COMPOSITION AND DIVERSITY OF *MEDICAGO TRUNCATULA* ROOT BACTERIAL ENDOPHYTE POPULATIONS RESULTING FROM GROWTH IN DIFFERENT OKLAHOMA SOILS

By

JAMES NEIL ENIS

Associate of Science in Agronomy
Eastern Oklahoma State College
Wilburton, Oklahoma
1999

Bachelor of Science in Plant & Soil Science
Oklahoma State University
Stillwater, Oklahoma
2001

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Thesis Approved:

Michael P. Anderson
Thesis Advisor

Shiping Deng

Robert V. Miller

A. Gordon Emslie
Dean of the Graduate College

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PREFACE

The endophytic bacterial populations of surface disinfected *M. truncatula* root tissues collected from plants grown in a commercial growing medium and six diverse soils from across the state of Oklahoma were evaluated for diversity and differences in composition resulting from plant growth in dissimilar soils by cloning and sequencing of near full-length 16S rDNA and temperature gradient gel electrophoresis (TGGE) of 16S rDNA fragments.

Cloning and sequencing of 16S rDNA revealed 36 genera of bacteria encompassing five phyla as putative *M. truncatula* root endophytes. Large differences in diversity were observed between endophyte populations originating from plants grown in different soils, with differences becoming increasingly pronounced at lower taxonomic levels. At the genus level, two acidic soils with a forest background and a commercial growing medium containing 15-25% ground pine bark yielded the highest endophyte diversity, while moderate diversity was observed in plants grown in managed agricultural soils. Root bacterial endophyte diversity was lowest in plants grown in soil collected from an undisturbed native tallgrass prairie.

The TGGE technique failed to adequately resolve the complex endophytic bacterial 16S rDNA fragments with respect to the level of diversity revealed by the cloning approach and 16S rDNA bands on the silver-stained TGGE gel were unable to be subsequently sequenced or cloned into plasmid vectors for identification.

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INTRODUCTION

Defining the Term "Endophyte"

The first observations of bacteria living within the tissues of a non-symptomatic plant were made by Pasteur in the 1870s (Hallmann et al., 1997a). In 1926, Perotti published a report detailing internal colonization of plants by bacteria that seemingly caused no harm. However, little attention was paid to these early reports, and such relationships between plants and internally-colonizing bacteria remained largely unstudied until 1951, when Hollis "rediscovered" bacteria within the tissues of healthy potatoes.

The term "endophyte" has a history dating back three decades and has seen a great deal of variation in meaning. There has been, and remains yet, disagreement as to the precise definition of the term. Perhaps the best means by which to understand the general meaning is to examine the various definitions that have been applied in published literature over the years.

In a 1996 commentary, Chanway reflects that from conception, the term "endophyte" was used almost exclusively to describe fungi that invaded the stems and leaves of plants without causing disease, beginning with a publication by Carrol (1988). However, the term was later applied to mycorrhizal fungi infecting roots by O'Dell and Trappe (1992).

In the early 1990s interest in internal bacterial colonizers of plants saw a rebirth, several published reports appeared in the literature, and the term "endophyte" began to be applied to bacteria as well as fungi. In 1992, Kado defined endophytic bacteria as those "bacteria that reside within living plant tissues without doing substantive harm or gaining benefit other than securing residency." Quispel (1992) defined bacterial endophytes as those establishing a symbiotic relationship with the plant, in which the host plant gained an ecological benefit such as growth promotion or enhanced stress tolerance. In the same year, Kloepper *et al.* (1992) considered any bacterium found within internal plant tissues to be an endophyte.

In 1995, Wilson proposed that the term "endophyte" be defined as meaning "fungi or bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease."

While not addressing the Kloepper et al. or Wilson definitions, Hallman et al. (1997a) expressed disagreement with Kado and Quispel's definitions because the former excluded endosymbionts and the latter excluded bacteria having no discernable effect on the host. Thus, Hallmann and associates defined endophytes as those bacteria that "can be isolated from surface-disinfested plant tissue or extracted from inside the plant, and [that do not] visibly harm the plant," a meaning strongly reminiscent of Wilson's but not microorganism-specific and expanded to include surface disinfection as part of the definition. In the same year, James and Olivares (1997) found themselves more in agreement with the Kloepper et al. definition and considered all bacteria found within internal plant tissues, including pathogens, to be endophytes.

Publications by Azevedo (1998) and Azevedo et al. (2000) were reflective of the Hallmann et al. definition and considered endophytic microorganisms to be "those that inhabit the interior of plants, especially leaves, branches, and stems, showing no apparent harm to the host." Also in 2000, Bacorn et al. gave a widely accepted definition of endophytes as "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects."

In a 2002 review article, Lodewyckx et al. effectively blended the Hallmann et al. definition with that of James and Olivares and defined endophytic bacteria as those that reside within the plant, "some of which are believed to impart a beneficial effect, whereas others are regarded to have a neutral or detrimental effect" on the host.

Additionally, Lodewyckx et al. (2002) pointed out that with respect to root endophytes, the distinction between a rhizoplane bacterium and an endophyte is often defined by the surface disinfection treatment applied to the tissue. In an effort to address this problem, Reinhold-Hurek and Hurek (1998) published criteria for recognizing "true" bacterial endophytes which required not only isolation from surface disinfected tissue, validation by microscopic observation of the organism inside the tissue, and capability to reinfect disinfected seedlings. Findings of potential endophytes not validated by the above manner are to be labeled as "putative." However, these requirements for isolation, culture, and microscopic validation are very often not fulfilled in many recent endophyte studies, primarily due to the emergence of molecular methods for analysis of bacterial communities such as 16S rDNA profiling which typically identify far more putative endophytes than do culture-based methods and do not require isolation and growth of bacteria in pure culture (Rosenblueth and Martinez-Romero, 2006).

The most recent published review of endophytic bacterial knowledge was made by Ryan et al. (2008). In this review, definition of the term "endophyte" seems to have come full-circle, being quite similar to the Hallmann et al. (1997a) definition, stating that endophytic bacteria are "those bacteria that colonize the internal tissue of the plant showing no external sign of infection or negative effect on their host."

It should be noted that while the symptomless nature of endophyte infection has created a trend to focus only on symbiotic or mutualistic host-endophyte interactions, the broad diversity of bacteria has included those that are aggressive saprophytes as well as latent and opportunistic plant pathogens (Strobel et al., 2004).

For the purposes of this study, we have considered the most recent and inclusive definition presented by Ryan et al. 2008 as the current definition of the term "endophyte".

Biodiversity of Bacterial Endophytes and Their Plant Hosts

Far from being a rare or even occasional occurrence, endophytic bacteria have been isolated from virtually all major plant tissues, including roots, stems, leaves, fruits, tubers, seeds, ovules, and legume nodules (Chanway, 1996; Hallmann et al., 1997a; Ryan et al., 2008; Sturz et al., 1997). Plants identified as harboring endophytic bacteria span a tremendous range of diversity and include both monocotyledonous and dicotyledonous species including both herbaceous and woody plants, including many of agronomic importance (Lodewyckx et al., 2002). In fact, after years spent bioprospecting for novel endophytes and useful natural products derived from them, Strobel and Daisy (2003) wrote that of the estimated 300,000 higher plant species known to exist on Earth, it could be safely assumed that every individual plant of the billions in existence on the planet is home to one or more endophytes.

Diversity among endophytic bacterial species is enormous as well. A review of endophyte research by Hallmann et al. (1997a), found over 129 identified endophytic bacterial species representing over 54 genera. The list has grown substantially larger since, encompassing hundreds of species, and includes both Gram-positive and Gram-negative bacteria (Lodewyckx et al., 2002). As interest in endophyte biodiversity has since grown considerably and several findings of new endophytic species are reported each year (Rosenblueth and Martinez-Romero, 2006), no review since has succeeded with respect to complete tabulation of all reported endophytic bacterial species. However, lists that are fairly extensive have been published in reviews of the subject made by Lodewyckx et al. (2002), Rosenblueth and Martinez-Romero (2006), and Berg and Hallmann (2006).

While endophytic bacterial species diversity is enormous, trends have been observed with respect to certain bacterial types such as *Pseudomonas, Bacillus, Agrobacterium, Azospirillum,* and *Enterobacter* among those most commonly identified and tending to predominate in endophytic populations (Chanway, 1996; Hallmann et al., 1997a; Kobayashi and Palumbo, 2000; Van Peer et al., 1990; Gardner et al., 1982). Species observed to occur most frequently within a population are generally termed "dominant" while species with lesser numbers are categorized as "rare" (Lodewyckx et al., 2002).

Additionally, while fungi and eubacteria are thus far the only forms of endophytic microorganisms reported, given the diversity of plant hosts and microbial colonizers observed thus far, is likely that other microorganisms may be identified in the future as

endophytic, possibly including organisms such as mycoplasmas, rickettsias, and members of the archaea (Strobel et al., 2004).

Evaluation of Endophytic Bacterial Populations

The inclusion of a reference to surface disinfection by Hallmann et al. (1997a) in the very definition of "endophyte" reflects the importance of experimental methodology to the study of this diverse population of microorganisms. A procedure must be devised for the recovery of the maximum diversity of bacteria from the internal tissues of the plant without contamination of the recovered population with rhizoplane organisms. Unfortunately, a protocol that accomplishes each of these goals with 100% effectiveness has yet to be devised (Hallmann et al., 1997a; Lodewyckx et al., 2002). Bacterial endospores present on the external tissue surfaces are highly resistant to chemical sterilization, rhizoplane and endophytic bacteria can be exchanged via wounds induced during sampling, and chemical disinfectants can penetrate into the interior of plant tissues, resulting in a loss of endophytic bacteria.

The most common method employed for surface sterilization is submersion of a collected tissue sample in a disinfection solution followed by several washes with sterile water or buffer solutions (Hallmann et al., 1997a; Lodewyckx et al., 2002). While no standardized surface-disinfection solution has emerged over the years, the most common disinfectants employed by far have been solutions containing ethanol and/or sodium hypochlorite (Lodewyckx et al., 2002). Other disinfecting agents do occasionally appear in the literature, including hydrogen peroxide (McInroy and Kloepper, 1994; Misaghi and Donndelinger, 1990) and mercuric chloride (Gagne et al., 1987; Hollis, 1951; Sriskandarajah et al., 1993). Additionally, when tissues are large and durable, externally-

applied flame has been utilized for durable tissues with large diameters such as sugar beets (Jacobs et al., 1985) and sugarcane (Dong et al., 1994). A further step involving aseptic excision of internal tissue following surface disinfection has been performed with large-diameter samples such as sugar beets (Jacobs et al., 1985), corn stems (Fisher et al., 1992), and grapevines (Bell et al., 1995).

If endophytic bacteria are to be isolated and grown in pure culture, some method must be used to retrieve bacterial inoculum from within the surface-disinfected tissue. Many methods for this have been described in literature including dilution plating of macerated tissue (Garbeva et al., 2001; Reiter and Sessitsch, 2006), vacuum extraction of sap (Bell et al., 1995; Gardner et al., 1982), pressure extraction of sap using a Scholander pressure bomb (Hallmann et al., 1997b), and extraction of sap by centrifugation (Dong et al., 1994).

However, a study of Crocus *(Crocus albiflorus*) bacterial endophytes by Reiter and Sessitsch (2006) in which the bacterial population was evaluated by culturing and dilution plating of macerated tissue as well as by whole-community fingerprinting and sequencing of 16S rDNA fragments, the culture collection differed significantly in scope and diversity from the 16S rDNA clonal library. Only three bacterial divisions representing 17 phylotypes were isolated by culturing, whereas six divisions representing 38 phylotypes were identified in the 16S rDNA clonal library, confirming a long-held suspicion that culture-based methods are capable of detecting only a subset of the total endophyte biodiversity (Ryan et al., 2008).

Due to the limited detection capability of culture-based methods, molecular approaches to endophyte population assessment have became increasingly prevalent in

more recent work. These molecular approaches are not culture-dependent and utilize total DNA extractions containing endophytic bacterial DNA made from surface disinfected plant tissues. Typically, bacterial 16S rDNA sequences are amplified from the total DNA extract by PCR (polymerase chain reaction) using "universal" bacterial primers and dissimilar amplification products separated by a number of molecular techniques for identification. The 16S rRNA gene is typically preferred as it has been well –documented for suitability in species identification and determination of phylogenetic and evolutionary relationships between microorganisms (Weisburg et al., 1991). Additionally, a tremendous volume of known 16S rDNA sequences have been stored electronically and are available for comparison in publicly-accessible databases. Techniques commonly encountered in more recent studies include gene cloning and sequencing, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (TRFLP) analysis (Ryan et al., 2008; Lodewyckx et al., 2002).

Another advantage of PCR-based approaches to endophytic population assessment is the ability to specifically amplify any gene of interest for assessment of community members possessing important traits such as *nif* genes for nitrogen fixation, or genes involved in the degradation of environmental pollutants (Lodewyckx et al., 2002).

Origin of Bacterial Endophytes

Because endophytes appear to colonize all plant organs as well as seed and legume nodules (Hallmann et al., 1997; Sturz et al., 1997; Benhizia et al., 2004; Rosenblueth and Martinez-Romero, 2006), planting and propagation materials including

seeds, vegetative cuttings, and grafting scions and rootstocks are also likely sources of endophytic bacteria (Hallmann et al., 1997; Rosenblueth and Martinez-Romero, 2006). Additionally, above-ground plant parts may be colonized by organisms from the phylloplane (Beattie and Lindow, 1995). Ashbolt and Inkerman (1990) and Kluepfel (1993) described transmission of endophytes via insect vectors. In 2004, Miyamoto et al. found a group of endophytic *Clostridia* in grass that was not present in the surrounding soil.

However, soil appears to be the primary reservoir for endophytic bacteria. Many comparative studies of rhizosphere and endophytic bacterial populations have found that endophytic bacteria represent a subset of the soil bacterial population (Mahaffee and Kloepper, 1997; Hallmann et al., 1997a; Sturz, 1995; Berg et al., 2005; Germida et al., 1998; Mavingui et al., 1992; Rosenblueth and Martinez-Romero, 2004). Mahaffee and Kloepper (1997) found that while the initial endophytic population closely resembles the rhizosphere community, rapid differentiation occurs following colonization resulting in a distinctly different community with fewer genera than the rhizosphere population. This differentiation is thought to occur because the internal plant tissue represents a complex microecosystem with environmental conditions distinctly different from the rhizosphere (Hallmann et al., 1997a; Lodewyckx et al., 2002). Additionally, there is some evidence suggesting adaptation by former rhizosphere bacteria following colonization and establishment as plant endophytes. In a study by van Peer et al. (1990), *in planta* and *ex planta* populations of *Pseudomonas* could be differentiated by biochemical differences.

Certainly, it could be advantageous for a bacterium to possess the capacity for endophytic colonization. The interior of a plant represents a more uniform and protected

environment than the rhizosphere with reduced exposure to harsh conditions such as temperature, ultraviolet radiation, and variations in osmotic potentials, as well as the danger of predation by soil protozoa (Lodewyckx et al., 2002).

Some endophytic species appear to be more aggressive colonizers than others. *Pantoea* sp. out-competed *Ochrobactrum* sp. in rice (Verma et al., 2004). Some strains of *Rhizobium etli* were observed to be more aggressive than others during colonization of maize (Rosenblueth and Martinez-Romero, 2004).

Additionally, differences in host genotype, age, tissue, season of isolation, and soil type and fertility also appear to influence measured endophyte diversity (Kuklinsky-Sobral et al., 2004). Conn and Franco (2004) found that soil type had a large influence on *Actinobacteria* diversity in wheat when plants were grown in three different soils. Herbicide applications (Kuklinsky-Sobral et al., 2005) and introduction of genetically modified endophytic bacterial strains (Andreote et al., 2004) also resulted in altered compositions of endophytic bacterial communities. Diminished colonization of sugarcane by *Gluconacetobacter diazotrophicus* was observed when plants were grow with high nitrogen fertilization, as compared to the population in plants grown under N-deficient conditions (Fuentes-Ramirez et al., 1999). Tan et al. (2003) observed rapid changes in the nitrogen-fixing endophyte population in rice within 15 days following nitrogen fertilization.

Plant Colonization by Bacterial Endophytes

The *Rhizobia* have long been known to possess the capacity for direct penetration of root hairs. However, this method of entry approach now appears to be utilized by other endophytes as well. Studies have shown penetration of seedlings grown without

disturbance on liquid media or water agar by endophytic bacteria prior to root emergence and some endophytic species in the *Azoarcus, Azospirillum,* and *Pseudomonas* genera have the ability to produce the necessary cellulytic and/or pectinolytic enzymes, which are synthesized during penetration of plant cell walls but not after (Lodewyckx et al., 2002).

Endophytic bacteria are also able to obtain access to internal plant tissues via any natural or artificially-induced opening in the plant's epidermal layer. Huang (1986) found that bacteria could penetrate the epidermal layer via natural openings including stomata, hydathodes, nectarthodes, and lenticels. Evidence was also presented for entry via wounds including broken trichomes, crevices in the epidermal layer resulting from lateral root emergence, and the junctions of root hairs with epidermal cells. A 1991 study by Sharrock et al., found that bacterial endophytes in fruit may have gained entry via flowers. Artificially induced wounds also allow entry of bacteria, with wounded roots exhibiting increased colonization as compared to intact roots (Gagne et al., 1987).

James et al. (2002) used a GUS (β-glucuronidase) – marked strain of *Herbaspirillum seropedicae* to visualize the colonization of rice seedlings and followed entry via epidermal cracks at points of lateral root emergence. Once within the seedling, *H. seropedicae* continued to colonize the intercellular spaces in of the aerenchyma and cortex, eventually penetrating the stele and gaining entry to the vascular tissues, resulting in colonization of the xylem vessels in the stem and leaves. Systemic migration of endophytes is quite rapid, requiring less than one day to migrate from exposed roots to aerial parts of plants (Rosenblueth and Martinez-Romero, 2006) and thought to occur via

capillary transport and/or active migration of bacteria via the conducting elements or the apoplast (Hallmann et al., 1997; Lodewyckx et al., 2002).

