolation. AG broth medium was made with 10 ml/L of seven stock solutions (A-G): soln A (130.0 g/L HEPES and 110.0 g/L MES), soln B (0.67 g/L $FeCl_3 \cdot 6H_20$), soln C (18.0 g/L $MgSO_4 \cdot 7H_2O$), soln D (1.3 g/L $CaCl_2 \cdot 2H_2O$), soln E (25.0 g/L Na_2SO_4), soln F (32 g/L NH_4Cl), soln G (12.5 g/L Na_2HPO_4). In addition to the seven stock solutions, the remaining components of AG media are: 1.0 g/L arabinose, 1.0 g/L gluconate, and 1.0 g/L yeast extract. AG media is pH modified to 6.9 (29). 15.0 g/L agar is added AG broth to make AG agar. Yeast mannitol agar (YMA) and yeast xylose agar (YXA) were also used to plate nodule suspensions. YMA was composed of: 15.0 g/L agar, 10.0 g/L mannitol, 0.5 g/L K_2HPO_4, 0.4 g/L yeast extract,

0.2 g/L $MgSO_4 \cdot 7H_2O$, and 0.1 g/L NaCl. Yeast xylose agar was made by substituting 10.0 g/L xylose for mannitol in the YMA medium.

3.3.2. Media for Seed Germination. Plain agar plates (15.0 g/L agar) were used as a substrate for seed germination.

3.3.3. Media for Symbiont Characterization. To characterize the symbiont, rhizobia minimal media broth was used (8). Rhizobia minimal media broth was composed of: 0.33g/L $(NH_4)2SO_4$, 10.47 g/L MOPS, 0.3g/L KH_2PO_4, 0.3 g/L Na_2HPO_4, 0.12 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $CaCl_2 \cdot 2H_2O$ and 1 ml/L of 1000x trace elements. Trace element solution was made from 1.0 g/100 mL H_3BO_3, 0.1 g/100 mL $ZnSO_4 \cdot 7H_2O$, 0.05 g/100 mL $CuSO_4 \cdot 5H_2O$, 0.05 g/100 mL $MnCl_2 \cdot 4H_2O$, 0.01 g/100 mL $NaMoO_4 \cdot 2H_2O$, 0.1 g/100 mL $FeCl_3$, and 0.5 g/100 mL $NiCl_2 \cdot 6H_2O$.

3.3.4. Media for Enrichments. Nitrate mineral salts medium was used for methane and methanol enrichments (3). Nitrate mineral salts medium was composed of: 1.0 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L KNO_3, 0.1419 g/L Na_2HPO_4, 0.272 g/L KH_2PO_4, 0.20 g/L $CaCl_2 \cdot 6H_2O$, 4.0 mg/L ferric ammonium citrate substituted for ferric ammonium EDTA, and 0.5 mL/L trace element solution. The nitrate mineral salts trace element solution was made of: 0.5 g/L disodium EDTA, 0.2 g/L $FeSO_4 \cdot 7H_2O$, 0.030 g/L H_3BO_3, 0.020 g/L $CoCl_2 \cdot 6H_2O$, 0.010 g/L $ZnSO_4 \cdot 7H_2O$, 3.0 mg/L $MnCl_2 \cdot 4H_2O$, 3.0 mg/L $Na_2MoO_4 \cdot 2H_2O$, 2.0 mg/L $NiCl_2 \cdot 6H_2O$, and 1.0 mg/L $CaCl_2 \cdot 2H_2O$.

3.3.5. Media for Transformations. LB agar composed of: 10.0 g/L tryptone, 5.0 g/L yeast extract, and 5.0 g/L sodium chloride supplemented with X-galactose and ampicillin. Circlegrow (40.0 g/L) broth was used to prepare cultures for plasmid preparation (MP Biomeicals, Solon, OH). SOC medium was also used after

electroporation (9). SOC was prepared with: 20 g/L Tryptone, 5.0 g/L Yeast Extract, 0.6 g/L NaCl, 0.2 g/L KCl, 2.0 g/L $MgCl_2$, 2.5 g/L $MgSO_4$ and after autoclaving, 20 ml of 1M Glucose.

3.3.6. Media for TCE Degradation Analysis. Cultures were grown in AG broth and AG broth supplemented with Malate (10 mM final concentration).

3.4. BACTERIAL ISOLATION

AG, YMA, and YXA plates were plated with 25-50 µL of nodule suspension. The agar plates were incubated at 30°C for 72 hours. After 72 hours, individual colonies were streaked for isolation on YMA and AG media. Individual colonies were maintained for further analysis. Potential symbionts were stored frozen at -80 C in AG broth plus 20% glycerol.

3.5. DNA EXTRACTION

DNA was extracted from nodule suspensions using TRIzol®Reagent Total RNA Isolation Reagent. The protocol for DNA isolation was followed; one exception to the protocol performed was the DNA pellet was resuspended in 100 µL sterile water. The purified DNA was used for further analysis.

3.6. PCR PRIMERS, SEQUENCING, AND PHYLOGENETIC ANALYSIS

Polymerase chain reaction was performed on the purified DNA samples, nodule suspensions, and cell suspensions. Protocols for PCR amplification can be found in appendix B.

Table 3-2. PCR Primers and Their Sequence

Primer	Sequence (5'-> 3')	Gene	Reference
27f	AGAGTTTGATCMTGGCTCAG	16S rRNA	(14)
1392r	ACGGGCGGTGTGTRC	16S rRNA	(14)
alr1f	ATCTCTACGGAACAACTCCG	*Mesorhizobium* sp.	
alr1r	TCCAGCCGAACTGAAGGAAA	*Mesorhizobium* sp.	
5354BSD	AGGAGACCCCATGAGTTCGAA	*M. loti* dehalogenase gene	(30)
5354H	CAGGCCAACTCGTCCGTC	*M. loti* dehalogenase gene	(30)

PCR products were run on a 0.8% agarose gel for verification of amplification. After a successful first round PCR, a second PCR was performed using the first PCR product as a DNA template. PCR cleanup was accomplished using the PCR Clean Up kit by IBI. Their protocol was followed with the exception that the wash step was performed two times before centrifugation. The cleaned up PCR product was analyzed for purity and DNA content using a Nanometer spectrophotometer. To prepare for sequencing, 1 µL of sequence primer (either forward or reverse), DNA PCR product to have 50 ng DNA, and deionized water to a volume of 8 µL was placed in a centrifuge tube. The sequencing reaction was given to the cDNA lab for sequencing.