However, internal colonization does not always progress in this manner. Other studies have observed endophytic bacteria remaining localized in specific plant tissues such as the root cortex (Hallmann et al., 1997; Lodewyckx et al., 2002), while others such as *Rhizobium* sp. and *Alcaligenes faecalis* have been observed within host cells, enveloped by specialized structures (You et al., 1983 and 1991).

BENEFICIAL ASPECTS OF ENDOPHYTIC ASSOCIATIONS

Plant Growth Promotion

Because the endophytic bacterial population is largely derived from the rhizosphere population, it is not surprising that many of the beneficial aspects of plant growth-promoting rhizobacteria (PGPR) (Kloepper et al, 1991a; Hoflich et al., 1994) seem to be conferred by endophytic populations as well (Lodewyckx et al., 2002). Plant growth promotion can occur either directly or indirectly. Direct promotion involves bacterial synthesis of compounds stimulating growth or enhancement of nutrient uptake, while indirect promotion occurs as a result of competition with or inhibition of activity of phytopathogenic organisms (Lodewyckx et al., 2002).

Direct growth-promoting activities observed in PGPR are known to include fixation of atmospheric nitrogen, synthesis of siderophores that can solubilize and sequester iron from the soil for plant uptake, synthesis of phytohormones that act to enhance growth at various plant developmental phases, solubilization of minerals such as phosphorous (Kuklinsky-Sobral et al., 2004) resulting in increased availability for plant uptake, and synthesis of other poorly-characterized low-molecular-mass compounds or enzymes influencing plant growth (Lodewyckx et al., 2002; Rosenblueth and Martinez-Romero, 2006).

In a 1995 study by Sturz, 10% of potato tuber bacterial endophytes were found to promote plant growth. Further studies by Sturz et al. (1998) found 21% of endophytic bacteria isolated from red clover and potatoes grown in rotation were capable of promoting plant growth, resulting in a 63% increase in shoot height, with corresponding increases in shoot and root weights of 66% and 55%, respectively.

The best-documented form of direct plant growth promotion is diazotrophy. Diazotrophic bacteria fix atmospheric nitrogen in exchange for carbon fixed by the host. Members of the order *Rhizobiales*, *Frankia* sp., *Azotobacter, Acetobacter* sp., *Herbaspirillum* sp., and *Azospirillum* sp. are common examples (Postgate, 1998; Vessey et al., 2005; Lodewyckx et al., 2002). However, not all PGPR are diazotrophic, and many, including some diazotrophs, fix only small amounts of nitrogen, insufficient to supply their own needs as well as the host plant's (Hong et al., 1991). Yet, endophytic bacteria live within the plant in a low O_2 environment, and are therefore in a better position to express nitrogenase and exchange fixed nitrogen and carbon with the host plant, as compared to rhizosphere organisms (Lodewyckx et al., 2002).

Stimulation of plant growth due to nitrogen fixation by endophytic bacteria has been documented (Hurek et al., 2002; Iniguez et al., 2004; Sevilla et al., 2001; Reiter et al., 2003; Riggs et al., 2001). While some studies found nitrogen-fixing *Rhizobia* to be the dominant endophyte in the population (Reiter et al., 2003), other studies have found that nitrogen-fixing bacteria constituted only a small percentage of the entire endophytic population (Barraquio et al., 1997; Ladha et al, 1983; Martinez et al., 2003) and thus the potential remains for increasing total nitrogen fixation by inoculation of plants with endophytic bacterial species. However, it should be noted that many of the studies finding nitrogen-fixing endophytes in the minority were conducted using culture-based methods and could be biased due to the faster growth rate of many betaproteobacteria when compared to alphaproteobacteria such as *Rhizobium* sp (Rosenblueth and Martinez-Romero, 2006).

Additionally, there remains controversy as the amount of nitrogen fixed by endophytes and supplied to plants (Giller and Merckx, 2003). Hong et al., (1991) found that many nitrogen fixing bacteria fix only small amounts insufficient to support the plant's nitrogen requirement in addition to their own. Estimates of total fixed nitrogen vary widely within a total range of 30 to 80 kg N/ha/year (Boddey et al., 1995). However, considering that in Brazil, sugarcane has been grown for many years in nitrogen-deficient soil with only small amounts of fertilizer and remains nonsymptomatic for nitrogen deficiency, at least some plants would seem to obtain fixed nitrogen from bacterial endophytes (Rosenblueth and Martinez-Romero, 2006).

Phytohormones including ethylene, auxins, and cytokinins are produced by strains of *Pseudomonas, Enterobacter, Staphylococcus, Azotobacter,* and *Azospirillum* (Lodewyckx et al., 2002). Barbieri and Galli (1993) observed trends in growth promotion in wheat after inoculation of roots with *Azospirillum brasilense* strains with mutations affecting auxin biosynthesis, and found that enhanced growth could be correlated with auxin synthesized by the bacterium.

Glick et al., (1995 and 1998) found that many plant growth-promoting bacteria synthesize 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which seems to serve no known biological function for the bacteria. However ACC deaminase does act to modulate the level of ethylene synthesized by plants. Because ethylene synthesized by plants as a stress response (Abeles et al., 1992; Hyodo 1991) is responsible for a significant amount of damage (van Loon, 1984), ACC deaminase released by endophytic bacteria may act to reduce stress ethylene synthesis and thus enhance plant growth (Glick et al., 1998).

Pirttila et al. (2004) identified endophytes in Scots pine that produced adenine ribosides which seemingly stimulated growth and reduced browning of pine callus tissues in culture. Ryu et al. (2003) demonstrated that some volatiles synthesized by bacteria such as 2-3 butanediol and aceotin enhanced the growth of *Arabidopsis*. However, it is not known whether such compounds are biosynthesized by endophytes *in planta* (Rosenblueth and Martinez-Romero, 2006).

Suppression of phytopathogens can be considered an indirect method of plant growth promotion. Pathogen suppression can be the result of several mechanisms including direct antagonism via production of antibiotics, induction of systematic host resistance, and competition for nutrients and suitable niches in the growth environment (Lodewyckx et al., 2002).

An interesting method of bacterial nutrient competition combined with plant growth promotion is through the production of siderophores with high affinities for iron. Siderophores secreted by some bacteria can bind Fe^{3+} in the rhizosphere, making it unavailable for use as a nutrient by competitors, including phytopathogens, thus limiting their growth potential (Castignetti and Smarrelli, 1986; O'Sullivan and O'Gara, 1992; Buysens et al., 1994). Simultaneously, some plants have the capability of binding, transporting, and releasing the iron from the bacterial iron-siderophore complex, thus ensuring adequate supplies if iron for plant growth (Wang et al., 1993).

Some endophytes are believed to induce a phenomenon known as induced systemic resistance (ISR) in the host upon colonization. ISR is a mobilization of the plant's defensive mechanism against a pathogen that does not result in visible symptoms such as the hypersensitive response which are associated with systemic acquired

resistance (SAR). Colonization by an endophyte may stimulate the response, which not only impacts the endophyte, but other pathogens which may not be recognized by the plant or have not yet reached populations sufficient to trigger SAR (Ryan et al., 2008).

Biological Pest Control

Because endophytes occupy an ecological niche similar to plant pathogens, much interest has arisen for their potential application as biological control agents. Additionally, if endophytes could be utilized as biocontrol agents, the consistency and effectiveness of biocontrol treatments might be increased due to the enhanced environmental stability of the endosphere as compared to the rhizosphere, which would theoretically seem to favor more temporally-stable colonization (Hallmann et al., 1997).

Reports of antagonism by endophytic and rhizosphere bacteria toward phytopathogens including both bacteria and fungi are frequent in published studies. A few selected examples would include antagonism by *Burkholderia cepacia* toward *Fusarium* sp. in maize (Bevivino et al., 1998; Hebbar et al., 1992), *Pseudomonas* sp. toward *Pythium ultimum* in sugar beet (Fenton et al., 1992), *P. fluorescens* and actinobacteria toward *Gaeumannomyces graminis* var. *tritici* in wheat (Bangera and Thomashow, 1996; Coombs et al., 2004), *Enterobacter cloca* toward *F. moniliforme* (Hinton and Bacon, 1995), and *P. fluorescens* 89B-27 and *Serratia marcescens* 90-166 toward *P. syringae* pv. *lachrymans* (Liu et al., 1995). Brooks et al. (1994) found inhibition of the oak wilt pathogen *Ceratocystis fagacearum* with 183 of 189 endophytic bacterial isolates.

Additionally, antagonism toward nematodes by endophytic bacteria has been documented (Hallmann et al., 1995; Kloepper et al., 1991b; Sturz and Kimpinski, 2004).

In 1988, Dimock et al. demonstrated use of endophytic bacteria against insect pests. In the years since, endophytic *Herbaspirillum seropedicae* and *Clavibacter xylii* have been genetically modified to excrete the δ-endotoxin of *Bacillus thuringiensis* for insect pest control (Downing et al., 2000; Turner et al, 1991).

Unfortunately, a great deal of difficulty is presented with respect to practical applications of endophytes for use as biological control organisms or agents of plant growth-enhancement in field settings. A host of extremely complex and poorly-understood interactions exist between the indigenous microbial community and plants, and introduced endophytes are often less effective competitors for appropriate ecological niches than indigenous species (Sturz et al., 2000). Environmental fluctuations can adversely affect inoculated endophyte populations (Sturz and Nowak, 2000). Additionally, given the diversity and abundance of rhizosphere microbes, inoculated endophytes may already be present and thus benefits of inoculation may not be observed in some locations (Rosenblueth and Martinez-Romero, 2006).

Endophytic inoculation could prove to be of more benefit to crops propagated by seed in greenhouse conditions or via tissue culture as opposed to those sown directly into the field. Micropropagated explants readily accept introduced endophytic organisms because there are few or no other microbes which offer competition (Rosenblueth and Martinez-Romero, 2006). Plants propagated, transplanted, or sown to sterile soilless growing media, inoculated with endophytes and grown in controlled environmental conditions also appear to be readily colonizable. Studies of plants grown in this manner and inoculated with endophytes at early stages in growth demonstrated increased drought

resistance, increased pathogen resistance, reduced transplanting shock, and lower mortality (Barka et al., 2000; Martinez et al., 2003; Sahay and Varma, 1999).

Biosynthesis of Natural Products

Many endophytes are members of genera such as *Pseudomonas, Burkholderia,* and *Bacillus* known to produce substances useful for humans including antibiotics, anticancer compounds, volatile organic compounds, antifungal agents, antiviral agents, insecticidal agents, and immunosuppressants. However, the reservoir of potential bioproducts remains relatively untapped and the opportunity for exploitation of this resource is tremendous (Ryan et al., 2008; Strobel et al, 2004).

In a review by Ryan et al. (2008), a list of natural products derived from or produced by endophytic bacteria was presented. The list includes anticancer, antimicrobial, antibiotic, antifungal, antiviral, and antimalarial agents derived from endophytes such as *Taxomyces andreanae, Pseudomonas viridiflava, Streptomyces* spp. (including strains NRRL 30562 and NRRL 30566), *Serratia marcescens, Paenibacillus polymyxa,* and *Cytonaema* sp.

Industrial products such as bioplastics are also being isolated from bacterial endophytes. An example of one such product is PHB (poly-3-hydroxybutyrate). First isolated from *Bacillus megaterium* by Lemoigne in 1926, PHB is now known to be produced by a wide range of bacterial species (Kalia et al., 2003). An extremely common diazotrophic endophyte, *Herbaspirillum seropedicae* has now been found to synthesize significant levels of PHB (Catalan et al., 2007). Thus the opportunity exists for the development of *H. seropedicae* as an inoculated endophyte for the purpose of large-scale, cost-effective bioplastic production in field settings (Aldor and Keasling, 2003).

Phytoremediation

Interest is also growing in manipulation of endophytic bacterial populations for the purpose of enhancing or creating plant ability for the removal and degradation of xenobiotic materials from contaminated sites. For large contaminated sites, bioremediation strategies are the only economically and socially acceptable methods for cleanup. Phytoremediation involves the combined action of plants and associated microorganism for the uptake, trapping, and/or degradation of xenobiotic pollutants in the environment (Lodewyckx et al., 2002). Many endophytic bacteria naturally possess metabolic pathways allowing for the degradation of complex organic xenobiotics. Additionally, when no known degradation pathway exists for a particular xenobiotic, successful genetic engineering of a bacterium to create the needed pathway is far easier to achieve than when attempted with a plant (Newman and Reynolds, 2005).

Ryan et al. (2008) presented a nonexhaustive list of endophytic bacteria that have been associated with phytoremediation strategies. Endophytic *Pseudomonas* spp. have been shown to degrade mono- and dichlorinated benzoic acids, 2,4-D (2,4-dichlorophenoxyacetic acid), MTBE (methyl tert-butyl ether), BTEX (benzene, toluene, ethylbenzene, and xylene), and TCE (trichloroethylene). *Methylobacterium populi* can degrade methane, TNT (2,4,6-trinitrotoluene), RDX (hexahydro-1,3,5-trinitro-1,3,5-triazene), and HMX (octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine). *Burkholderia cepacia* has been shown to degrade toluene and several volatile organic compounds. Finally, species of *Herbaspirillum* can degrade TCP (2,3,4,6-tetrachlorophenol) and PCBs (polychlorinated biphenyls).

Endophytes are preferred over soil bacteria for phytoremediation purposes for several reasons. Firstly, endophytic bacterial populations have an opportunity to reach higher numbers than soil bacteria due to reduced competition. Secondly, toxic xenobiotics are broken up *in planta* when degraded by endophytes. For this reason, phytotoxic effects and the potential for poisoning of herbivores inhabiting the site are reduced. Siciliano et al. (2001) found that bacteria possessing the required degradatory pathways were more abundant among endophyte populations than among rhizosphere populations. Additionally, studies by Taghavi et al. (2005) and Ryan et al., (2007) indicated that horizontal gene transfer occurs frequently among endophytic populations. Thus xenobiotic-degradative plasmids can be rapidly exchanged throughout the endophyte population, eliminating the need for long term establishment of the inoculated organism.

ENDOPHYTES AS EMERGING PATHOGENS

Burkholderia, Enterobacter, Herbaspirillum, Klebsiella, Nocardia, Mycobacterium, Ochrobactrum, Pseudomonas, Ralstonia, Staphylococcus and *Stenotrophomonas* have all been identified as endophytic bacteria. All of these genera include species that are known pathogens to animals or plants (Ryan et al., 2008; Rosenblueth and Martinez-Romero, 2006). In 1995, Ponka et al. identified *Salmonella* among endophytes from alfalfa sprouts and *Salmonella* outbreaks resulting from ingested alfalfa sprouts have occurred in North America, Asia, and Europe since this finding. Guo et al. (2002) found *Salmonella* in hydroponically-grown tomatoes.

Burkholderia cepacia has been commonly isolated as an endophytic bacterium from several plants, as a known human pathogen responsible for sometimes-fatal pulmonary infections in cystic fibrosis patients, these findings are reason for concern. Holmes et al. (1998) and Parke and Gurian-Sherman (2001) proposed a moratorium on the agricultural use of *Burkholderia* until further study could be made, in order to reduce the risk to consumers of raw fruits and vegetables.

Nocardia spp. are known to cause human nocardiasis, a severe infection in the feet and legs of humans sometimes requiring amputation. *Mycobacterium leprae* is the pathogen responsible for human leprosy. Several *Pseudomonas* species are opportunistic human pathogens, and *Klebsiella pneumonia* is responsible for human bacterial pneumonia.

Unfortunately, it is often difficult to distinguish harmless environmental isolates from pathogenic clinical isolates. Additionally, the opportunity always exists for the

conferrance of virulence genes from a pathogen to a non-pathogenic endophyte via horizontal gene transfer (Rosenblueth and Martinez-Romero, 2006).

Unexpected interactions between endophytes and plants have been documented as well. Van Peer et al. (1990) found that bacterial endophytes isolated from healthy tomato plants caused growth inhibition when reinoculated into tomato seedlings. Sturz et al. (1997) found that inoculations of two or more bacteria known to individually inhibit plant growth sometimes resulted in enhanced growth. Apparently, the order in which endophytes are inoculated and become established can have an impact on the ultimate effect upon host health. It is likely that there is an equilibrium that is established within the endophytic community under certain environmental conditions that, when upset, can be detrimental to the bacteria and/or the plant host (Rosenblueth and Martinez-Romero, 2006).

Interestingly, there seems to be some potential for using certain endophytes as agents against other potentially pathogenic endophytes. For example, Cooley et al. (2003) found that *Enterobacter absuriae* could out-compete *Salmonella enterica* and *Escherichia coli* O157:H7 in *Arabidopsis thaliana* seeds.

The recognition that some endophytes are potential plant, animal, and/or human pathogens is cause for concern, especially when one considers the quantity of plant products consumed in an uncooked state. In a time with so much interest in the potential benefits of endophytic bacterial applications in agricultural settings, the consideration of potential adverse health consequences should not be neglected when endophytic bacteria are evaluated for potential usefulness.

Parke and Gurian-Sherman (2001) wrote that "It is not coincidental perhaps that many of the most effective biocontrol agents of plant diseases are also opportunistic human pathogens. [They] are fiercely competitive for nutrients and may produce antimicrobial metabolites and may themselves be resistant to multiple antibiotics."

RESEARCH OBJECTIVES

As soil seems to be the primary reservoir for endophytic bacteria, serving to support the population of rhizobacteria from which the endophytic bacterial population is derived, the physical and chemical properties of soil are likely to have a substantial effect on the endophytic bacterial diversity of plants grown within that soil. Soil factors such as pH, salinity, texture, nutrient availability, chemical composition, and adsorption capacity are known to alter the rhizosphere bacterial community, and thus effectively preselect the endophytic bacteria potentially available for colonization of plants (Hallmann et al., 1997; Quadt-Hallmann and Kloepper, 1996).

In a 1996 study by Quadt-Hallmann and Kloepper, various soils were drenched with a suspension containing *Enterobacter asburiae*. Recovery of the inoculated bacterium was higher from siliceous sand, loamy sand, and ground clay than for sandy loam and a peat-based soilless substrate. Mahaffee and Kloepper (1996) reported higher colonization of *Pseudomonas fluorescens* in common beans grown in sandy soils than in soils with finer textures. Studies by Samish et al. (1963) and Bell et al. (1995) yielded seemingly opposite results. The former found differences in endophytic bacterial populations of crops grown in different fields. The latter found no differences in the endophytic populations of grapevines grown at different vineyards. Hallmann et al., (1999) found that the addition of 1% chitin to soil modified both the rhizosphere and endophytic bacterial populations of cotton roots. Conn and Franco (2004) found that soil type had a large influence on endophytic diversity in wheat plants grown in three different soils. However, only *Actinobacteria* were evaluated in this latest work.

The objectives of this study were threefold. The primary objective was to evaluate the total root bacterial endophyte population of *Medicago truncatula*, currently the model legume for plant-microbe interaction studies. While some *M. truncatula* endophytes had been previously isolated, to this author's knowledge, no comprehensive evaluation of the endophytic population of *M. truncatula* using a culture-independent molecular approach had yet been attempted.

The second objective of this study was to evaluate the influence of soil on endophytic bacterial diversity and structure by comparing the root bacterial endophyte populations of *M. truncatula* grown in diverse soils.

The final objective of the study was to compare the efficacy of two commonly used molecular approaches for examination of environmental bacterial communities: cloning and sequencing of PCR-amplified 16S rDNA fragments, and temperature gradient gel electrophoresis (TGGE) of amplified 16S rDNA fragments. This evaluation of methodology was made by analysis of the same endophytic bacterial populations from *M. truncatula* roots grown in diverse soils using both approaches, followed by a comparison of results achieved with each technique.

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IDENTIFICATION OF ROOT BACTERIAL ENDOPHYTES FROM *MEDICAGO TRUNCATULA* GROWN IN DIVERSE SOILS BY CLONING AND SEQUENCING OF PCR-AMPLIFIED 16S rRNA GENES

ABSTRACT

The endophytic bacterial populations of surface disinfected *M. truncatula* root tissues collected from plants grown in a commercial growing medium and six diverse soils from across the state of Oklahoma were evaluated for diversity and differences in composition resulting from plant growth in dissimilar soils.

Endophytic bacteria were identified by PCR amplification of near full-length bacterial 16S rRNA genes using "universal" bacterial primers, followed by "shotgun" cloning and mass sequencing of inserts.

Thirty-six genera of bacteria were putatively identified as *M. truncatula* root endophytes, encompassing five phyla (*Proteobacteria, Firmicutes, Actinobacteria, Planctomycetes,* and *Bacteroidetes*). Large differences in diversity were observed between endophytic bacterial populations originating from plants grown in different soils, with differences becoming increasingly pronounced as taxonomic level was decreased from phylum to genus. At the genus level, two acidic soils with a forest background and a commercial growing medium containing 15-25% ground pine bark yielded the highest endophyte diversity, while moderate diversity was observed in plants grown in managed agricultural soils. Root bacterial endophyte diversity was lowest in plants grown in soil collected from an undisturbed native tallgrass prairie.

INTRODUCTION

The diversity of endophytic bacteria and their potential for beneficial plant-microbe interactions is enormous and has been the subject of numerous studies examining a host of diverse plant species. It is thought that every plant species on Earth is host to one or more endophytes (Strobel et al., 2004). Endophytic bacteria have been found to provide many benefits to their hosts including growth promotion, accelerated seedling emergence and establishment even under adverse environmental conditions, disease suppression through production of antimicrobial compounds, and activity as biocontrol agents (Ryan et al., 2007).

While many studies of endophytic bacterial populations have been conducted by culturing bacteria isolated from surface disinfected plant tissues, molecular-based approaches which do not require culturing, especially those utilizing amplification and sequencing of bacterial genes, provide for a much greater detection capability and have become increasingly favored as methods for study of diverse endophytic bacterial populations (Rosenblueth and Martinez-Romero, 2006).

In general, molecular approaches toward endophyte identification utilize amplification of the bacterial 16S rRNA gene from macerates of surface disinfected plant tissues, followed by various techniques used to separate the amplification products including terminal restriction fragment length polymorphism analysis (TRFLP), denaturing gel electrophoresis (including temperature gradient gel electrophoresis and denaturing gradient gel electrophoresis), and “shotgun” cloning of 16S rDNA products (Ryan et al., 2007). Once separated, the amplification products can then be sequenced and identified by comparison to databases of known sequences.

Successful surface disinfection of plant tissue is a critical step in endophyte studies, as inadequately disinfected tissues will yield organisms from the rhizosphere/rhizoplane as well as true endophytes. While many different protocols for surface disinfection have been employed, none have emerged as a standard for use across many plant species and many types of tissue, due to great variation in tissue size, degree and form of external contamination, durability, and chemical permeability. Surface disinfection protocols must strike a balance between being sufficiently mild to prevent destruction of tissues and endophytes, while sufficiently lethal to surface bacteria. To date, no surface disinfection protocol yet devised could be expected to result in the complete destruction of 100% of surface bacteria (Lodewyckx et al., 2002).

A review of surface disinfection protocols by Lodewyckx et al. (2002) revealed bleach and ethanol as the most common antimicrobial agents used for surface disinfection. Hydrogen peroxide and mercuric chloride were also occasionally employed. Externally-applied flame has been utilized for durable tissues with large diameters such as sugar beets (Jacobs et al., 1985). Aseptic excision of internal tissue following surface disinfection has been performed with large-diameter samples such as sugar beets (Jacobs et al., 1985), corn stems (Fisher et al., 1992), and grapevines (Bell et al., 1995).

Endophytes have been found within all plants tissues, including seed (Ryan et al., 2008). However, the highest densities are usually observed in roots, and decrease progressively from the stem to the leaves (Lodewyckx et al., 2002). This phenomenon is thought to occur largely because the roots serve as the primary entry point for endophytic organisms from the rhizosphere through wounds occurring either naturally due to growth or via the activity of soil pests and pathogens. Root hairs and epidermal junctions have

also been identified as entry points for endophytic microorganisms (Sprent and de Faria, 1988).

Medicago truncatula (barrel medic) has been adopted as the model legume for plant-microbe interaction studies for some time now. *M. truncatula* is favored as a model species because it is an easily transformed diploid with a relatively small genome. Additionally, the genome of *M. truncatula*'s most well-known nitrogen fixing endosymbiont, *Sinorhizobium meliloti* has been sequenced while sequencing of the *M. truncatula* genome itself is nearing completion, with version 2.0 of the *M. truncatula* genome released by the *Medicago* Genome Sequence Consortium (MGSC) in August 2007.

Despite its status as a model legume, very little investigation has been made of bacterial endophyte diversity within *M. truncatula.* A study by Zakhia et al. (2006) evaluated bacterial endophytes in naturally-occurring Tunisian *M. truncatula* root nodules, and found species of *Sinorhizobium, Pseudomonas,* and *Ornithinicoccus*. But to this author's knowledge, a comprehensive evaluation of *M. truncatula* bacterial endophyte diversity has never been made.

Additionally, while many studies of bacterial endophyte diversity have been made with respect to other host plant species, most studies have been site-specific, examining only plants grown at one specific location. While it is certainly recognized that soils influence rhizosphere and therefore endophytic bacterial populations, to this author's knowledge, few studies have directly examined the soil-bacterial endophyte relationship by evaluating endophytic bacterial diversity in the same plant species grown in a number

of different soils. Conn and Franco (2004) examined endophytic diversity in wheat plants grown in three different soils, but only *Actinobacteria* were evaluated.

Thus, the objective of this research was to evaluate differences in the diversities of bacterial endophyte populations from surface disinfected *M. truncatula* roots grown in a selection of different soils collected from across the state of Oklahoma, using a molecular strategy consisting of PCR (polymerase chain reaction) amplification of near full-length bacterial 16S rRNA genes using "universal" primers followed by "shotgun" cloning and sequencing of the bacterial 16S rDNA inserts.

MATERIALS AND METHODS

Development of Surface Disinfection and Total DNA Extraction Protocol

In order to ensure that only endophytic bacteria were being examined in this research, it was critical to develop an effective procedure for the elimination of rhizosphere bacteria inhabiting the surface of *M. truncatula* root tissue, without causing harm to the endophytic bacterial population within the same tissue. Additionally, since it was intended that PCR be used to amplify endophytic bacterial DNA in order to detect non-culturable species, the surface disinfection protocol had the added requirement of rendering any residual rhizosphere bacterial DNA or RNA unamplifiable. The four experiments described below were conducted to develop and test procedures for surface disinfection and extraction of total DNA from *Medicago truncatula* root tissue.

Surface Disinfection Experiment 1

For the purpose of developing the surface disinfection protocol, *M. truncatula* plants grown in a field plot on the OSU Agronomy Farm were utilized. Three replications of the following experiment were performed in the initial attempt at surface disinfection.

M. truncatula plants were collected from the field plot area using a spade, with care taken to leave the root system intact. Collected plants were brought to the laboratory where the roots were cleaned thoroughly by hand under a stream of RO water so that all soil and debris were removed. From the cleaned plants, 0.1 g samples of root tissues were cut from the shoots with clean scissors and added to individual 2.0 mL microcentrifuge tubes. With the exception of the "no exposure" control tubes, all tubes were then filled completely with surface disinfection solution (a filter-sterilized solution consisting of

10% commercial bleach and 10% ethanol in QH_2O). The "no exposure" control tubes were filled with sterile QH_2O rather than surface disinfection solution. Tubes were then vortexed for varying lengths of time (5, 10, 15, 20, 25, and 30 minutes, respectively) at 1400 RPM (25°C) using an Eppendorf® Thermomixer R (Eppendorf North America, Westbury, NY) in order to test required exposure time for effective surface disinfection. The "no exposure" control tubes were vortexed for 1 minute in order to bring any surface bacteria into suspension.

Following vortexing, the rinsate was aspirated off aseptically within a laminar flow hood. Each sample was then rinsed thrice with sterile QH_2O in order to remove all residual surface disinfection solution. Each rinse was performed by filling the tube completely with sterile QH_2O, vortexing at 1400 RPM (25°C) for 1 minute, and aseptically aspirating off the resulting rinsate. A 1.0 mL aliquot of the third (final) rinsate from each tube was collected into a sterile 1.5 mL microcentrifuge tube and stored at -20°C for use as template for evaluation of surface disinfection efficacy by PCR.

Each 20 µL PCR reaction mixture contained the following: 2 µL of Qiagen® 10X PCR buffer, 4 µL of Qiagen® 5X Q Solution, 2 µL of 25 mM $MgCl_2$, 0.4 µL of dNTP mix (10 mM each), 0.2 µL of 10 µM primer F968, 0.2 µL of 10 µM primer R1401/1378GC, 0.1 µL of Qiagen® *Taq* DNA polymerase, 1 µL of "final wash" template, and sterile QH_2O to volume. Positive and negative control reactions were also included which substituted an equal volume of either a *Bacillus megaterium* genomic DNA solution or sterile QH_2O, respectively, for the 1 µL of "final wash" template. Reactions were performed using an MJ Research® PTC-200 thermal cycler (MJ Research Inc., Waltham, MA) programmed for an initial denaturation step of 94°C for 3 min,

followed by 35 amplification cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min. To increase specificity, a simulated "hot start" was performed by delaying loading of reaction tubes until the sample block had reached the initial denaturation temperature of 94°C.

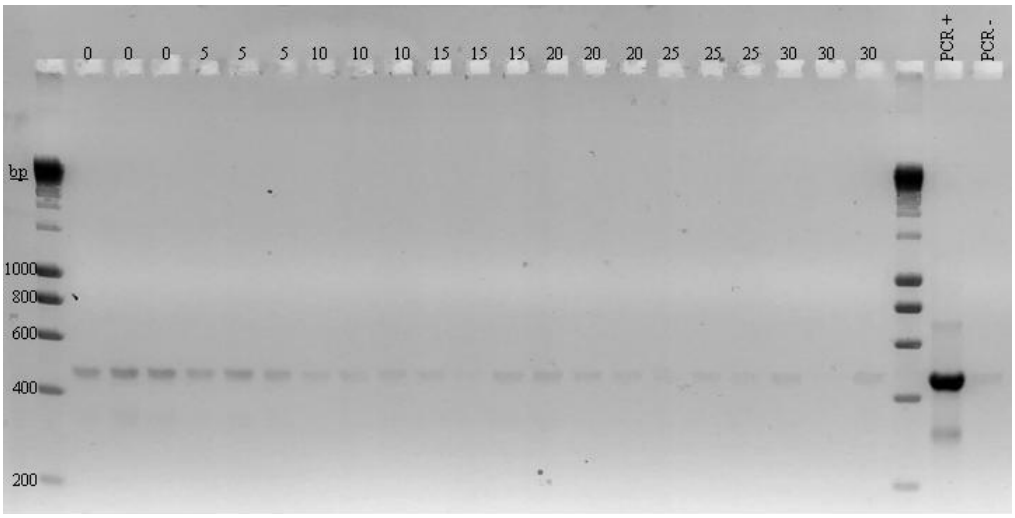

Figure 1: Electrophoresis of 12 µL aliquots of "final wash" solution PCR amplification products. Three replications of the experiment were performed. Numbers above each lane indicate time of root tissue exposure to surface disinfection solution in minutes. Ladder lanes contain GeneChoice® DNA Ladder 1 (GeneChoice Inc., Frederick, MD). Lanes containing PCR control products are designated as PCR + (positive control) and PCR – (negative control). Electrophoresis was performed at 200 V for 50 min using a 1.5% agarose, 1X sodium borate (pH 8.5) gel containing 0.5 µg/mL EtBr (ethidium bromide).

These results indicated that exposure of root tissues to the surface disinfection solution for durations of 10 min or longer reduced PCR amplification to a level not more than the background present in the PCR negative control, suggesting that the surface disinfection protocol was effective. However, it was as yet unknown whether the protocol had been sufficiently mild to prevent destruction of endophytic bacterial DNA within the tissue.

Surface Disinfection Experiment 2

Following the initial test of a novel protocol for surface disinfection of *M. truncatula* root tissue, a second experiment was needed to verify that amplifiable endophyte DNA was still obtainable following surface disinfection. *M. truncatula* root tissue samples were collected and surface disinfected by the procedure described above. Three replicates of this experiment were performed, with root tissue samples exposed to the surface disinfection solution for durations of 0, 5, 10, and 15 min. Again, "final wash" aliquots from each sample were collected and stored at -20°C for use as PCR template.

Following surface disinfection, total DNA was extracted and purified from the treated root tissues using a Qiagen® DNeasy® Plant Mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. All portions of the protocol which required exposure of the tissue to the laboratory environment were carried out aseptically within a laminar flow hood. Purified total DNA extracts were stored at -20°C.

A 1 µL volume of each "Final Wash" rinsate from three replicate 0, 5, 10, and 15 minute-exposure surface disinfection experiments (described above) and 1 µL volumes of the respective total DNA elutions from these tissues were used as templates for PCR. Reaction conditions were as described previously. Conditions for the 20 µL PCR reactions using primers F968 and R1401/1378GC were as described previously in Surface Disinfection Experiment 1. Following PCR, 10 µL volumes of the PCR products were examined by electrophoresis

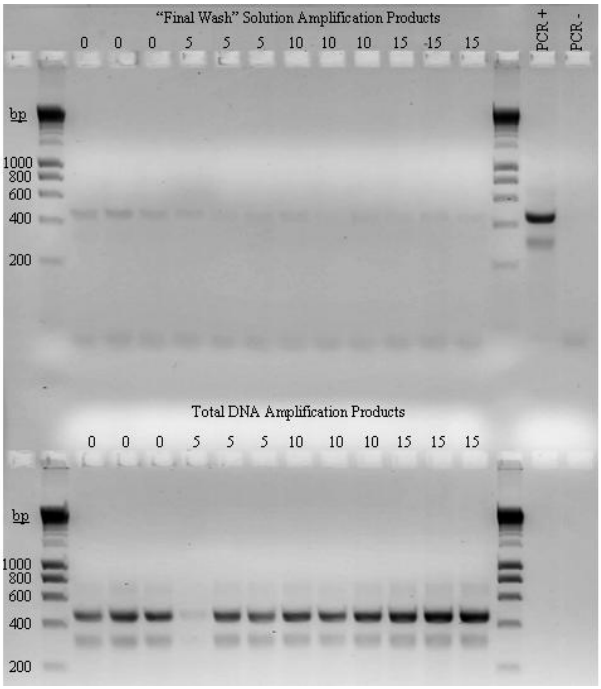

Figure 2: Electrophoresis of three replications of "final wash" solution (upper lanes) and total DNA (lower lanes) PCR amplification products (10 µL amplification product per lane). Numbers above each lane indicate time of root tissue exposure to surface disinfection solution in minutes. Ladder lanes contain GeneChoice® DNA Ladder 1. Lanes containing PCR control products are designated as PCR + (positive control) and PCR – (negative control). Electrophoresis was performed at 200 V for 50 min using a 1.5% agarose, 1X sodium borate (pH 8.5) gel containing 0.5 µg/mL EtBr.

The surface disinfection procedure did not appear to have any adverse effect on amplifiable total DNA from the tissue lysate. PCR yields from the total DNA solution were high regardless of time of exposure to the surface disinfection solution. Additionally, the surface disinfection protocol appeared fairly effective, as PCR yields in the 10 and 15 minute exposure samples were lower than the yields in the 0 min exposure samples. However, it would be far more satisfactory to see a complete absence of amplification products in these samples. It was hypothesized that these faint amplification products could be resulting from residual plant or microbial DNA and/or

RNA remaining on the surface of the tissue following surface disinfection. In order to test this hypothesis, it was decided that DNAse and RNAse treatments should be incorporated into the surface disinfection protocol to remove any residual surface nucleic acids following exposure to the surface disinfection solution.

Surface Disinfection Experiment 3

The surface disinfection protocol described above above (see Surface Disinfection Experiment 1) was amended to include a DNAse / RNAse treatment in an attempt to further reduce amplifiable nucleic acids remaining on the exterior of the root tissue following surface disinfection. Three replications of the following procedure were performed to test the efficacy and digestion time needed for effective DNAse / RNAse treatment.