3.7. SEED STERILIZATION AND GERMINATION

Crotolaria spectabilis purchased from Onalee's Home Grown Seeds were sterilized in H_2SO_4 for 10 minutes. After five rinses with sterile water, the seeds were rubbed between two pieces of sterile sand paper. The seeds were placed on 1.5% agar plates and kept in the dark for 72 hours for germination. This procedure was modified from the procedure used by Sy et. al. (36). *C. spectabilis* seeds were used in place of *M.*

truncatula, incubated for 72 hours on 1.5% agar plates vs. 48 hours on Fahreus medium plates.

3.8. SEED INOCULATION AND PLANTING

The bacterial culture was grown in AG broth for 72 hours. The germinated seedlings were incubated in the broth suspension in a petri dish on a rocking table for one hour. The inoculated seedlings were planted in sterile soil and placed in a covered container under lights (16 hour/8 hour light cycle). The seedlings remained covered until they reached ~15 cm in height.

3.9. NODULE HARVEST

After four to five months, the plants and their roots were removed from the containers. Soil was gently removed from the roots and any remaining soil was rinsed off the roots. Nodules were removed from the plant and placed in a microcentrifuge tube for further analysis.

3.10. CHARACTERIZATION OF SYMBIONT

Isolated symbiont was grown in minimal broth media supplemented with various carbohydrates to create a 10 mM carbohydrate suspension. Carbohydrate solutions were autoclaved. A saline suspension was created by inoculating 0.85% NaCl saline with colonies from a pure culture plate. The saline suspension was used to inoculate each carbohydrate medium. An AG broth medium was used as a control to verify that the saline solution contained sufficient inoculum.

3.11. DENATURING GRADIENT GEL ELECTROPHORESIS

Denaturing gradient gel electrophoresis (DGGE) was used to analyze 16S rRNA gene PCR products from nodule suspensions and colony cell suspensions. The DGGE gel contained a 30% to 60% denaturant gradient (urea and formamide).The gel ran for 14 hours at 58 milliamps. The gel was later stained in SYBR green for 30 minutes and destained in deionized water. Analysis of bands was viewed using UV light.

3.12. ENRICHMENTS

Methane and methanol enrichments were made with nitrate mineral salt (NMS) media. The methane enrichments contained a 5% methane atmosphere. The methanol enrichments contained a 50 mM methanol concentration. Cultures were incubated at 30°C and shaken. Absorbance at 600nm using a Nanodrop (Thermo Scientific, Wilmington, DA) was observed daily for eight days to measure growth. Uninoculated methane and methanol controls were maintained. Experimental samples were inoculated with 25 µL of nodule suspension.

3.13. 16S LIBRARY PRODUCTION

16S rRNA gene PCR products were inserted into a Topo vector plasmid. The Topo protocol was followed. Electroporation was used to transform competent *Escherichia coli* cells. SOC broth media was added after electroporation. SOC culture was plated on LB plates with X-gal and ampicillin. Blue/white screening was performed for successful transformations. Successful transformation colonies were prepared in Circlegrow broth media for plasmid isolation. IBI Scientific kit was used to isolate the

plasmid. Restriction enzyme EcoRI (New England BioLabs, Ipswich, MA) was used to verify the presence of an insert of the appropriate size. The verified plasmid was then prepared for sequencing using the standard sequencing primers.

3.14. TCE DEGRADATION

Mesorhizobium species isolates were grown in AG broth and AG broth supplemented with malate. Cultures were placed in sterile amber colored serum bottles with a Mini-Nert for repeated headspace removal. TCE was added to create a 34 ppb concentration. Inverted serum bottles were incubated at room temperature with a shaking speed of 80 rpm. Headspace samples were taken with a 250 µL syringe. The 250 µL samples were injected into an Agilent 7890 gas chromatograph (GC) machine equipped with a micro-electron capture detector (µ-ECD). The CombiPAL headspace syringe auto sampler (CTC Analytics, Zwingen, Switzerland) was used for samples of headspace from the standards. The temperature for the injector was set at 230°C, with purge flowing occurring after 0.75 minutes. Average column velocity was 33 cm/s using nitrogen as the carrier gas in constant flow mode. The column was a VOCOL column with dimensions of 10m x 200 µm x 1.2 µm (Supelco, Bellefonte, PA). The oven temperature was held at 40°C for 0.75 minute and then ramped at 20°C/min until 160°C was reached, which was the termination of the run. The µECD detector was set at 250°C. GC procedure modified from Limmer et. al. (15).

Calibration was obtained by using 10 mL of water placed in a 20 mL vial and dosed with known concentrations of TCE. Headspace samples (250 µL volume) of 5 different standards were sampled and were used to create a linear calibration plot using

three standard sets. The concentrations and peak areas were log-transformed to ensure equal variances for least-square regressions. Standard samples were run once during the experiment. Experimental samples were analyzed a total of 10 times on the GC. The calibration graph with equation can be seen in Figure A-8.

4. RESULTS AND DISCUSSION

4.1. NODULE DESCRIPTION

Nodules were received from Dr. Lee Newman who was at the Savannah River Site National Laboratory. All nodules were cleaned with water before mass and size were annotated; although, nodules did not have much exterior soil. The nodules are dark brown in color and spherical; the spherical shape is indicative of determinate nodules. An image of the nodules is shown in Figure 4-1. Nodules varied in mass and size; the smallest of the nodules had an approximate mass of 1.2 mg and diameter of 10 mm. The largest nodules had a mass of 90 mg and diameter of 90 mm. Although the nodules from TCE exposed plants tended to be larger both in weight and diameter, there were fewer nodules per plant. The nodules had an average mass of 3 mg and average diameter of 42 mm. A chart of the average mass and diameters of the various nodules can be seen in Figure A-2.