Root tissues were disinfected according to the protocol described previously in Surface Disinfection Experiment 1. Tissues were exposed to the surface disinfection solution for 10 min. Aliquots (20 uL) of each final sterile QH_2O wash were removed aseptically to sterile microcentrifuges tubes as templates representative of no DNAse / RNAse treatment. The remaining volume of the final rinsate was aseptically removed from each disinfected tissue sample by aspiration.

A 2.0 mL volume of sterile DNAse buffer solution (an aqueous solution containing 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 25 mM KCl, sterilized by autoclaving and stored at RT) was added to each surface disinfected root tissue sample, followed by 4 µL of Qiagen® RNAse A (7,000 U/µL) and 2 µL of Qiagen® DNAse I (153 U/µL). Samples were vortexed briefly then incubated at 37°C for 120 min. At 15 min intervals, tubes were inverted to ensure adequate mixing

and a 20 µL aliquot of the buffer solution was aseptically removed to a fresh sterile 1.5 mL microcentrifuge tube and immediately incubated at 80°C for 10 min to inactivate the DNAse and RNAse. Aliquots were then stored at -20°C, thus creating a series of samples representing DNAse / RNAse treatment times of 15, 30, 45, 60, 75, 90, 105, and 120 minutes, respectively.

A 1 µL volume of each "final wash" rinsate (representing 0 min of DNAse / RNAse treatment) and 1 µL volumes of each final DNAse / RNAse treatment aliquot were used as templates for PCR. Reaction conditions using primers F968 and R1401/1378GC were as described previously in Surface Disinfection Experiment 1. Following PCR, 10 µL volumes of the products were examined by electrophoresis.

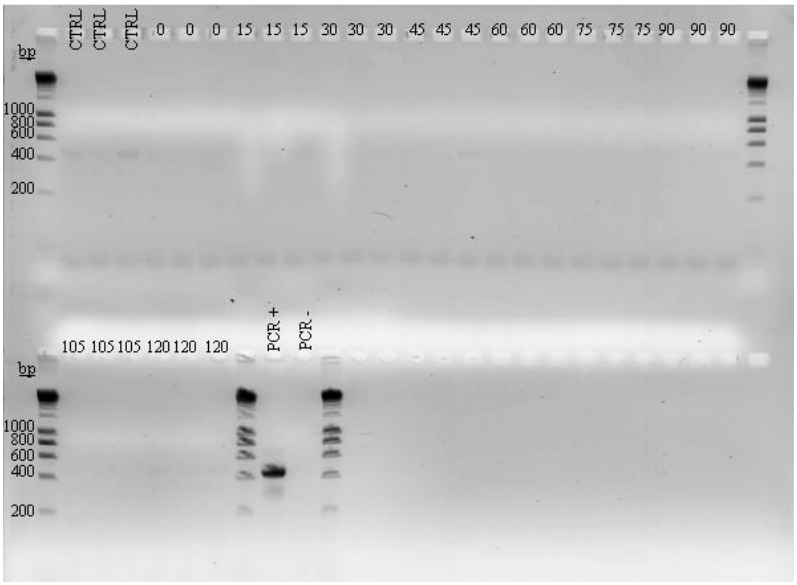

Figure 3: Electrophoresis of PCR amplification products of three replications of "final wash" solution with varying times of DNAse / RNAse treatment. Numbers above each lane indicate time of root tissue exposure to DNAse and RNAse in minutes. Control (CTRL) lanes represent root tissue exposed to neither surface disinfection nor DNAse / RNAse treatment. Ladder lanes contain GeneChoice DNA Ladder 1. Lanes containing PCR control products are designated as PCR + (positive control) and PCR – (negative control). Electrophoresis was performed at 200 V for 50 min using a 1.5% agarose, 1X sodium borate (pH 8.5) gel containing 0.5 µg/mL EtBr.

Faint bands of amplification products remained detectable in the 0 minute through 30 minute DNAse / RNAse treatment lanes. However, only one replicate of the 45 min DNAse / RNAse treatment produced an amplification product. No amplification products were detectable in the 60, 75, and 90 min treatment lanes, suggesting that an incubation time of 60 min is sufficient for complete digestion of residual external DNA and RNA contamination. Some faint product bands appeared in the 105 and 120 min treatment lanes, which were thought to possibly result from migration of endophytic microbes from within the root tissue back into the DNAse buffer solution during this extended treatment time period.

Because the 60, 75, and 90 min DNAse / RNAse treatments were highly successful with respect to elimination of PCR amplification products from the exterior of surface disinfected roots, far greater confidence was placed in the efficacy of the surface disinfection protocol, and a 75 minute DNAse / RNAse treatment was incorporated into all further surface disinfections.

Surface Disinfection Experiment 4

A final experiment was still needed to verify that the DNAse / RNAse treatment incorporated into the disinfection protocol did not have a negative impact on the ability to amplify total DNA from surface disinfected root tissue. Three replicate samples of *M. truncatula* root tissues were collected and subjected to the surface disinfection protocol described previously, using a a 10 min exposure to surface disinfection solution as well as a 75 min DNAse / RNAse treatment followed by heat inactivation of the DNAse and RNAse by incubation at 80°C for 10 min. Following heat inactivation, aliquots of the

DNAse-treated rinsate were collected aseptically and stored at -20°C for use as template for PCR verification of surface disinfection efficacy.

Total DNA was extracted from the three replicates of surface-disinfected root tissues using a Qiagen® DNeasy® Plant Mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

Templates for 20 µL PCR reactions consisted of 1 µL from each of the replicate aliquots of DNAse-treated rinsate and 1 µL of extracted total DNA from each replicated sample. PCR reaction conditions using primers F968 and R1401/1378GC were as described previously in Surface Disinfection Experiment 1. Following PCR, 10 µL volumes of the PCR products were examined by electrophoresis.

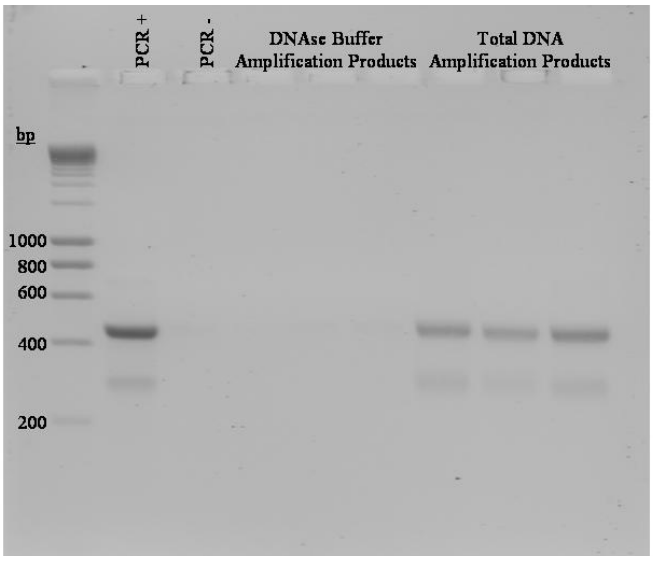

Figure 4: Three replicates of PCR amplification products of final DNAse buffer solutions and total DNA extraction products (10 µL amplification product per lane). The ladder lane contained GeneChoice® DNA Ladder 1. Lanes containing PCR control products are designated as PCR + (positive control) and PCR – (negative control). Electrophoresis was performed at 200 V for 50 min using a 1.5% agarose, 1X sodium borate (pH 8.5) gel containing 0.5 µg/mL EtBr.

These results confirmed the efficacy of the surface disinfection and total DNA extraction protocols. No amplification occurred when the final DNAse-treated rinsate was used as a template for PCR, indicating that all detectable DNA and RNA had been removed from the exterior of the root tissues. Excellent amplification was still achieved using the total DNA extracted from homogenized root tissues.

Verification of Surface Disinfection Efficacy by Culturing

Although experiments described above amply demonstrated the ability to surface disinfect *M. truncatula* root tissue to the extent that no contamination was detectable by PCR, as a final proof-of-concept, it was desirable to verify the efficacy of the procedure utilizing a live-culture approach.

To accomplish this, roots from five *M. truncatula* plants grown in unamended Stillwater soil were collected and cleaned as described above. Total root mass of each plant was measured followed by subdivision of the roots into 0.1 g samples as before. This data is provided in Table 1, below.

Plant	Root Mass (g)	Number of 0.1 g Samples Collected	Identification Codes Assigned to Samples
1	0.31	3	1A, 1B, 1C
2	0.25	2	2A, 2B
3	0.22	2	3A, 3B
4	0.22	2	4A, 4B
5	0.17	1	5A

Table 1: Identification key for *M. truncatula* root samples collected for surface disinfection efficacy verification using live bacterial culturing methods.

These 0.1 g root samples were surface disinfected using the protocol described above, modified slightly to allow for collection of bacterial inoculum at critical stages in the disinfection process. As before, the 0.1 g root tissue samples were collected into sterile 2.0 mL microcentrifuge tubes. Samples were vortexed at 1400 RPM in 2.0 mL of sterile Q H_2O for 1 min at 25°C using a Thermomixer R. Aliquots (200 µL) of the rinsate were collected to a fresh sterile 1.5 mL microcentrifuge tubes and saved as inoculum sources representing contamination present prior to surface disinfection. These aliquots were designated as series "A" and stored at 4°C. The remaining rinsate volumes were aspirated off.

Surface disinfection solution (2.0 mL) was then added to each tube, followed by vortexing at 1400 RPM for 10 min at 25°C using a Thermomixer R. The rinsates were then aspirated off aseptically. A 2.0 mL volume of sterile Q H_2O was then added to each sample and vortexed at 1400 RPM for 1 min at 25°C. Aliquots (200 µL) of these rinsates were collected to fresh sterile 1.5 mL microcentrifuge tubes and saved as inoculum sources representing contamination immediately following exposure to the surface disinfection solution. These aliquots were designated as series "B" and stored at 4°C. The remaining rinsate volumes were aspirated off aseptically.

The samples were now washed thrice using 2.0 mL of sterile QH_2O with vortexing of each wash performed at 1400 RPM for 1 min at 25°C. Aliquots (200 µL) of the third Q H_2O wash rinsate were collected to a fresh sterile 1.5 mL microcentrifuge tubes and saved as inoculum sources representing contamination following all water washes. These aliquots were designated as series "C" and stored at 4°C. The remaining rinsate volumes rinsate were aspirated off aseptically.

Finally, 2.0 mL of DNAse buffer solution was added to each sample, followed by 4 µL of Qiagen® RNAse A, and 2 µL of Qiagen® DNAse I. The samples were vortexed briefly to ensure adequate mixing, then incubated at 37°C for 75 min using a Thermomixer R. Tubes were mixed by inversion at 15 min intervals during incubation. A final incubation was performed at 80°C for 10 min to inactivate the DNAse and RNAse in the samples. Aliquots (200 µL) of the DNAse-treatment rinsate were withdrawn and stored in 1.5 mL microcentrifuge tubes as inoculum sources representing contamination present following the entire surface disinfection procedure. These aliquots were designated as series "D" and stored at 4°C.

From each stored aliquot, 100 µL was withdrawn aseptically and used to inoculate 1 mL of sterile trypticase soy broth (TSB) in 12 X 75 mm culture tubes. Five additional control tubes were also created at this time. A negative control tube was inoculated with 100 µL of sterile Q H_2O. Three positive control tubes were inoculated from bacterial stock cultures stored at -80°C. These stock organisms were, respectively: *Bacillus megaterium*, *Escherichia coli* XL-10 Gold, and *Agrobacterium tumefaciens* AGL-1. Following inoculation, the cultures were incubated at 28°C with orbital agitation at 275 RPM. Cultures were examined for growth at 12 and 24 hours of elapsed incubation time. Growth observations are presented in Table 2, below.

Culture Growth Observations After 12 hr Incubation at 28C

Sample Series	Sample Series Description	Sample Number									
		1A	1B	1C	2A	2B	3A	3B	4A	4B	5A
A	Pre-sterilization	-	+	-	+	-	+	-	+	-	+
B	Post-sterilization solution treatment	-	-	-	-	-	-	-	-	-	-
C	Post-washing	-	-	-	-	-	-	-	-	-	-
D	Post-DNAse/RNAse treatment	-	-	-	-	-	-	-	-	-	-
+ Control	*B. megaterium*	Growth									
+ Control	*E. coli*	Growth									
+ Control	*A. tumefaciens*	Growth									
- Control	Sterile Q H_2O	No Growth									

Culture Growth Observations After 24 hr Incubation at 28C

Sample Series	Sample Series Description	Sample Number									
		1A	1B	1C	2A	2B	3A	3B	4A	4B	5A
A	Pre-sterilization	+	+	+	+	+	+	+	+	+	+
B	Post-sterilization solution treatment	+	+	+	+	+	+	+	+	+	+
C	Post-washing	+	+	+	+	+	+	+	+	+	+
D	Post-DNAse/RNAse treatment	+	+	+	+	+	+	+	+	+	+
+ Control	*B. megaterium*	Growth									
+ Control	*E. coli*	Growth									
+ Control	*A. tumefaciens*	Growth									
- Control	Sterile Q H_2O	No Growth									

Table 2: Observations of bacterial growth in TSB cultures inoculated with rinsates collected during surface disinfection of *M. truncatula* roots after incubation at 28°C for 12 and 24 hours. Negative signs (-) indicate tubes with no observable bacterial growth, while tubes with growth are designated by plus signs (+).

After 12 hours of incubation at 28°C, growth was observed in 50% of the "series A" tubes which were inoculated with rinsate collected prior to surface disinfection. This confirmed the presence of root epiphytic bacteria prior to surface disinfection. No growth was observed in any of the tubes inoculated with rinsate collected at steps following the treatment of root samples with the surface disinfection solution. These results strongly supported the efficacy of the surface disinfection protocol.

However, after 24 hours of incubation at 28°C, growth was observed in all of the inoculated tubes, including those inoculated with rinsates collected after root sample treatment with the surface disinfection solution. This was hypothesized to be the result of the germination of bacterial endospores present on the root surface which would not have been killed by the bleach/EtOH surface disinfection treatment. While this unarguably exposes a limitation to the efficacy of the treatment, it must be emphasized that the resistance of bacterial endospores to chemical sterilization is well known. By definition, the term "disinfection" refers to a treatment which destroys vegetative bacterial cells, but not endospores. As discussed in the literature review, other published methods used to surface "sterilize" or disinfect likely would not have destroyed most bacterial endospores. Therefore, considering that: 1) the principal basis for endophyte detection and identification used for this work is PCR amplification-based; 2) the PCR amplification method used did not detect this contamination; and 3) endophytes have (of necessity) been rather loosely defined in other published works as being microorganisms that are detectable within plant tissues following a surface disinfection procedure, the decision was made to proceed with this work using the surface disinfection protocol as described, with the understanding that characterization of any spore-forming bacteria detectable by this method as "endophytic" should be considered to be strictly putative.

Identification of Unknown Bacteria Surviving Surface Disinfection

The result of the experiment described above created a necessity to determine the identity of the disinfection-resistant bacteria for comparison to endophytes identified later in the course of this work. Additionally, identification was needed in order to be certain that our characterization of these organisms as spore-forming bacteria was correct. Thus,

all of the "series D" broth cultures (representing organisms that had survived the complete surface disinfection treatment) were pooled. From the pooled culture, four 500 µL aliquots removed to sterile 2.0 mL screw-capped microcentrifuge tubes. Each aliquot was then prepared for storage by adding an equal volume of sterile glycerol freezing solution (a filter-sterilized aqueous solution stored at 4°C consisting of 0.025 M Tris-HCl pH 8.0, 0.1 M $MgSO_4$-$7H_2O$, and 65% glycerol by volume). Tubes were vortexed briefly to mix, then stored at -80°C for future identification.

An additional volume of the poolued culture was utilized to extract total bacterial DNA to provide template for PCR amplification and sequencing for identification purposes. This protocol is described in the following paragraphs.

A 1.0 mL aliquot of the broth culture was removed to a sterile 2.0 mL screw-capped microcentrifuge tube and centrifuged at 21,000 X *g* for 10 min. Following centrifugation, the supernatant was decanted carefully so not as to disturb the bacterial cell pellet. This procedure was performed thrice in the same 2.0 mL screw-capped tube so that the final pellet consisted of bacterial cells from a 3.0 mL volume of broth culture. After the final decantation, cells were washed by resuspension in 1.0 mL of sterile PBS (phosphate-buffered saline, an aqueous solution consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 in Q H_2O, adjusted to pH 7.4 with HCl, sterilized by autoclaving, and stored at RT) followed by centrifugation at 21,000 X *g* for 10 min and decantation of the supernatant. Cells were then resuspended in 100 µL of sterile Q H_2O, and a small volume of sterile 0.1 mm glass beads added to the tube. Cells were then lysed by agitation in a Mini-BeadBeater-1™ (BioSpec Products Inc., Bartlesville, OK) for 1 min at 4200 RPM. The lysate was centrifuged for 30 sec at 21,000 X *g* to

pellet the cellular debris. Supernatant containing the total bacterial DNA was aseptically removed by pipetting into a fresh sterile 1.5 mL microcentrifuge tube and buffered by the addition of 0.5X TE buffer (Tris-EDTA buffer, an aqueous solution consisting of 100 mM Tris-Cl pH 8.0 and 10 mM EDTA pH 8.0 in Q H_2O, sterilized by autoclaving and stored at RT).

Bacterial DNA was purified from the crude lysate by phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by chloroform:isoamyl alcohol (24:1) extraction and ethanol precipitation as described by Sambrook and Russell (2001).

16S rRNA gene fragments were amplified by PCR from the purified total DNA extract. Amplification, sequencing, and identification of this DNA was performed in parallel with like procedures involving endophytic bacterial DNA isolated from *M. truncatula* roots. To avoid repetition, discussion and results of these procedures are detailed later in this work.

Soil Collection and Analysis

Six samples of natural soil were collected from diverse locations across the state of Oklahoma (Woodward, Goodwell, Wilburton, Kansas, Pawhuska, and Stillwater). Sampling locations were selected to ensure that the collection contained a wide range of soil types with marked differences in texture, fertility, utilization history, and biology. Additionally, a commonly used soilless growing medium, Scott's® MetroMix® 366 (Scotts Co., Marysville, OH) was selected for inclusion in the study to further broaden the scope of investigation.