Figure 4-1. Picture of Nodules

4.2. NODULE STERILIZATION

Nodules were surface sterilized to ensure that soil bacteria would not interfere with future analysis of the nodules. Three different methods were tested to sterilize the nodule surface. The first two methods did not remove most of the exterior bacteria on the

nodules. The great variety of bacteria on the AG plates after incubate at 30°C for 48hours made it difficult to determine which bacterial colonies were from the nodule and which were surface contaminants. The plates from the first two sterilization methods contained cream, tan, and white colored colonies and sizes of the colonies varied from pinpoint colonies to about a diameter of 10 mm. The third sterilization method proved to be more successful at reducing the background contamination and dominant colony morphologies could be observed on the plates. An image of the initial plating from a nodule suspension is shown in Figure 4-2. The dominant colony morphology had a circular form with a smooth edge and was whitish cream in color. The colonies were opaque, with flat elevation but appeared to have a smooth and glistening surface texture. Consistent with legume symbiosis, the absence of a dominant species on culture plates may be due to the nature of the nodule. Determinant nodules from older plants may not always have viable bacteria present. Increased age in plants and storage time in the freezer can negatively influence the viability of the bacteria present in the sample and make them more difficult to culture.

Figure 4-2. Initial Plating from Nodule Suspension

4.3. ENRICHMENT ANALYSIS

Previous literature had shown that many *Crotalaria* species were nodulated with *Methylobacterium nodulans*. *M. nodulans* has the ability to grow on single carbon substrates such as methane and methanol. Methane and methanol enrichments were done to encourage the growth of the expected symbiont. In both the methane and methanol enrichments, no significant change in absorbance was observed in 8 days. Therefore, it appeared that the nodule suspension did not contain bacterial species that were capable of metabolizing single carbon compounds and may be a different symbiont than predicted.

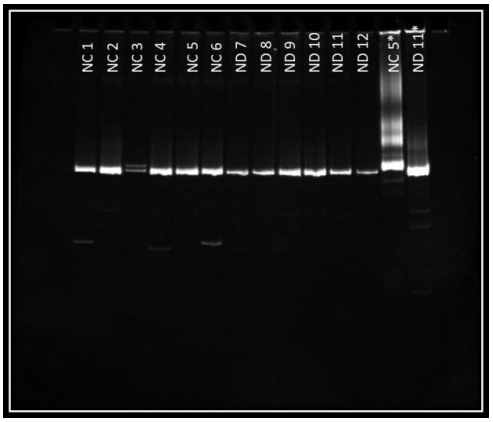
4.4. NODULE CLONE LIBRARY

A 16S rRNA gene clone library was constructed to determine whether the nodule contained a pure bacterial culture. Competent *E. coli* cells were transformed with a TOPO vector using nodule suspension clones of 16S rRNA gene from PCR amplification using 27F and 1392R primers (14). After plating transformed cells on LB plates supplemented with X-gal and ampicillin, white colonies were chosen and streaked for isolation. Individual colonies were then used to inoculate Circlegrow medium for plasmid isolation. Plasmid isolation was performed using the High Plasmid Prep Kit (IBI Scientific, Peosta, IA). Plasmids were prepared and submitted for sequencing. Results were inconclusive and did not provide meaningful data. Direct sequencing of the 16S rRNA gene was performed in place of cloned genes.

4.5. DGGE ANALYSIS

The DGGE gel was run for 14 hours at 58 mA and later observed under UV light. Shown on the gel are PCR products from DNA extracts of nodules suspensions and from colony suspensions (indicated by an asterisk following the nodule number). It is evident that one species is predominant throughout all the nodules and cell suspensions (Figure 4-3). As expected because nodules are formed as one bacterial species 'infects' the root hair of the legume and nodulation occurs. The nodule suspension should be a pure bacterial suspension as there should be only one bacterial species present in a nodule and all should be nodulated with the same species. NC 3 appears to be the only nodule that may have more than one bacterial species present indicated by the double band. This could be from inefficient sterilization of the exterior of the nodule before crushing or it could be that this was a non-functional nodule which did not contain a bacterial species and only the DNA of exterior bacterial species were amplified or nodulated with an atypical species. The DGGE gel did show that almost all of the nodule suspensions most likely had one bacterial species and direct sequencing could be performed on PCR products produced from them.

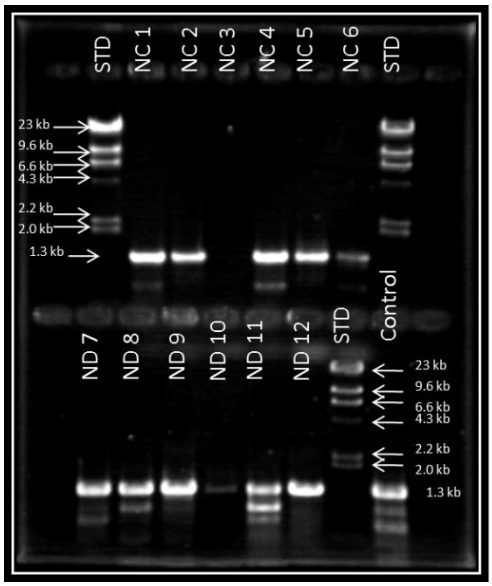
Figure 4-3. Image of DGGE Gel



4.6. IDENTIFICATION OF SYMBIONT

PCR products of nodule suspensions using 16S rRNA gene primers, 27F and 1392R (14), run on a 0.8% agarose gel gave the following results shown in Figure 4-4. All nodule suspensions except NC 3 showed amplification of the 16S rRNA gene in Figure 4-4. In subsequent 16S rRNA PCR amplification of NC 3, PCR was successful and did produce product, which was verified on 0.8% agarose gel. The control sample in figure 4-4 was *Bradyrhizobium japonicum* chromosomal DNA.

Figure 4-4. Image of 16S rRNA Gene Amplification of Nodule Suspensions



Mesorhizobium species is the predominant genera in most of the nodules. The three samples not showing *Mesorhizobium* species as their predominant genera may have non-functional nodules or PCR may have amplified DNA from the nodule surface that was not damaged during sterilization. There may also have been sequencing error

introduced during PCR amplification. A BLAST search of the 16S rRNA sequence results gave the results shown in Table 4-1 as being the most predominant species.

Representative BLAST searches (2) for NC 2 and NC 3 16S rRNA sequences (approximately 900 base pairs), using 27F primers (14), show the top 10 genera sequences that best match in Figures A-3 and A-4, respectively. NC 2 was chosen as a representative for the *Mesorhizobium* species and BLAST results for the other *Mesorhizobium* species were similar to the NC 2 BLAST results. A non-Mesorhizobium species, represented by NC 3, was also chosen to show the dissimilarities between the *Mesorhizobium* sp. and the non-*Mesorhizobium* sp. BLAST results.

Table 4-1. Nodule Sequence Results Based on BLAST Search

Nodule	Genus
NC 1	*Rhizobium sp.*
NC 2	*Mesorhizobium sp.*
NC 3	*Pseudomonas sp.*
NC 4	*Mesorhizobium sp.*
ND 7	*Mesorhizobium sp.*
ND 8	*Mesorhizobium sp.*
ND 9	*Mesorhizobium sp.*
ND 11	*Mesorhizobium sp.*

4.7. CREATION OF *Mesorhizobium* sp. SPECIFIC PRIMERS

To ensure that colonies cultured from nodule suspensions were from the infecting bacterium, primers were developed to their suspected 16S rRNA gene sequences. After BLAST results of the sequences indicated that most of the nodules contained a *Mesorhizobium* sp., a ClustalW alignment was performed on the sequences (37). The ClustalW alignment was used to compare sequences that were *Mesorhizobium* sp. and those that were not *Mesorhizobium* sp.. Locations in the sequence which aligned for the *Mesorhizobium* sp. but not for the non-*Mesorhizobium* sp. were chosen as possible primer locations. In the initial colony screenings, *Paenibacillus* sp. was a common contaminant and had similar colony morphology to the *Mesorhizobium* sp. The symbiont may be less viable making this step necessary.

Therefore, it was also included in the ClustalW alignments to ensure that it would not be amplified along with the *Mesorhizobium* sp. cultures. Two locations were chosen, 68 nucleotides in for the forward primer and 917 nucleotides in for the reverse primer. These primers were named alr1f (forward primer) and alr1r (reverse primer) and used on cell suspensions from plated nodule suspensions. The ClustalW alignment for determining the forward primer and reverse primer are shown in Figures A-5 and A-6, respectively. PCR products using these primers during amplification verified on a 0.8% agarose gel indicating that the colony was the bacteria of interest. Absence of product indicated that the colony was not the organism of interest and culturing from nodule suspensions needed to continue.

4.8. ISOLATION OF SYMBIONT

Bacterial isolates were needed for identification and characterization. Isolates were also used for inoculating germinated *C. spectabilis* seedlings and for TCE degradation analysis. To obtain symbiont isolates, nodule suspensions were plated on typical rhizobial media: AG, YMA, and YXA plates. Colony morphology of *Mesorhizobium* sp. on YMA and YXA plates showed a circular form with a smooth edge and off white in color. The colonies were opaque, with raised elevation and appeared to have a slimy surface texture. The colony morphology of *Mesorhizobium* sp. on AG agar plates had a circular form with a smooth edge and was whitish cream in color. The colonies were opaque, with flat elevation but appeared to have a smooth and glistening surface texture. After growth, isolated colonies on AG plates were used to create a cell suspension. The cell suspension was used as a DNA template for PCR using alr primers. An isolate from NC 5 and ND 11 were chosen as representative isolates of *Mesorhizobium* sp. and used for further analysis. A suspension of the NC 5 *Mesorhizobium* sp. isolate was used as an inoculant for germinated seedlings.

4.9. CHARACTERIZATION OF SYMBIONT

Carbohydrate characterization of the symbiont was performed to better understand the carbohydrates the symbiont was capable of using. Isolated *Mesorhizobium* sp. culture from NC 5 was used to make a turbid saline (0.85 % NaCl) suspension which had an approximate absorbance of 0.500 at 600 nm. The saline suspension was used to inoculate 14 various 10 mM carbohydrate media and an AG broth control. The 14 carbohydrates tested were: arabinose, fructose, galactose, glucose, gluconate, glycerol, inositol, malate, mannitol, mannose, sorbitol, succinate, sucrose, and xylose. The AG

broth medium was used to verify the saline suspension had sufficient inoculum. Turbid growth in the broth cultures indicated utilization of the carbohydrate by the bacterium. All turbid broth cultures were compared to a negative control (a minimal media broth that was not inoculated). The NC 5 *Mesorhizobium* sp. isolate was able to utilize all 14 carbohydrates as shown in Figure A-7.

4.10. NODULATION OF *C. spectabilis* by *Mesorhizobium* sp. ISOLATE

Three day old germinated seedlings were inoculated with NC 5 *Mesorhizobium* sp. isolate and planted in sterile soil. The seedlings were kept covered until they were approximately 15 cm in height and replanted in larger containers. At 51 days, plants were removed from the soil, excess soil was gently removed from the roots, and nodules were harvested. Only one plant appeared to have nodules present, all other plants did not have an extensive root system like the plant that contained the nodules. The nodules were removed from the plant and placed in a centrifuge tube for further analysis.

4.11. TCE METABOLISM

Both uninoculated media and media inoculated with *Mesorhizobium* sp. cultures were exposed to TCE at a concentration of 33.8 ppb. The first set of samples was exposed to TCE after 72 hours of incubation. Figure A-9 shows ppb concentration of sample vs. time since the culture was exposed to TCE for the first set of samples. There appears to be no TCE metabolism occurring. TCE degradation may only be possible when the bacteria are in association with the plant. The symbiotic relationship may influence TCE degradation. The second set of samples was exposed to TCE after 24 hours of incubation.