A volume of natural soil sufficient to fill one clean 5 gal plastic tub was collected from each sampling site using a clean spade. Samples were collected from the uppermost

portion of the soil, to a depth not in excess of 0.5 m. To the greatest possible extent, vegetation was removed and discarded from the soil samples during collection.

Subsamples of each natural soil and the soilless growing medium were submitted to the Soil, Water, and Forage Analytical Laboratory (SWFAL) at Oklahoma State University for analysis of texture and fertility. Findings of these analyses can be seen in the "Results and Discussion" section of this chapter.

Growth of *Medicago truncatula*

M. truncatula cv. Jemalong A-17 seed was hulled by hand, then mechanically scarified by gentle abrasion between two sheets of fine grit sandpaper. Seeds were sown in black polypropylene nursery "cone-tainers" (1.5" diameter X 8.25" deep) cleaned by scrubbing and immersion in a 1:4 dilution of commercial bleach in RO H_2O followed by six rinses with sterile RO H_2O. Once filled with samples of the soils and the soilless growing medium described above, five seeds were sown to each cone-tainer to ensure successful germination of at least one seedling in each. Upon germination, seedlings were thinned to one plant per cone-tainer and grown for 1 month under controlled environmental conditions in a growth chamber set to provide a 16/8 h (day/night) photoperiod with 25°C/21°C (day/night) temperatures at 70% RH. Plants were watered with RO water as needed to keep the soil moist, but not saturated (generally every two days). No fertilizers, soil amendments, or pesticides were applied to the plants at any time. Fly paper strips were hung directly over the cone-tainers in the growth chamber to monitor for pest infestations, however no pests were ever observed on the fly paper or the plants themselves. At time of collection, the plants were healthy and approximately 4" to

6” in height. Roots were well established, with most plants having root systems extending to the bottoms of their cone-tainers.

Collection of Root Tissue Samples

Due to time requirements, it was not possible to collect and disinfect root tissue samples from all of the plants simultaneously, therefore sampling had to be performed in daily “batches”. The first sampling batch consisted of five plants grown in the commercial growing medium (Scott’s® MetroMix® 366). Root tissue samples were collected by first cutting the cone-tainers in half longitudinally with scissors so that plants could be removed without damaging the root system. Once removed, all soil was cleaned from the root system by hand under a stream of RO water. Finally, clean scissors were used to cut two 0.1 g samples of root tissue from each plant. These tissue samples were placed in 2.0 mL sterile microcentrifuge tubes and immediately subjected to the surface disinfection protocol described below.

After the MetroMix® 366 samples were collected and surface disinfected, concern was raised regarding the order of sample collection. If each daily sampling batch consisted of all of the plants from the same soil or growing medium, then some soils would be represented by samples taken many days later than other soils. Thus, a change in the growth chamber environment during the sampling collection period could affect tissue samples collected from plants grown in some soils, but not others. Due to this concern, it was decided that the constitution of the daily sampling batches should be changed to one plant from each soil rather than all the plants from a single soil. Therefore, all plants grown in the six unamended natural soils were sampled in a series of eleven batches (taken over eleven days, one batch per day), with each sampling batch

consisting of a single plant representative of each soil. Root samples collected from these plants were taken as described above for the MetroMix® 366-grown plants. When possible, two 0.1 g root samples were collected from each plant and surface disinfected as described below. In a few rare cases, the entire root mass of a plant was less than 0.1 g. In this situation, the entire root system was collected as a single sample representative of that plant.

Surface Disinfection of Root Tissue

Each 0.1 g root tissue sample was placed into sterile 2.0 mL microcentrifuge tube. A 2.0 mL volume of surface disinfection solution (a filter-sterilized aqueous solution containing 10% commercial bleach and 10% EtOH in QH_2O) was then added to the tube. The root tissue was washed in this solution with agitation by vortexing at 1400 RPM for 10 minutes at 25°C using a Thermomixer R. Once complete, the rinsate was removed aseptically by aspiration in a laminar flow hood.

The tissue was then rinsed thrice with 2.0 mL of sterile QH_2O, added aseptically in the laminar flow hood. Each rinse was performed by vortexing at 1400 RPM for 1 minute at 25°C using a Thermomixer R. Each volume of rinsate was removed aseptically by aspiration within a laminar flow hood.

Following the third QH_2O wash, 2.0 mL of DNAse buffer solution was added aseptically to the tube. Next, 4 µL of Qiagen® RNAse A and 2 µL of Qiagen® DNAse I were added to the tube and mixed by vortexing briefly. Tubes were then incubated at 37°C for 75 minutes to allow for sufficient endonuclease activity. Finally, a subsequent incubation at 80°C for 10 minutes was used to inactivate the DNAse and RNAse present in the tube.

To allow for verification of surface disinfection efficacy by PCR, a 1.0 mL aliquot of the final DNAse-treatment rinsate was collected aseptically into a sterile 1.5 mL microcentrifuge tube and stored at -20°C. The remaining volume of DNAse-treatment rinsate was removed aseptically by aspiration.

Extraction of Total DNA from Surface Disinfected Root Tissue

Following surface disinfection, total DNA was extracted and purified from the treated root tissues using a Qiagen® DNeasy® Plant Mini kit according to the manufacturer's instructions. All portions of the protocol which required exposure of the tissue to the laboratory environment were carried out aseptically within a laminar flow hood. Final eluted DNA solutions were stored at -20°C.

Verification of Surface Disinfection Efficacy by PCR

Surface disinfection was verified by lack of amplification resulting from PCR utilizing a 1 µL aliquot of the final DNAse-treatment rinsate taken by sterile pipette at the conclusion of the surface disinfection protocol as template. Reaction conditions using the primers F968 and R1401/1378GC were as described previously in Surface Disinfection Experiment 1. Positive control reactions were included which substituted equal volumes of genomic DNA purifications from *Bacillus megaterium, Escherichia coli* XL-10 Gold, *Agrobacterium rhizogenes* ATCC 15834, and *Agrobacterium tumefaciens*, respectively, for the final DNAse buffer solution template. A negative control reaction was also included which lacked any added template.

An aliquot (10 µL) of each PCR amplification product was examined by electrophoresis for the presence of bands amplified from disinfected tissue samples. Any

such bands would indicate insufficient surface disinfection of that sample. Tables 3 and 4, below, summarize the results. Images of the agarose gels can be found in Appendix 1.

Metro Mix 366-Grown Plants
(Sampling Batch 1)

	Plant				
Sample No.	**A**	**B**	**C**	**D**	**E**
1	-	-	-	-	-
2	-	-	-	-	-

Table 3: Results of PCR-based verification of surface disinfection efficacy for plants grown in MetroMix® 366. A "-" sign indicates no amplification. None of the samples yielded amplification from PCR utilizing the final DNAse-treatment rinsate as template, indicating that all were sufficiently surface disinfected.

Unamended Natural Soil-Grown Plants

		Sampling Batch										
Soil	**Sample No.**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
WLB	1	+	+	+	-	-	-	-	-	-	-	-
	2	n/a	+	+	-	-	-	-	-	-	-	n/a
WDW	1	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-
KNS	1	+	+	+	-	-	-	-	-	-	-	-
	2	+	+	+	-	-	-	-	-	-	-	-
STW	1	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-
PAW	1	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	n/a	n/a
GDW	1	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-

Table 4: Results of PCR-based verification of surface disinfection efficacy for plants grown in unamended natural soils. A "-" sign indicates no amplification, and indicates successful surface disinfection of the sample. A "+" sign indicates the presence of an amplification product following PCR, thus indicating unsatisfactory results for surface disinfection of the sample. The "n/a" designation indicates a sample not obtainable due to insufficient root mass. Soils are abbreviated as follows: Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), and Goodwell (GDW).

Samples which yielded an amplification product following PCR (indicated by a "+" in Tables 3 and 4, above) were discarded due to unsatisfactory surface disinfection. A 5 µL aliquot of the total root DNA extract was withdrawn from each satisfactory sample and pooled with like aliquots (those originating from plants grown in the same soil) and stored at -20°C for use as template for PCR amplification of the bacterial 16s rRNA gene.

Amplification of Full-Length 16S rDNA from Surface Disinfected Roots

The pooled total DNA extracts were used as templates for PCR using the 16S-27f (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and 16S-1525r (5'-AAGGAGGTG(AT)TC CA(AG)CC-3') domain *Bacteria*-specific "universal" primers designed for sequencing of the 16S rRNA gene described by Lane (1991). Primers were synthesized by Integrated DNA Technologies Inc., Coralville, IA. Figure 5, below depicts the annealing sites for these primers with respect to the *E. coli* 16S rRNA gene.

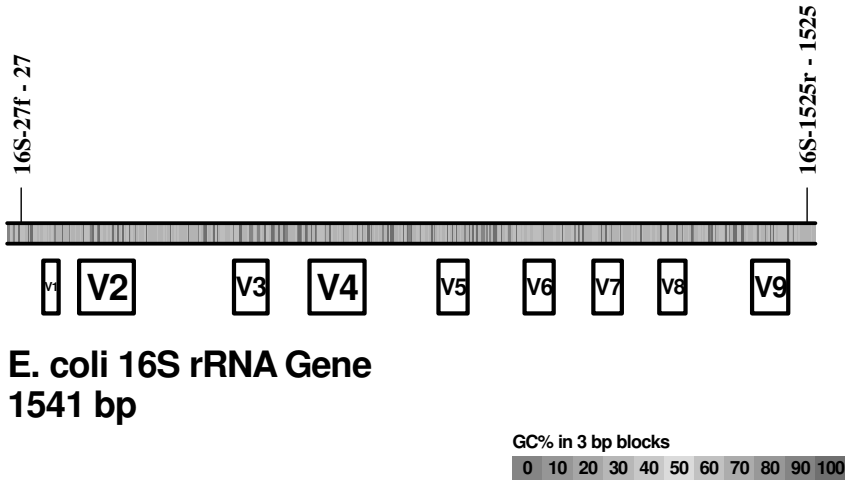

Figure 5: Location of annealing sites for the 16S-27f / 16S-1525r primer pair with respect to the *Escherichia coli* 16S rRNA gene sequence reported by Ehresmann et al. (1972). The approximate locations of the nine hypervariable regions valuable for identification and phylogenetic purposes are also indicated (Neefs et al., 1990; Chakravorty et al., 2007). Illustration created using pDraw32 (Acaclone Software, 2007).

PCR was performed in 100 µL reaction volumes containing the following: 10 µL of BioLine® 10X KCl reaction buffer (BioLine USA, Inc., Taunton, MA), 12 µL of 25 mM $MgCl_2$, 8 µL of dNTP mix (2.5 mM each), 12 µL of 2.5 µM primer 16S-27f, 12 µL of 2.5 µM primer 16S-1525r, 4 µL of BioLine® *Taq* DNA polymerase (5 U/µL), 5 µL of total DNA template, and sterile QH_2O to volume. Additionally, one reaction substituting 5 µL of DNA template extracted from the unknown bacteria surviving surface disinfection was prepared, as were positive control reactions which substituted 1 µL of template from purified genomic DNA solutions of *B. megaterium*, *E. coli*, *A. rhizogenes*, and *A. tumefaciens*. A negative control reaction containing no added template was also included in the PCR.

Reactions were performed using an MJ Research® PTC-200 thermal cycler programmed for an initial denaturation step of 94°C for 3 min, followed by 35 amplification cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min with a final extension at 72°C for 10 min. To increase specificity, a simulated "hot start" was performed as described previously. Aliquots (10 µL) of the PCR amplification products were examined by electrophoresis.

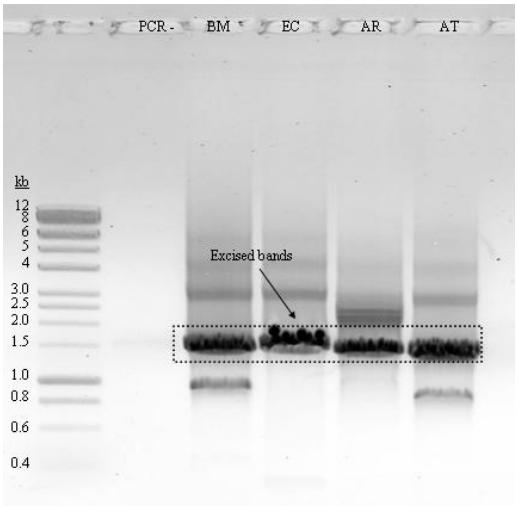

Figure 6a: Full-length 16S rDNA amplification products. The standard lane contained GeneChoice® DNA Ladder I. Dotted lines indicate major product bands that were excised and eluted. This gel included the PCR negative control (PCR -) and positive controls using templates from *E. coli* (EC), *B. megaterium* (BM), *A. rhizogenes* (AR), and *A. tumefaciens* (AT). Electrophoresis was performed at 200 V for 25 min using a 0.5% agarose, 1X sodium borate (pH 8.5) gel containing 0.5 µg/mL EtBr.

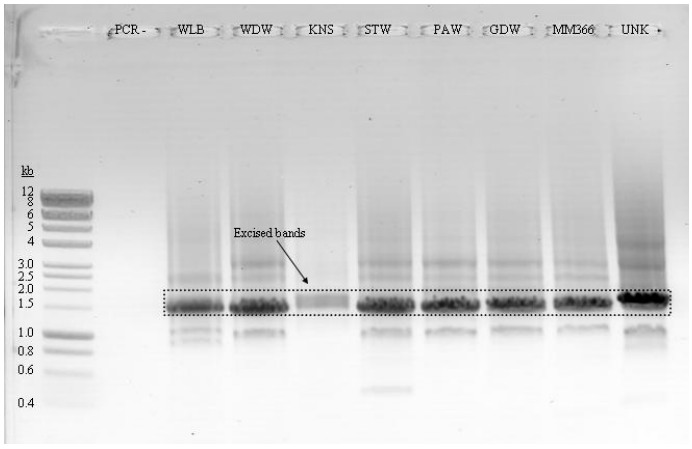

Figure 6b: Full-length 16S rDNA amplification products. The standard lane contained GeneChoice® DNA Ladder I. Dotted lines indicate major product bands that were excised and eluted. This gel included the PCR negative control (PCR -) and amplification products from total DNA extracts of surface disinfected *M. truncatula* roots grown in Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), and Goodwell (GDW) soils, as well as the MetroMix® 366 (MM366) soilless growing medium. "UNK" indicates the amplification product from the unknown surface disinfection-resistant bacteria. Electrophoresis was performed at 200 V for 25 min using a 0.5% agarose, 1X sodium borate (pH 8.5) gel containing 0.5 µg/mL EtBr.

While amplification yields were excellent, additional bands were present as a result of non-specific amplification. Thus, it was necessary to purify the major product band from each reaction by band excision and elution using a Qbiogene® GeneClean® *Turbo* kit (MP Biomedicals, Irvine, CA) according to the manufacturer's instructions. Excised bands are represented by dotted lines in Figures 6a and 6b, above. Gel elution products were stored at -20°C.

The remaining 90 µL volumes of amplification products were purified by the method described above. Each volume was divided into two equal aliquots and loaded onto consecutive lanes for electrophoresis, followed by band excision and DNA elution. Like bands were pooled into a single product during the elution procedure. Finally, these elution products were pooled with like samples previously eluted (see above) and stored at -20°C. Excised bands are represented by dotted lines in Figure 7, below.

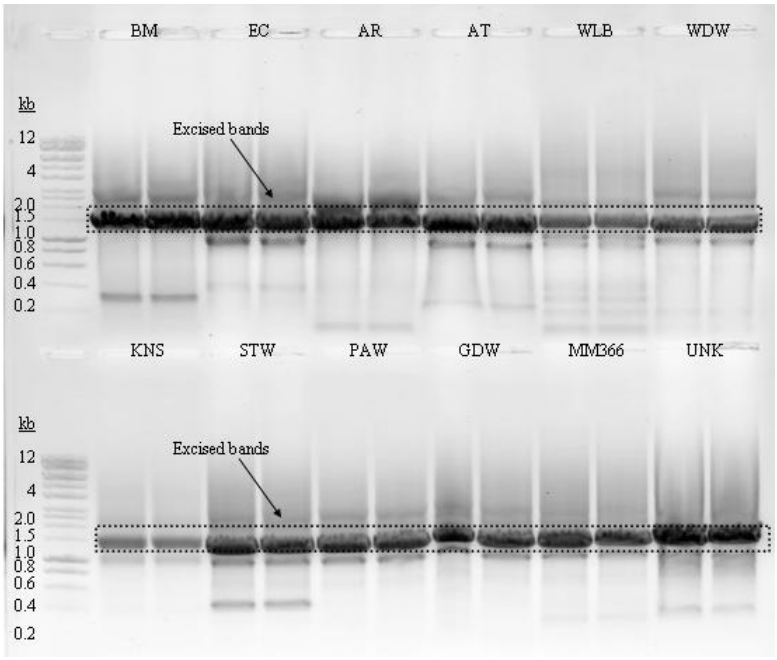

Figure 7: Full-length 16S rDNA amplification products. Standard lanes contained GeneChoice® DNA Ladder I. Dotted lines indicate major product bands that were excised and eluted. This gel included the PCR negative control (PCR -), positive controls using templates from *E. coli* (EC), *B. megaterium* (BM), *A. rhizogenes* (AR), and *A. tumefaciens* (AT), and total DNA extracts taken from surface disinfected *M. truncatula* roots grown in Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), and Goodwell (GDW) soils, as well as the MetroMix® 366 (MM366) soilless growing medium. "UNK" indicates the unknown surface disinfection-resistant bacteria. Electrophoresis was performed at 200 V for 25 min using a 0.5% agarose, 1X sodium borate (pH 8.5) gel, containing 0.5 µg/mL EtBr.

A 1 µL aliquot was removed from each pooled elution product and evaluated for quality by agarose gel electrophoresis (see Figure 8, below). An additional 1 µL aliquot was used for analysis of DNA purity and concentration using a NanoDrop™ ND-1000 UV/Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA).