There appeared to be TCE metabolism in all samples, including the controls; this is shown in Figure A-10.

After TCE analysis on the GC was complete, cultures were plated on AG agar plates and incubated at 30°C for 36 hours. Visual inspection of the plates showed that control samples had contaminants present. All other samples had contaminants present as well as what appeared to be the bacterial culture of interest. The decrease in TCE concentration could not be attributed to the bacterial symbiont. There was contamination present on all plates, so even if TCE degradation was occurring because of bacterial metabolism, the degradation could not be directly linked to the bacterial symbiont. Since all samples were contaminated and had a decrease in TCE concentration, there was no way to distinguish if TCE metabolism was occurring or if there was a leak in the seal.

5. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

5.1. CONCLUSIONS

Crotalaria spectabilis is shown to be nodulated by a *Mesorhizobium* species. This symbiotic relationship has been not shown before in previous literature and appears to be a novel symbiosis. Preliminary research results demonstrate Koch's postulates that the *Mesorhizobium* sp. isolated from *C. spectabilis* was able to nodulate *C. spectabilis* germinated seedlings. Further analysis of this will need to be performed to verify that Koch's postulates were indeed fulfilled. TCE metabolism by the symbionts appears to be possible but further analysis will need to be completed before declaring that the symbionts are capable of TCE metabolism.

5.2. RECOMMENDATIONS FOR FUTURE WORK

Further isolation of *Mesorhizobium* sp. from nodule suspensions would provide a larger sample size to further analyze TCE degradation and future nodulation of *C. spectabilis*. A more complete investigation in TCE degradation should be done to determine if the symbiont is able to metabolize TCE. A method in which to monitor TCE degradation while minimizing the risks of contamination will ensure accurately if TCE is being degraded by the symbiont. Completing Koch's postulates would bring the symbiosis full circle; to do this sequencing of the symbionts in the new nodules would need to be performed. Identification of the gene responsible for TCE degradation would be ideal if TCE metablism is occurring by the symbiont. Analysis of TCE metabolism of *C. spectabilis* nodulated with *Mesorhizobium* sp. isolated from original nodules would complete this project.

APPENDIX A

TABLES AND GRAPHS

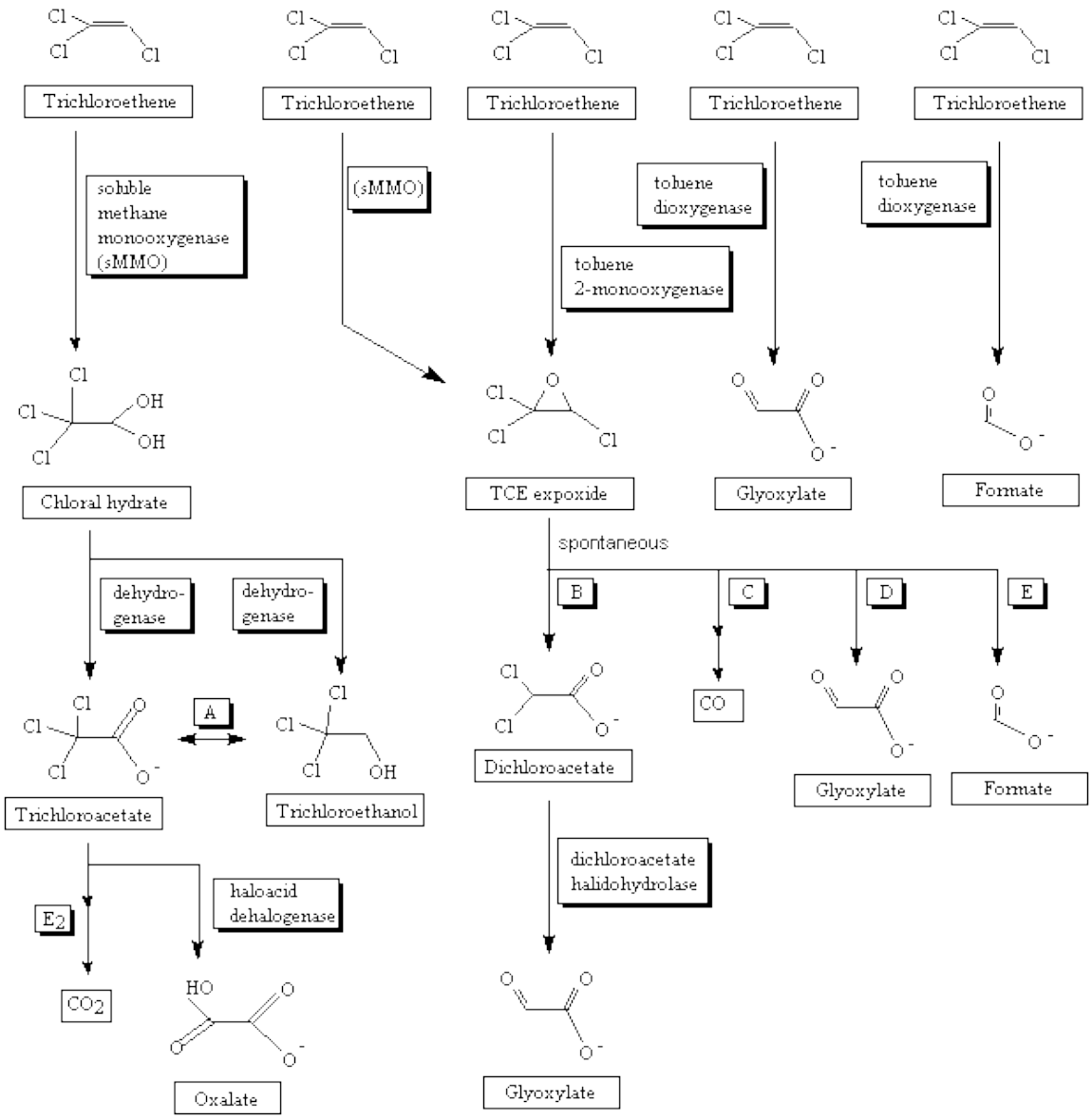


Figure A-1. Metabolic Pathways for TCE Degradation (23)