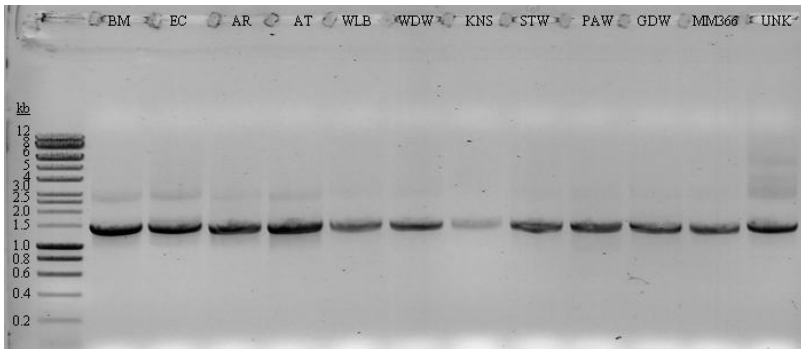

Figure 8: Electrophoresis of pooled elution products. Sample lanes contained 1 µL of product. GeneChoice DNA Ladder I was used as a standard. Abbreviation used in lane titles are as follows: *E. coli* (EC), *B. megaterium* (BM), *A. rhizogenes* (AR), *A. tumefaciens* (AT), Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), and Goodwell (GDW), MetroMix® 366 (MM366), and unknown surface disinfection-resistant bacteria (UNK). Electrophoresis was performed at 200 V for 25 min using a 0.5% agarose, 1X sodium borate (pH 8.5) gel, containing 0.5 µg/mL EtBr.

Production of Competent Cells for Cloning

The XL-10 Gold® ultracompetent genotype of *Escherichia coli* (Stratagene Cloning Systems Inc., La Jolla, CA) was selected as a host for the plasmid vector. Competent cells were prepared from a -80°C stock culture by the heat-shock method described by Hanahan (1983). Competent cells produced by this method were divided into 50 µL aliquots in sterile screw-capped microcentrifuge tubes, flash frozen over liquid N_2 and stored at -80°C.

A-Tailing of PCR Amplified Full-Length 16S rRNA Gene Inserts

A-tailing of each of the gel-eluted full-length 16S rDNA PCR amplification products was performed in a 10 µL reaction volume containing 0.5 µL of 50 mM $MgCl_2$, 1 µL of 2.5 mM dATP, 1 µL of BioLine® *Taq* DNA polymerase (5U/µL), 1 µL of BioLine® 10X KCl reaction buffer, a volume of amplification product solution sufficient

to provide 23.3 ng/µL in the final reaction mixture, and sterile Q H_2O to volume. Reactions were incubated for 30 minutes at 70°C, then stored at 4°C until used for the ligation performed shortly thereafter.

Ligation of A-Tailed Inserts to the pGEM®-T Easy Cloning Vector

The Promega® pGEM®-T Easy cloning vector (Promega Corp., Madison, WI) was chosen for this work because it is supplied by the manufacturer pre-prepared for ligation by cutting with *Eco*R V and addition of a 3' terminal thymidine overhang to each blunt end (Promega Corp., 2003), thus eliminating the need to perform restriction and T-tailing in the laboratory. A map of the vector is presented below in Figure 9.

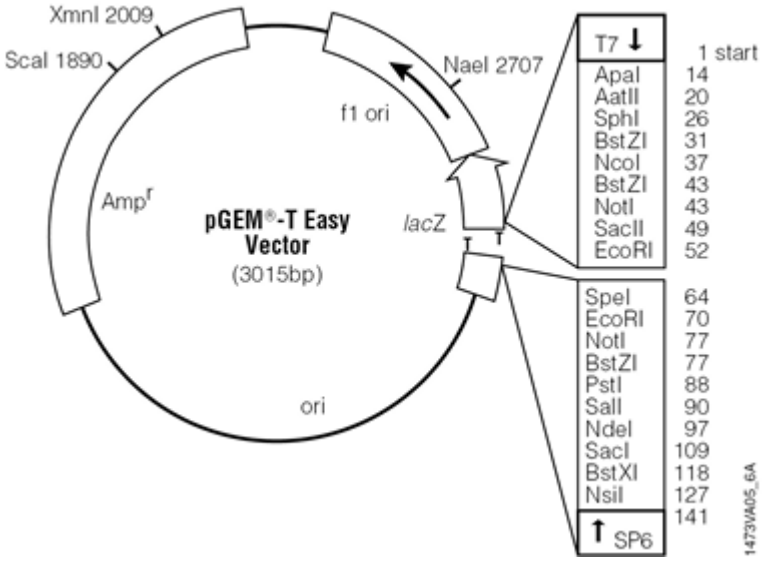

Figure 9: Map of the Promega pGEM®-T Easy vector. Courtesy of Promega Corp (2003).

Ligation reactions were performed as directed by the manufacturer's instructions, using a 1:1 insert:vector molar ratio. A positive control reaction was included in the ligation which substituted 2 µL of Promega® Control Insert DNA (kit supplied) for the A-

tailed insert. A negative control reaction containing no insert DNA was also included. Reactions were mixed by pipetting then incubated overnight at 4°C.

Transformation of *E. coli* XL-10 Gold® with pGEM®-T Easy Constructs

E. coli XL-10 Gold® was transformed with the pGEM®-T Easy vector ligation products. Transformation was performed by the heat-shock method. For each transformation, a 50 µL aliquot of competent *E. coli* XL-10 Gold was thawed on ice and mixed with a 5 µL aliquot of ligation product. Cells suspensions were incubated on ice for 60 min, then heat-shocked by transfer to a 42°C water bath for 90 sec, followed by a return to ice for 2 min. A 0.8 mL volume of SOC medium (2% tryptone, 0.5% yeast extract, 8.5 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose at pH 7) was then added to the suspension, followed by an incubation period at 37°C for 1 hour with gentle agitation at 800 RPM using a Thermomixer R. Two control transformations were included in the transformation procedure. A "vector control" transformation was performed using 1 µL of the pGEM®-5Zf(+) midipreparation product, and a "no-vector control" transformation was created by omitting the addition of any plasmid to one competent cell culture.

Following incubation, 100 µL aliquots were removed from each cell suspension and spread to four LB+tet50, cam20, amp200 (Luria-Bertani media containing 50 µg/mL tetracycline, 20 µg/mL chloramphenicol, and 200 µg/mL ampicillin) plates having surfaces treated with 2 µL of 20% IPTG and 100 µL of 2% X-Gal for blue/white screening.

An additional 100 µL inoculation from each cell suspension was made to a control plate containing media identical to that described above, except lacking

ampicillin. These plates were included to verify that the competent cells remained viable through the heat-shock protocol, regardless of transformation success. All inoculated plates were incubated at 37°C for 24 hours followed by screening for blue/white colonies.

Blue/White Screening of *E. coli* XL-10 Gold® pGEM®-T Easy Clones

Following incubation, the inoculated plates were observed for growth and screened for white colonies indicating successful transformation with vector containing the full-length 16S rRNA gene insert. Screening results are summarized below in Table 5. Plates were sealed with Parafilm M® (Alcan Packaging, Inc., Neenah, WI) and stored at 4°C during construction of the clonal library described below.

Sample	Plate Number							
	LB + tet50, cam20, amp200				LB + tet50, cam20			
	1	2	3	4	1	2	3	4
BM	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
EC	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
AR	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
AT	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
WLB	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
WDW	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
KNS	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
STW	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
PAW	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
GDW	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
MM366	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
UNK	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white	blue/white
Promega Control Insert	blue/white				blue/white			
No Insert Control	blue				blue/white			
Vector Control	blue				blue/white			
No Vector Control	no growth				white			

Table 5: Blue/white screening of *E. coli* XL-10 Gold®. The following abbreviations are used in the chart: *E. coli* (EC), *B. megaterium* (BM), *A. rhizogenes* (AR), *A. tumefaciens* (AT), Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), Goodwell (GDW), MetroMix® 366 (MM366), and unknown surface disinfection-resistant bacteria (UNK).

Verification of Successful Ligation and Transformation by PCR

To make absolutely certain that transformation and ligation were successful prior to library construction, as well as to verify that inserts of the appropriate size were ligated into the pGEM®-T easy vector, stabs were made of seven white colonies representing clones putatively carrying an *A. rhizogenes* 16S rDNA insert using a sterile pipette tip. The pipette tips were then swirled briefly in 20 µL PCR reaction mixtures to provide bacterial cells for direct PCR amplification using the FpGEM (5'-CGACTCACTATAGGGCGAATTG-3') and RpGEM (5'-CTCAAGCTATGCATCCAACG-3') primers provided by Dr. Anderson.

These primers are designed specifically for sequencing of inserts cloned into pGEM®-5Zf(+)-based vectors and anneal to sites flanking the MCS of the pGEM®-T Easy vector (see Figure 10, below). With no insert present, these primers would amplify a 92 bp region of the vector which includes the MCS and *Eco*R V recognition site. If present, any insert would be amplified along with short segments of vector DNA flanking the MCS. Figure 10, below, is provided to illustrate the primer annealing sites with respect to the pGEM®-T Easy vector.

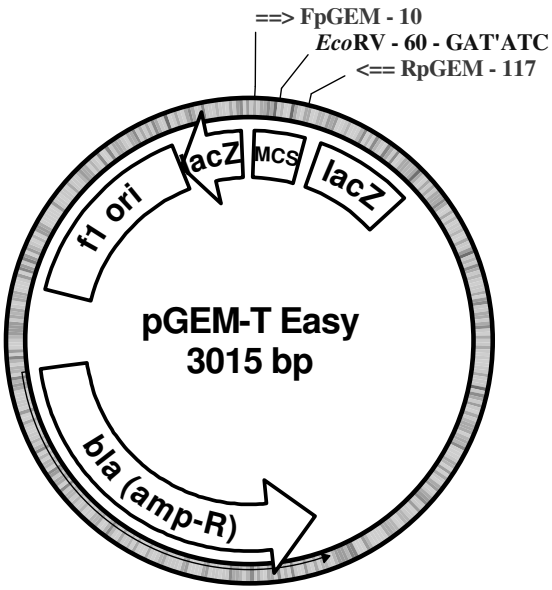

Figure 10: Annealing sites of the FpGEM / RpGEM primer pair to the pGEM®-T Easy Vector. Image was created using pDraw32 (Acaclone Software, 2007).

Each 20 µL PCR reaction mixture contained 2.0 µL of BioLine® 10X KCl Reaction Buffer, 2.4 µL of 25 mM $MgCl_2$, 1.6 µL of dNTP mix (2.5 mM each), 2.4 µL of 2.5 µM primer FpGEM, 2.4 µL of 2.5 µM primer 16S-1525r, 0.8 µL of BioLine® *Taq* DNA polymerase (5 U/µL), and sterile QH_2O to volume.

Additional control reactions were also included. The first utilized 1 µL of a cell suspension of untransformed *E. coli* XL-10 Gold® taken from a stock culture as template. This control was included to ensure that the primer pair did not amplify any *E. coli* genomic DNA similar in size to the insert. A second control reaction contained the pGEM®-5Zf(+) vector with no insert as template. This vector is almost identical in sequence to the pGEM®-T Easy vector used for transformation, and was included to provide an amplified sequence from the vector with no insert as a reference point for

comparison during examination by agarose gel electrophoresis. The final control reaction was a PCR negative reaction to which no template was provided.

Reactions were performed using an MJ Research® PTC-200 thermal cycler programmed for an initial denaturation step of 94°C for 3 min, followed by 35 amplification cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min with a final extension at 72°C for 10 min. To increase specificity, a simulated "hot start" was performed by the method described previously. Amplification products were examined by electrophoresis.

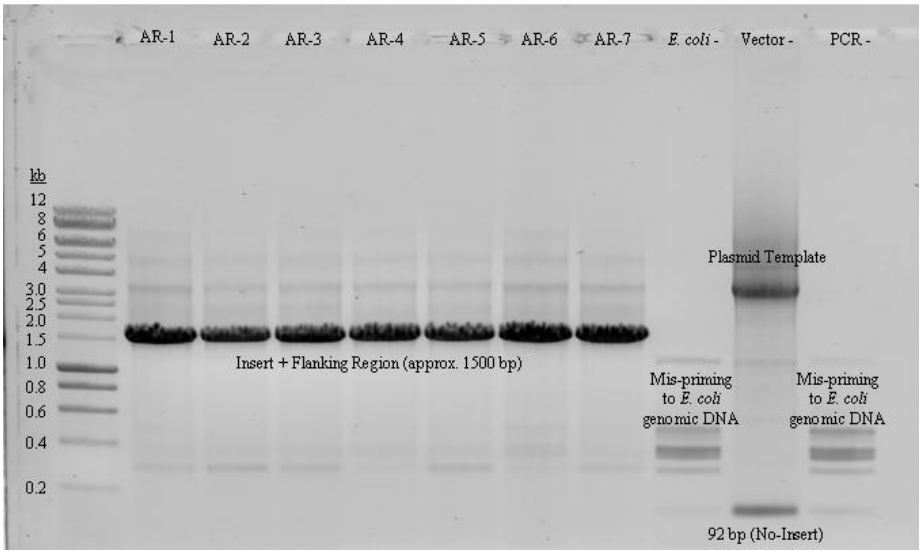

Figure 11: Agarose gel electrophoresis of products (10 µL each lane) from PCR amplification of inserts in the pGEM®-T Easy vector. Seven white colonies of *E. coli* XL-10 Gold putatively transformed with pGEM®-T Easy containing *A. rhizogenes* 16S rRNA gene inserts were selected at random during blue/white screening and picked to provide template for direct PCR (lanes designated AR 1 through 7). Control reactions included in the experiment were created using untransformed *E. coli* XL-10 Gold (*E. coli* -) and pGEM®-5Zf(+) vector with no insert (vector -), respectively, as templates. No template was provided in the PCR negative control reaction (PCR -). The standard lane contains GeneChoice® DNA Ladder I. Electrophoresis was performed at 200 V for 25 min using a 0.5% agarose, 1X sodium borate (pH 8.5) gel containing 0.5 µg/mL EtBr.

Electrophoresis provided confirmation of successful ligation and transformation. Bands indicative of an appropriately sized insert and flanking DNA (approximately 1500 bp) can be seen in each of the seven "AR" sample lanes. The untransformed *E. coli* XL-10 Gold control yielded only minor amplification from mispriming to genomic DNA, as did the PCR negative control reaction containing no template with the exception of *E. coli* genomic DNA typically present in the *Taq* DNA polymerase solution itself. Amplification of pGEM®-5ZF(+) containing no insert yielded the expected low molecular weight band representing 92 bp of DNA flanking the *Eco*R V recognition sequence within the vector MCS. Together, these results indicated that ligation and transformation were successful, and construction of the clonal library was commenced.

Clonal Library Construction

Library construction was performed by selecting 200 isolated white colonies at random from the four plates of LB + tet50, cam20, amp200 (Luria-Bertani media containing 50 µg/mL tetracycline, 20 µg/mL chloramphenicol, and 200 µg/mL ampicillin) representing each cloned insert. Each colony was picked under a laminar flow hood using a sterile toothpick to a 10 mL culture tubes containing 2.0 mL of LB + tet50, cam20, amp200. The 2.0 mL broth cultures were incubated overnight at 37°C with mild agitation in a platform incubator/shaker.

Following overnight growth, a 1.0 mL aliquot was transferred from each 2.0 mL broth culture to sterile a 1.5 mL microcentrifuge tube. An equal volume of sterile glycerol freezing solution was then added to each culture and mixed by vortexing. Cell suspensions were then stored at -80°C.

For automated sequencing purposes, it was necessary to transfer the clonal library to 384 well microplates. Each 2.0 mL cell suspension was removed from -80°C storage, thawed on ice, and vortexed briefly to ensure adequate mixing. Two 75 µL aliquots were removed and transferred to duplicate sterile 384 well microplates. The microplates were then stored at -80°C. In this manner, two duplicate copies of the library were created. One copy was used for sequencing purposes, while the second was retained on-site as a backup in -80°C storage in case of future need. The identification keys for the stored 384 well microplate library can be found in Appendix 2.

Sequencing of Full-Length 16S rDNA Inserts

As Oklahoma State University did not possess the high-throughput sequencing (HTS) capacity for a sample set of this magnitude, it was necessary to have the sequencing performed elsewhere. Therefore, automated lysis, purification, contig construction, bi-directional cycle sequencing using the FpGEM and RpGEM primers, as well as initial BLAST® searching of the insert sequences was performed by Dr. Bruce A Roe's laboratory in the Biochemistry Division of the University of Oklahoma.

Each 16S rDNA insert was PCR-amplified from the *E. coli* XL-10 Gold® clonal library and incorporated into two contigs for mass sequencing. The paired contigs were then sequenced using the FpGEM and RpGEM primers, thus providing a forward and reverse sequence for each insert, each originating from one of the two contigs. Note that the terms "forward" and "reverse" are used here with respect to the full-length 16S rDNA insert. "Forward" sequences are those extending inward from the 5' end of the full-length 16S rDNA gene, while "reverse" sequences extend inward from the 3' end of the

gene. Thus, the "forward" and "reverse" sequences contain different regions of the 16S rDNA insert, and are not reverse-complements of each other (see Figure 12, below).

Sequences obtained from both primers were typically 650 – 750 bp in overall length, including 30 – 50 bp of 5' flanking vector sequence, thus providing approximately 600 – 700 bp of actual 16S rDNA insert sequence. The region sequenced using the FpGEM primer typically contained the V1 – V4 hypervariable regions and the 16S-27f primer annealing site. Use of the RpGEM primer typically provided sequence data for the V6 – V9 hypervariable regions and the 16S-1525r primer annealing site. The sequence of an interior region approximately 200 bp in length containing the V5 hypervariable region was not usually obtained. Figure 12, below, illustrates the approximate regions that were sequenced within each insert.