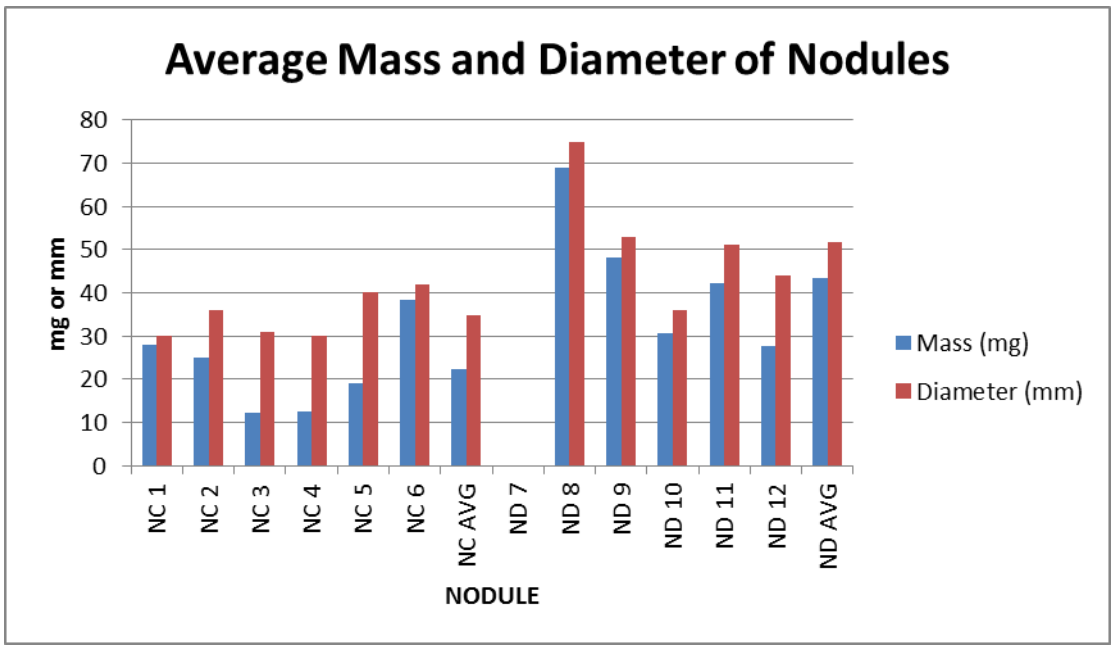


Figure A-2. Chart of Nodule Mass and Diameter

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU130444.1	Mesorhizobium sp. CCBAU 11231 16S ribosomal RNA gene, partial sequence	1807	1807	100%	0	99%
FJ827044.1	Mesorhizobium sp. WSM3872 16S ribosomal RNA gene, partial sequence	1805	1805	99%	0	99%
FJ025126.1	Mesorhizobium amorphae strain SEMIA 6392 16S ribosomal RNA gene, partial sequence	1805	1805	99%	0	99%
EU881339.1	Uncultured bacterium clone KMS200711-026 16S ribosomal RNA gene, partial sequence	1805	1805	99%	0	99%
AY528710.1	Mesorhizobium sp. phym.2a 16S ribosomal RNA gene, partial sequence	1805	1805	99%	0	99%
DQ100060.1	Mesorhizobium plurifarium strain CCBAU 51471 16S ribosomal RNA gene, partial sequence	1801	1801	100%	0	99%
AB636289.1	Mesorhizobium sp. NL123 gene for 16S rRNA, partial sequence	1799	1799	99%	0	99%
DQ859040.1	Mesorhizobium plurifarium strain ORS 3369 16S ribosomal RNA gene, partial sequence	1799	1799	99%	0	99%
DQ859039.1	Mesorhizobium plurifarium strain ORS 3365 16S ribosomal RNA gene, partial sequence	1799	1799	99%	0	99%
DQ859038.1	Mesorhizobium plurifarium strain ORS 3359 16S ribosomal RNA gene, partial sequence	1799	1799	99%	0	99%

Figure A-3. Representative BLAST search of NC 2 using 16S rRNA forward primer (27F) (2)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JN055435.1	Pseudomonas sp. N26 16S ribosomal RNA gene, partial sequence	1757	1757	99%	0	99%
HQ849997.1	Pseudomonas sp. 4AG6 16S ribosomal RNA gene, partial sequence	1753	1753	99%	0	99%
FM202487.1	Pseudomonas tolaasii partial 16S rRNA gene, strain IEXb	1753	1753	99%	0	99%
AY236959.1	Pseudomonas sp. PCL1171 16S ribosomal RNA gene, partial sequence	1753	1753	99%	0	99%
AY599720.1	Pseudomonas sp. TB2-3-I 16S ribosomal RNA gene, partial sequence	1753	1753	99%	0	99%
GU569131.1	Uncultured Pseudomonas sp. clone PBXB2 16S ribosomal RNA gene, partial sequence	1751	1751	99%	0	99%
GU563771.1	Uncultured Pseudomonas sp. clone Bmc70 16S ribosomal RNA gene, partial sequence	1751	1751	99%	0	99%
EU537676.1	Uncultured bacterium clone nbt75g04 16S ribosomal RNA gene, partial sequence	1751	1751	99%	0	99%
FJ483527.1	Pseudomonas sp. ICMP 11284 16S ribosomal RNA gene, partial sequence	1748	1748	99%	0	99%
FJ483526.1	Pseudomonas sp. ICMP 11283 16S ribosomal RNA gene, partial sequence	1748	1748	99%	0	99%

Figure A-4. Representative BLAST search of NC 3 using 16S rRNA forward primer (27F) (2)

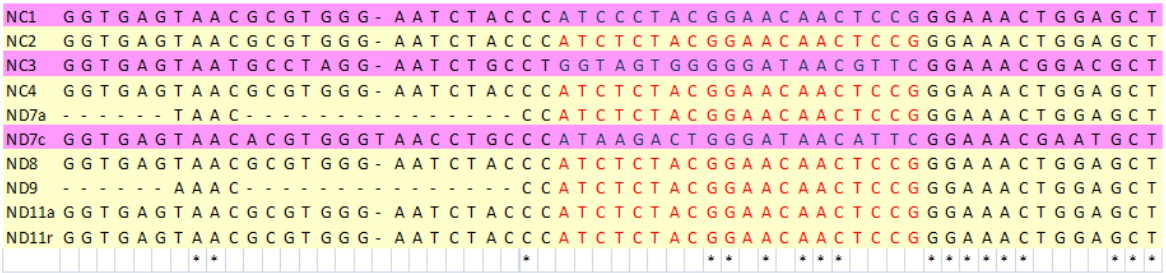


Figure A-5. ClustalW sequence alignment showing forward primer (68 nucleotides into sequence) in red (37)

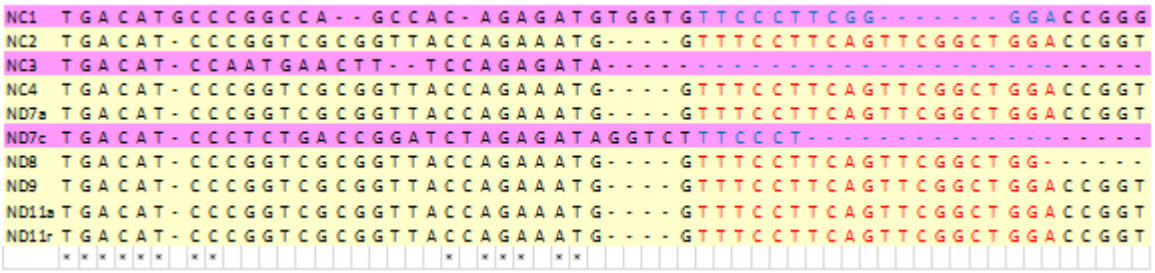


Figure A-6. ClustalW sequence alignment showing complement base pair to Reverse Primer (917 nucleotides into sequence) in red (37)

	Arabinose	Fructose	Galactose	Glucose	Gluconate	Glycerol	Inositol	Malate	Mannitol	Mannose	Sorbitol	Succinate	Sucrose	Xylose	AG control
Mesorhizobium isolate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Figure A-7. Carbohydrate Utilization Chart