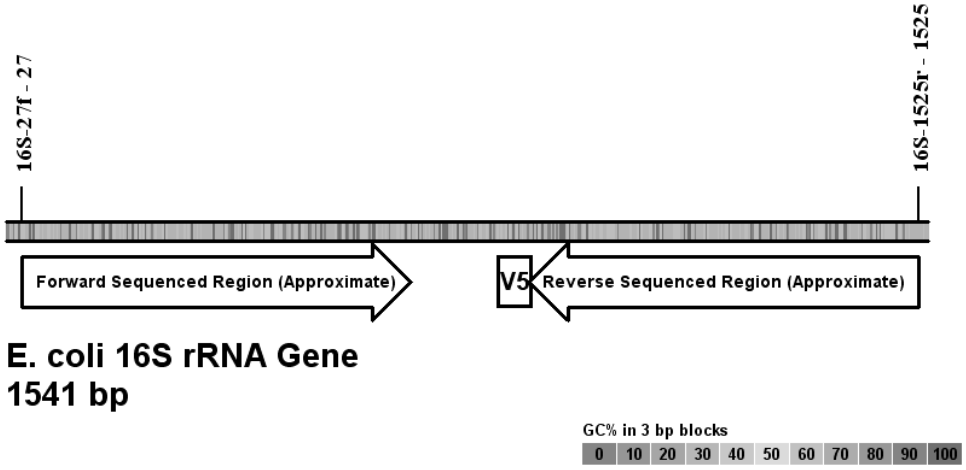

Figure 12: Typical sequenced regions of 16S rDNA inserts obtained using the FpGEM and RpGEM primers, with respect to the *Escherichia coli* 16S rDNA sequence reported by Ehresmann et al. (1972). Illustration created using pDraw32 (Acaclone Software, 2007).

Putative Identification of 16S rDNA Inserts

Following automated sequencing, the 16S-rDNA inserts in the clonal library were putatively identified by searching for positive matches to known sequences in the NIH (National Institutes of Health) GenBank® genetic sequence database maintained by the NCBI (National Center for Biotechnology Information) using the BLAST® (Basic Local Alignment Search Tool) algorithm (Altschul et al., 1997). Initial automated database searching was performed by Dr. Bruce Roe's laboratory at the University of Oklahoma.

Once results were received from Dr. Roe's laboratory, the sequencing and BLAST® data were reviewed for quality. Any insert sequence not yielding a BLAST® hit (so-called "No Hit" sequences), or producing hits less than 97% identity were rejected. These rejected insert sequences were then manually edited to remove any regions of vector and/or low-quality sequence data. The edited sequences were then queried against the GenBank® non-redundant database using the BLAST® algorithm. If an edited sequence yielded a new BLAST® hit with 97% or greater identity, that hit was considered valid and used for identification. However, if the edited sequence did not yield a BLAST® hit with a 97% or greater identity, then the sequence was classified as "No Hits" for identification purposes. Thus, identities of all sequences in the database were assigned based on BLAST® hits to database sequences sharing at least 97% identity with the queried insert sequences.

If BLAST® hits to the forward and reverse sequences of the same insert yielded the same identification with both hits having 97% or greater identities, then the insert was identified accordingly. However, because some sequencing reactions failed ("No Data" sequences) and because the strongest BLAST® hits to the forward and reverse sequences

of the same insert were not always in agreement, a set of rules had to be established for resolving such ambiguities when assigning a final identification to the insert. These rules are outlined below in Table 6.

Sequence A		Sequence B		Insert Identification Assigned
No Data	Versus	No Data	=	No Data
No Data		No Hits		No Hits
No Data		Hit (>97% ID)		Hit
No Hits		No Hits		No Hits
No Hits		Hit (>97% ID)		Hit
Unknown Hit (>97% ID)		Known Hit (>97% ID)		Known Hit
Known Higher Taxon (>97% ID)		Known Lower Taxon (>97% ID)		Lower Taxon
Genus A, Species A (>97% ID)		Genus A, Species B (>97% ID)		Genus Only
Hit A (>97% ID)		Hit B (>97% ID)		Hit with higher %ID If equal %ID, then hit with higher score. Verify by Clustal W Alignment.

Table 6: Rules for assigning overall identity to 16S rDNA inserts given various combinations of BLAST® results from queries of the forward and reverse sequences of each insert.

All final 16S rDNA insert identity assignments were checked by generating cladograms from ClustalW2 multiple sequence alignments (Larkin et al., 2007) of the forward and reverse sequences of each sample series (WLB, WDW, KNS, STW, PAW, GDW, MM366, and UNK). The cladograms can be seen in Appendix 4. Identifications appearing misplaced on both the forward and reverse sequence cladograms were manually edited to remove any regions of vector and/or low-quality sequence data. The edited sequences were then queried against the GenBank® non-redundant database using the BLAST® algorithm. Questioned identities were then altered or left unchanged as deemed appropriate, with the final decision dictated by BLAST® results and the rules for identity assignment described above in Table 6.

RESULTS AND DISCUSSION

Physical and Chemical Properties of Experimental Soils

The soils and growing medium used for this study varied widely with respect to origin and usage history. The Wilburton soil (WLB) was collected from the Jim Enis Farm on a steep mountainside in the Kiamichi range supporting a native undisturbed mixed pine/hardwood forest ecosystem. The soil was classified as a Carnasaw-Clebit-Denman clay loam (37.5% sand, 35.0% silt, 27.5% clay).

The Woodward (WDW) soil sample was collected from an improved pasture for beef cattle production created from native rangeland on the USDA/ARS Southern Plains Range Research Station. This soil was classified as a Pratt loamy fine sand, hummocky (70.0% sand, 17.5% silt, 12.5% clay).

The Kandas (KNS) soil sample was collected from the boundary between a beef cattle pasture and a native mixed hardwood forest on the Bill Smith Farm. However, the pasture itself was originally part of the forest, but had since been cleared, converted, and managed as rangeland for livestock production. This soil was classified as a Clarksville stony silt loam (27.5% sand, 62.5% silt, 10% clay).

The Stillwater (STW) soil sample was collected from an area on the Oklahoma State University Agricultural Resarch Station used for alfalfa production. The soil was classified as an Easpur loam (42.5% sand, 37.5% silt, 20.0% clay).

The Pawhuska (PAW) soil sample was collected from a historical native prairie on the Tallgrass Prairie Preserve. This preserve is managed for conservation of the native tallgrass prairie ecosystem that once existed across the midwestern United States. Herds

of bison are allowed to graze the prairie. The soil was classified as a Verdigris clay loam (32.5% sand, 40.0% silt, 27.5% clay).

The Goodwell (GDW) soil sample was collected from a plot area on the Oklahoma Panhandle Research and Extension Center used for the production of alfalfa overseeded with wheat. The soil was classified as a Richfield clay loam (30.0% sand, 42.5% silt, 27.5% clay).

Finally, Scott's® MetroMix® 366 (MM366) is a commercially-produced soilless growing medium. The precise blend of this peat-lite medium is proprietary, but is stated to include 35-50% sphagnum peat moss, 30-40% vermiculite, 15-25% pine bark, nutrients, and ground limestone.

Figures 13 through 25 show the results of fertility analysis of the soils and commercial growing medium as reported by the Soil, Water, and Forage Analytical Laboratory (SWFAL) at Oklahoma State University.

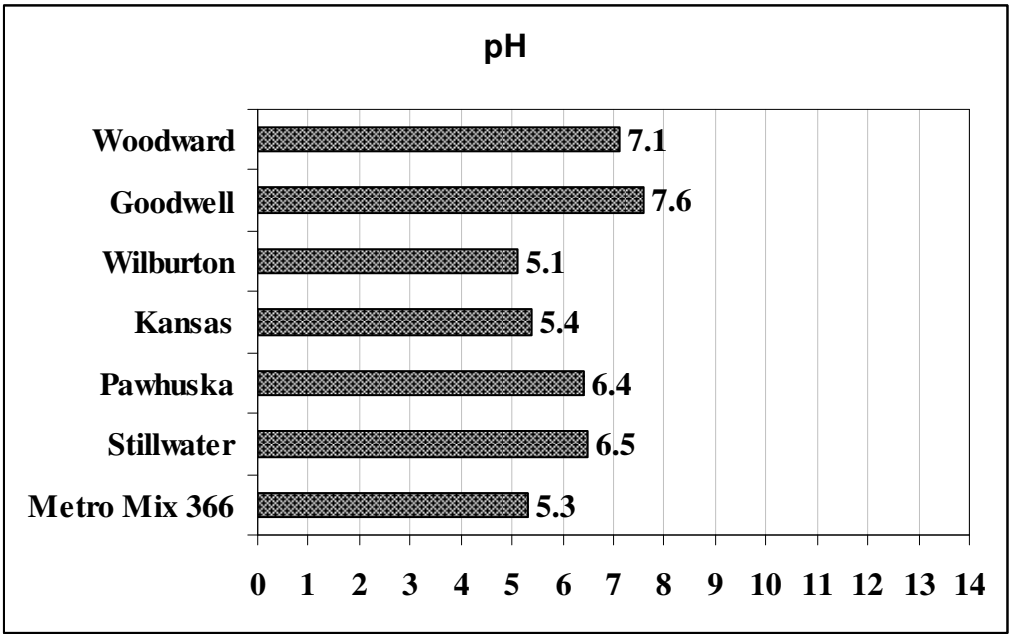

Figure 13. pH measurements of soil and soilless medium samples as reported by SWFAL.

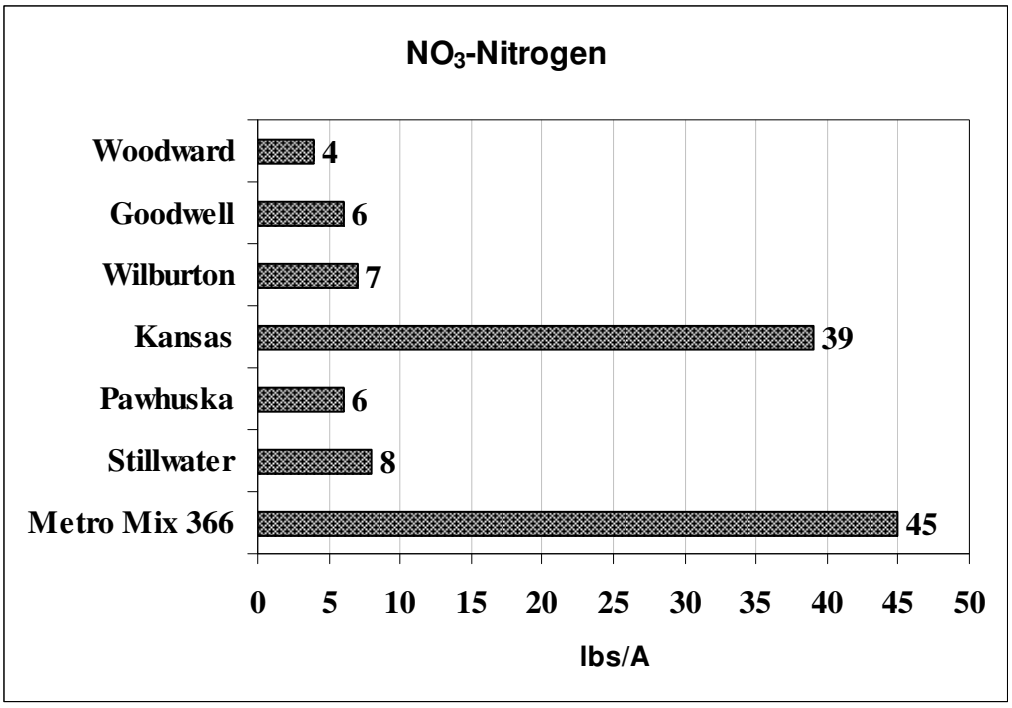

Figure 14. Analysis of nitrogen in the nitrate (NO_3) form in soil and soilless medium samples as reported by SWFAL (lbs/A = pounds per acre).

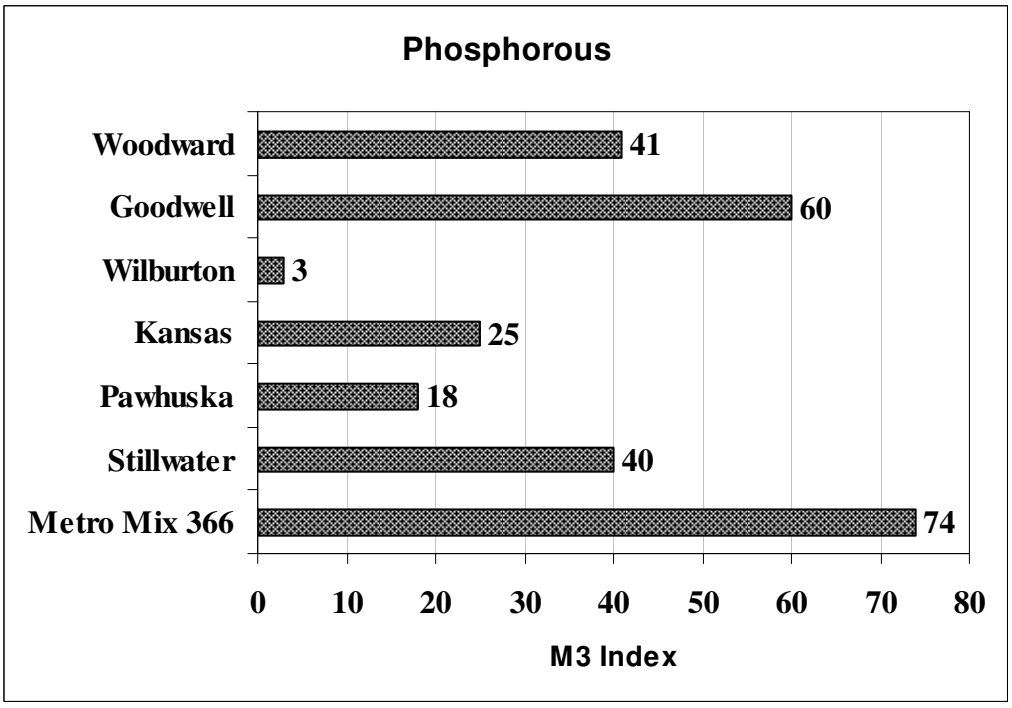

Figure 15. Analysis of plant-available phosphorous in soil and soilless medium samples using the Mehlich 3 test method as reported by SWFAL.

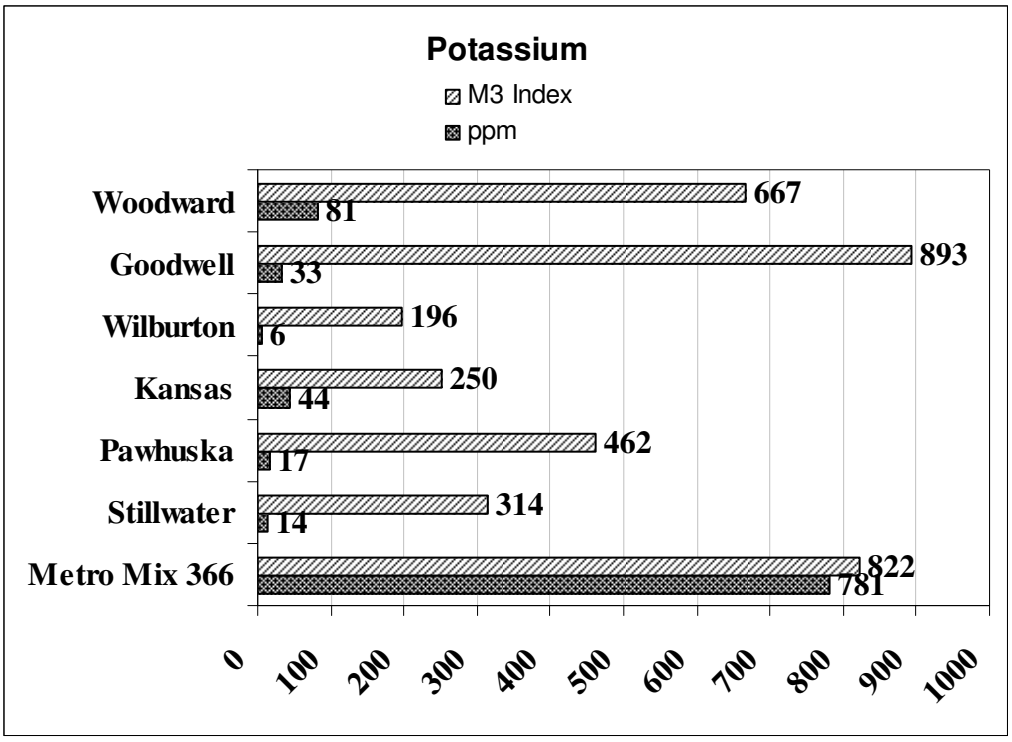

Figure 16. Analysis of plant-available (Mehlich 3 test method) and total soluble potassium in soil and soilless medium samples as reported by SWFAL (ppm = parts per million).

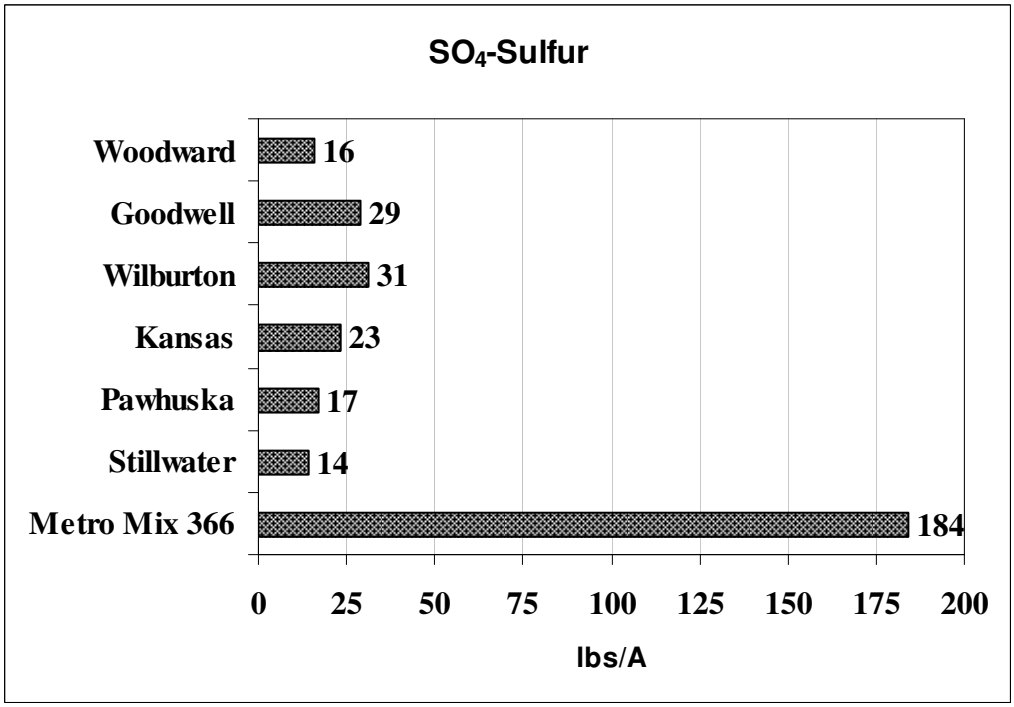

Figure 17. Analysis of sulfur in the sulfate (SO_4) form in soil and soilless medium samples as reported by SWFAL (lbs/A = pounds per acre).