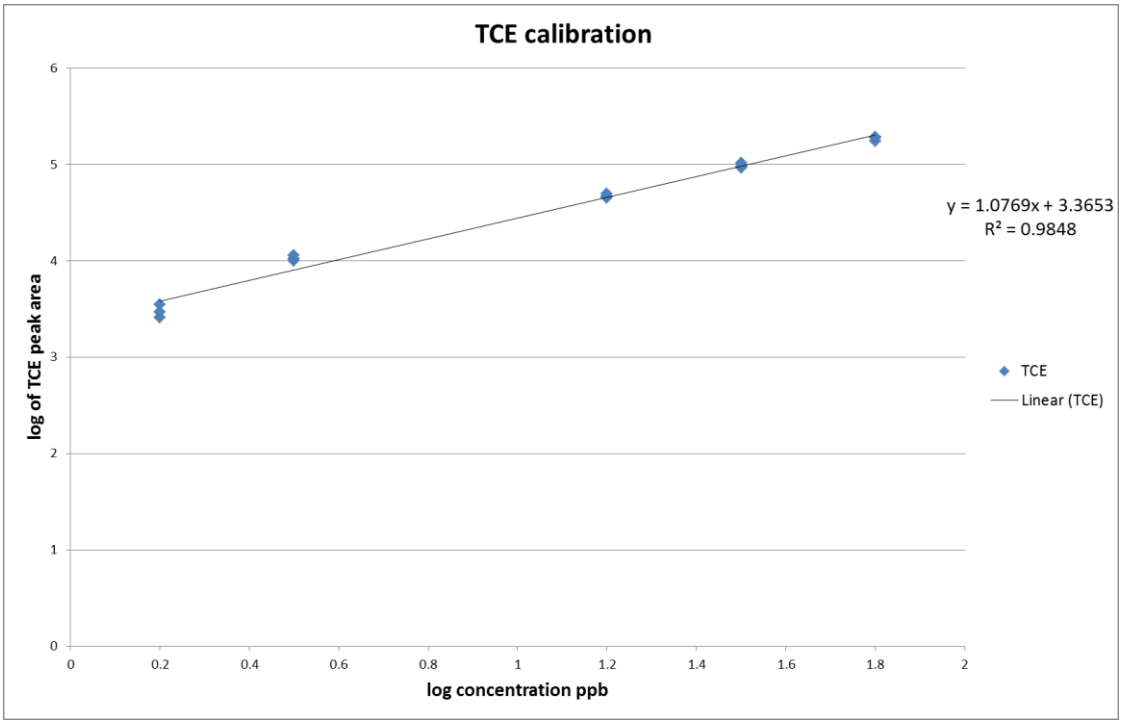


Figure A-8. TCE Calibration Graph

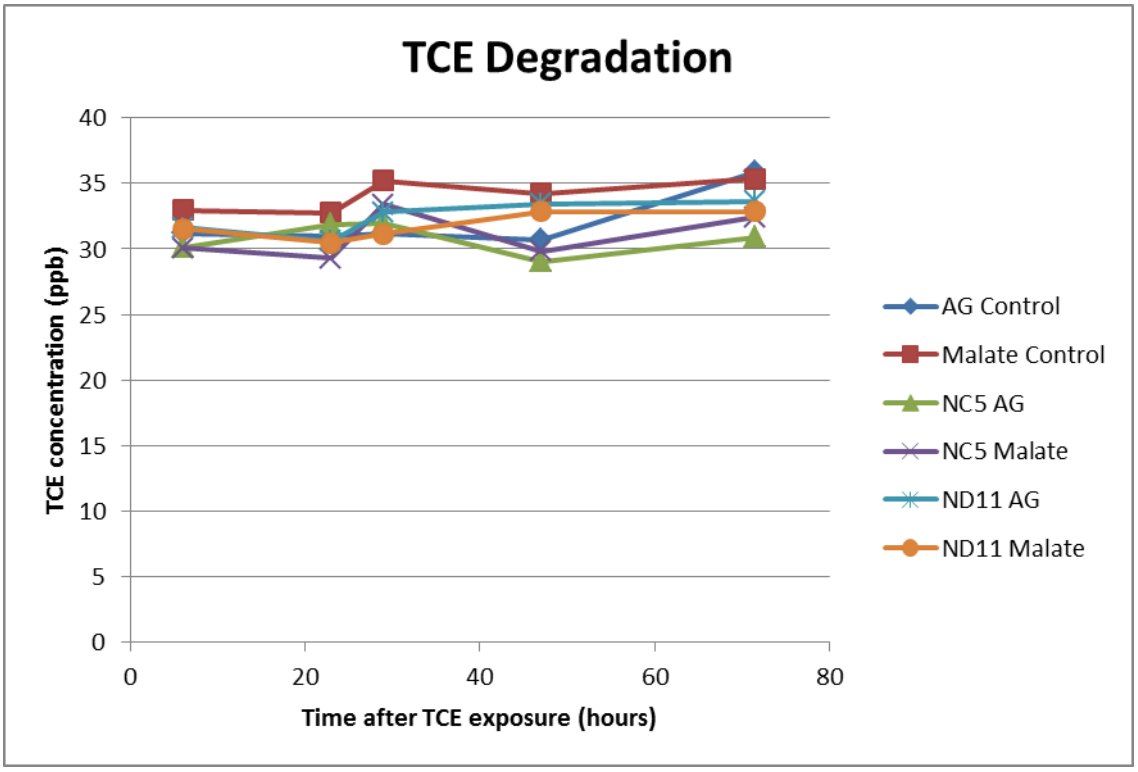


Figure A-9. TCE degradation with 72 hour culture

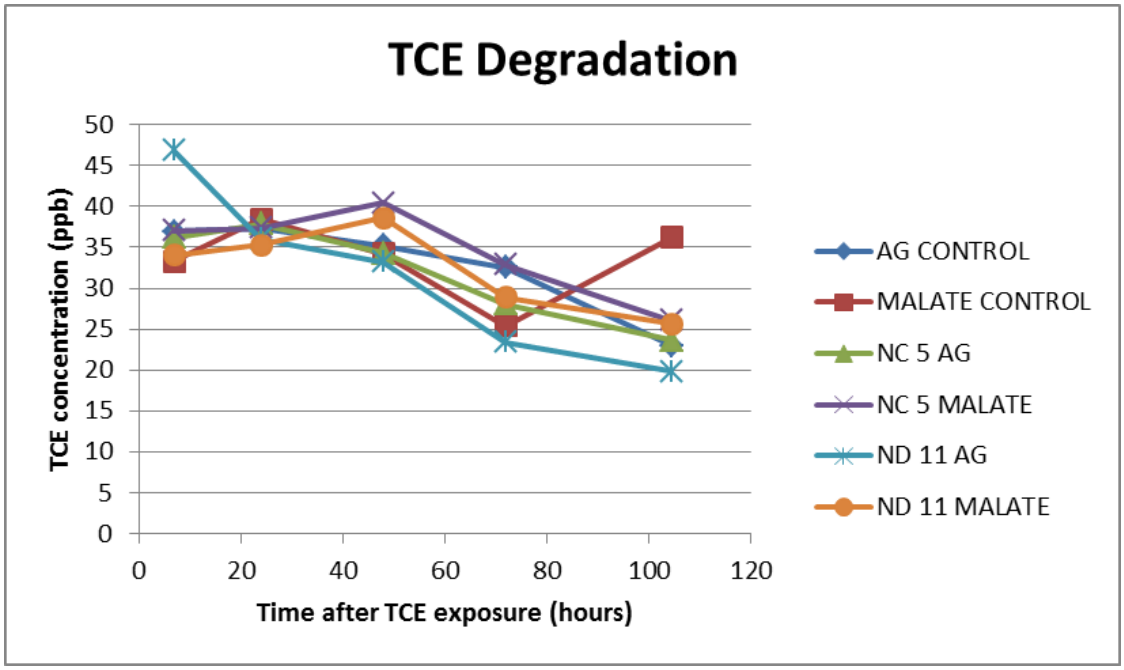


Figure A-10. TCE degradation with 24 hour culture

APPENDIX B

STANDARD OPERATING PROCEDURES

Standard Procedure for Polymerase Chain Reaction (PCR) Amplification

Materials and Instrumentation:

200 µL thin walled PCR tubes
10 µL pipette man
2 µL pipette man
1.0-200.0 µL pipette tips
0.1-10 µL pipette tips

Preparation of PCR samples:

Add to each 200 µL PCR tube:

22.5 µL Pfx Supermix
1.0 µL forward primer
1.0 µL reverse primer
1.0 µL DNA template

Polymerase Chain Reaction Programs

Pfx Supermix Program

Step 1: 95°C for 5 minutes
Step 2: 95°C for 15 seconds
Step 3: 56°C for 30 seconds
Step 4: 68°C for 1.5 minutes
Repeat steps 2-4 for 35 cycles.
Step 5: 68°C for 5 minutes
Step 6: hold at 10°C

PCR products stored at 4°C.

Pfx ALR Program (for primers targeting *Mesorhizobium* genera)

Step 1: 95°C for 5 minutes
Step 2: 95°C for 15 seconds
Step 3: 53°C for 30 seconds
Step 4: 68°C for 1.5 minutes
Repeat steps 2-4 for 30 cycles.
Step 5: 68°C for 5 minutes
Step 6: hold at 4°C

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VITA

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