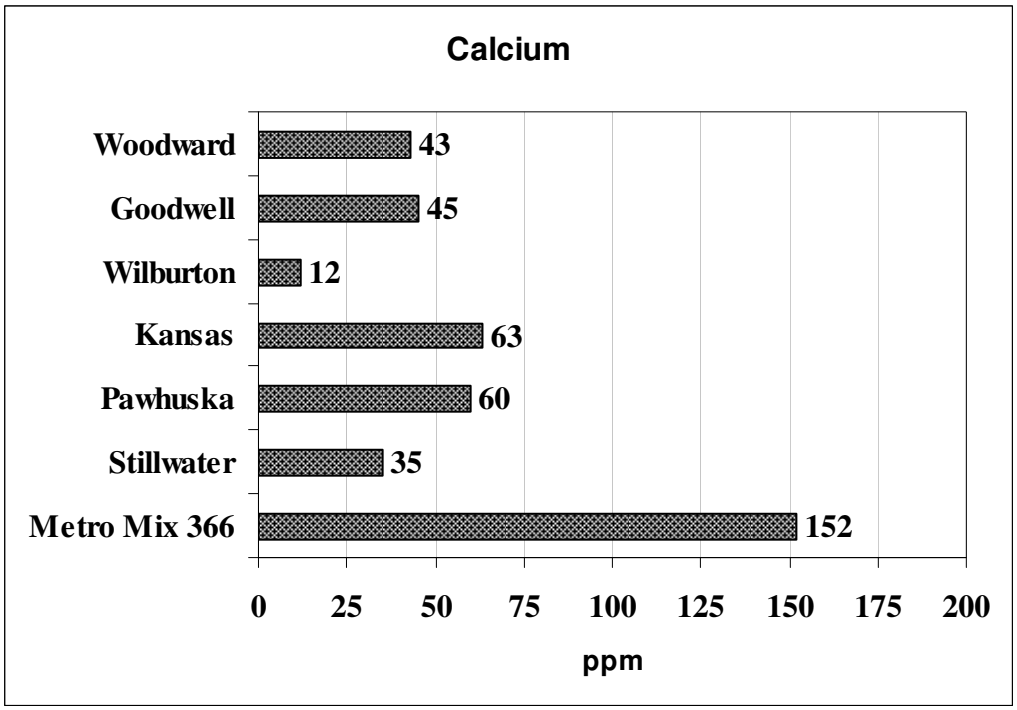

Figure 18. Analysis of calcium in soil and soilless medium samples as reported by SWFAL (ppm = parts per million).

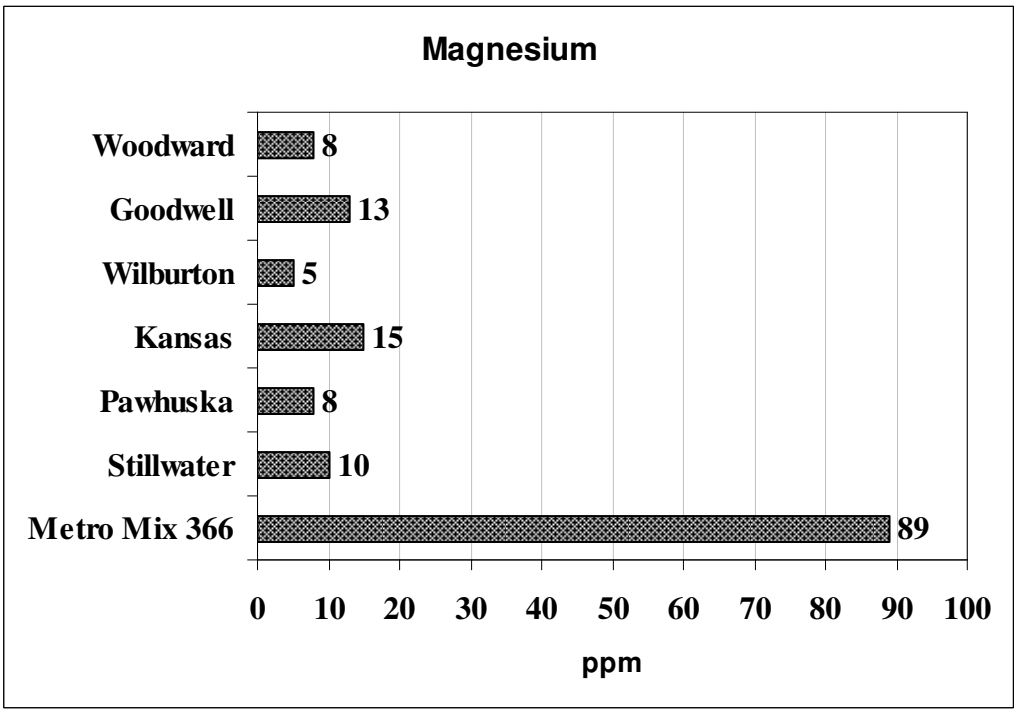

Figure 19. Analysis of magnesium in soil and soilless medium samples as reported by SWFAL (ppm = parts per million).

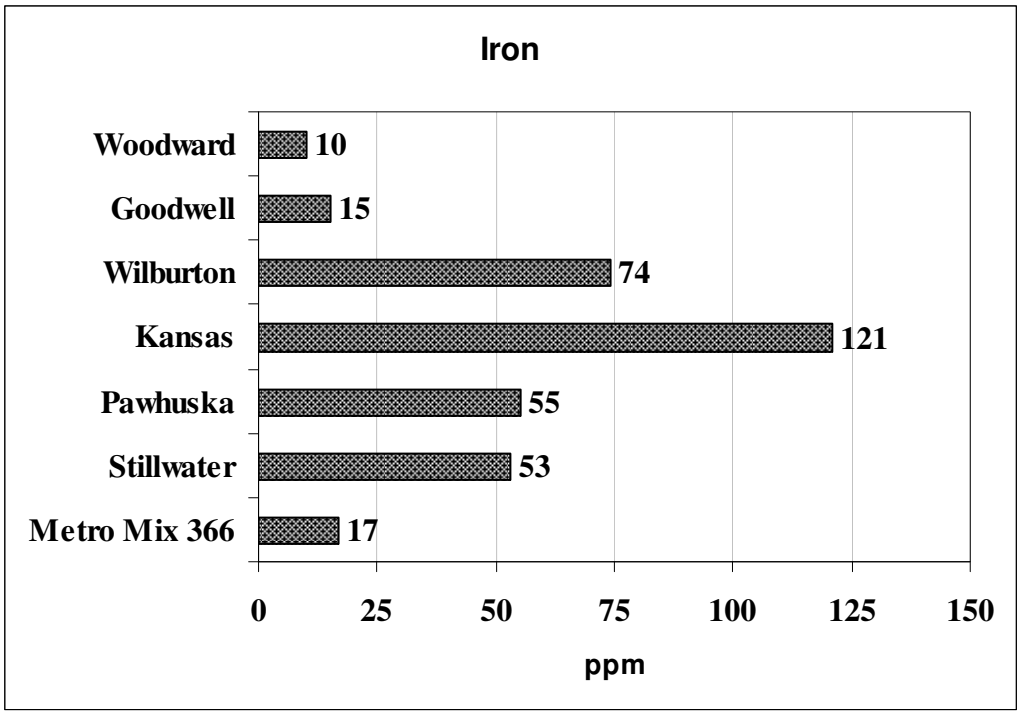

Figure 20. Analysis of iron in soil and soilless medium samples as reported by SWFAL (ppm = parts per million).

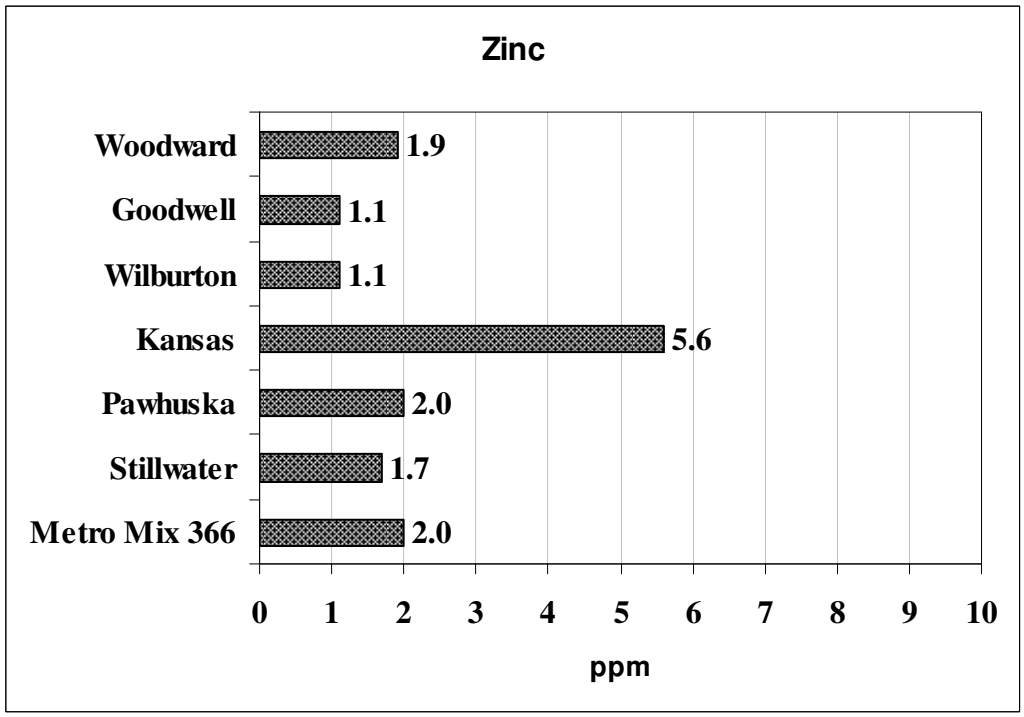

Figure 21. Analysis of zinc in soil and soilless medium samples as reported by SWFAL (ppm = parts per million).

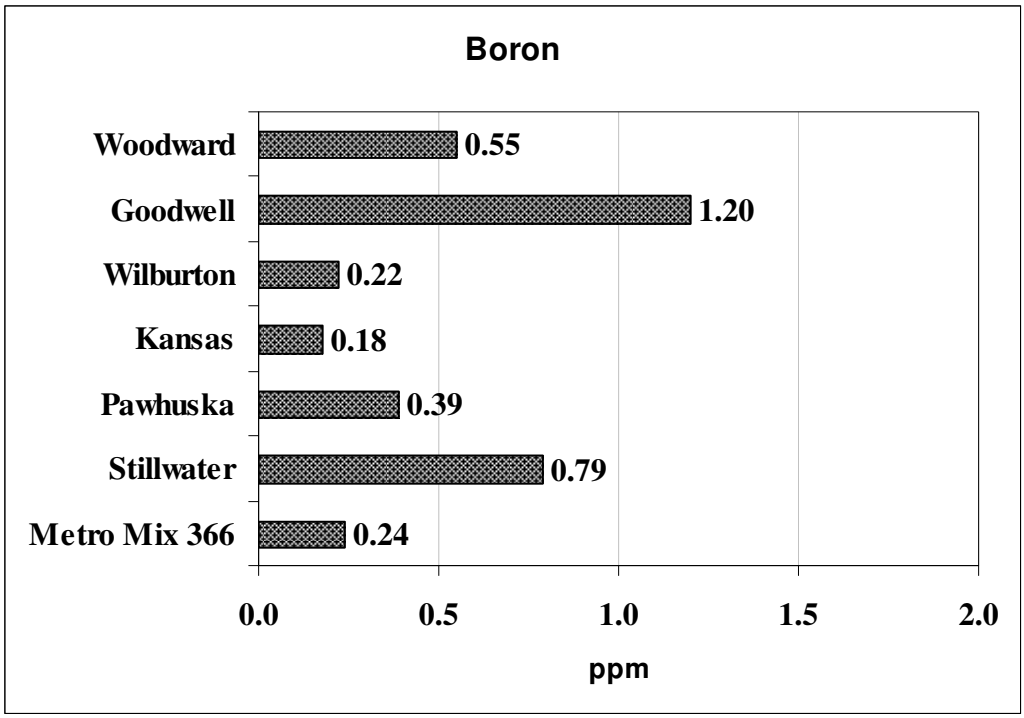

Figure 22. Analysis of boron in soil and soilless medium samples as reported by SWFAL (ppm = parts per million).

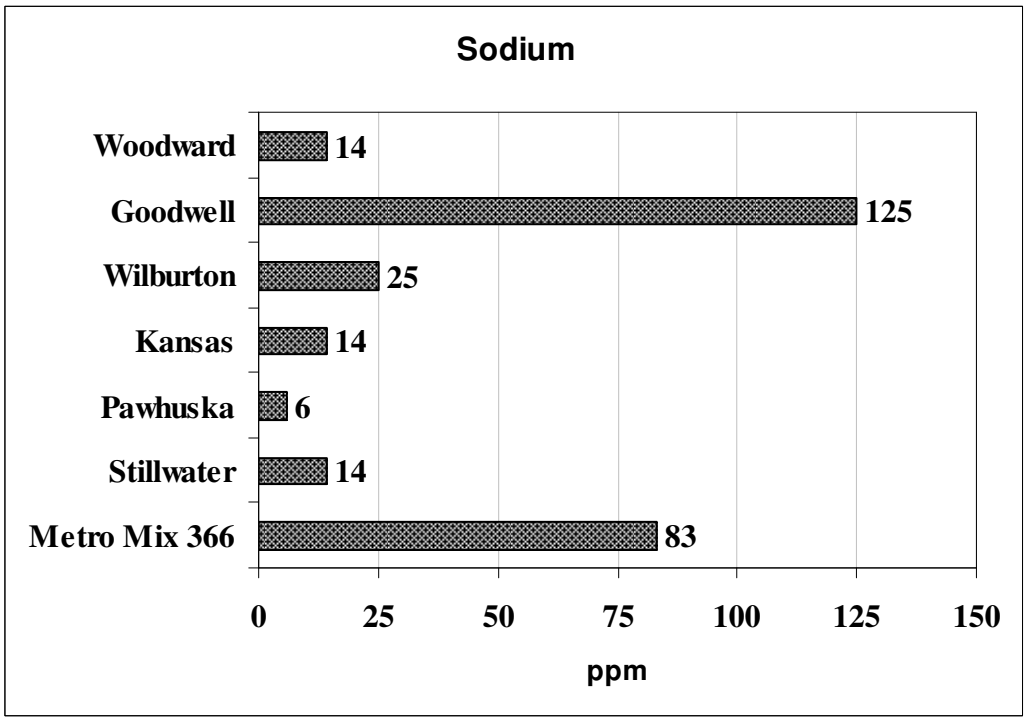

Figure 23. Analysis of sodium in soil and soilless medium samples as reported by SWFAL (ppm = parts per million).

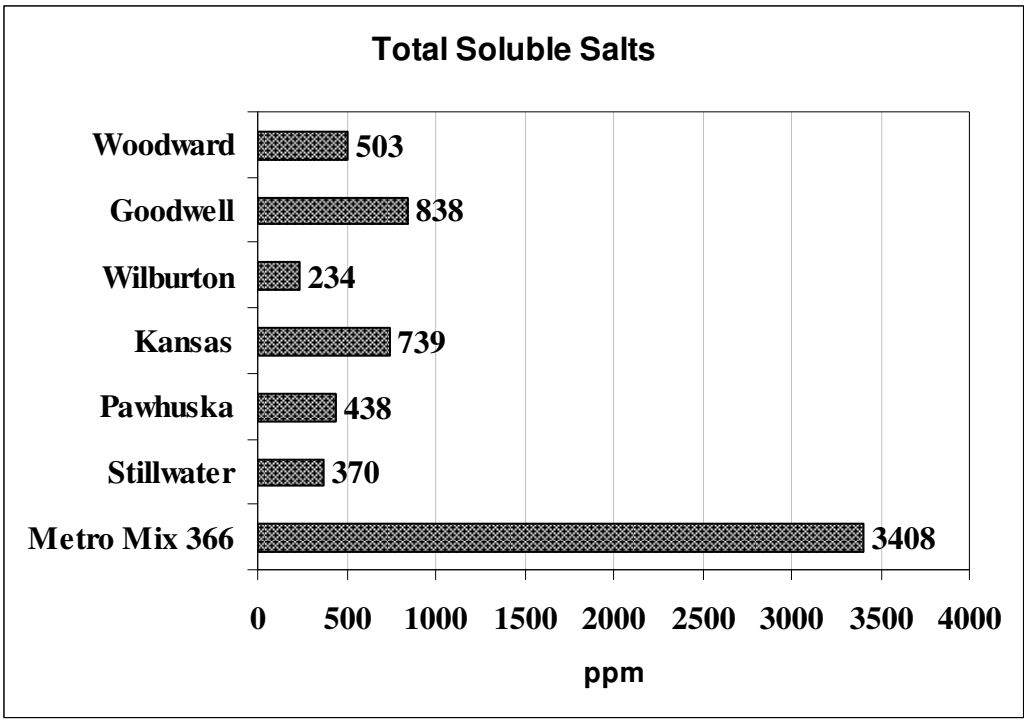

Figure 24. Analysis of total soluble salts (TSS) in soil and soilless medium samples as reported by SWFAL (ppm = parts per million).

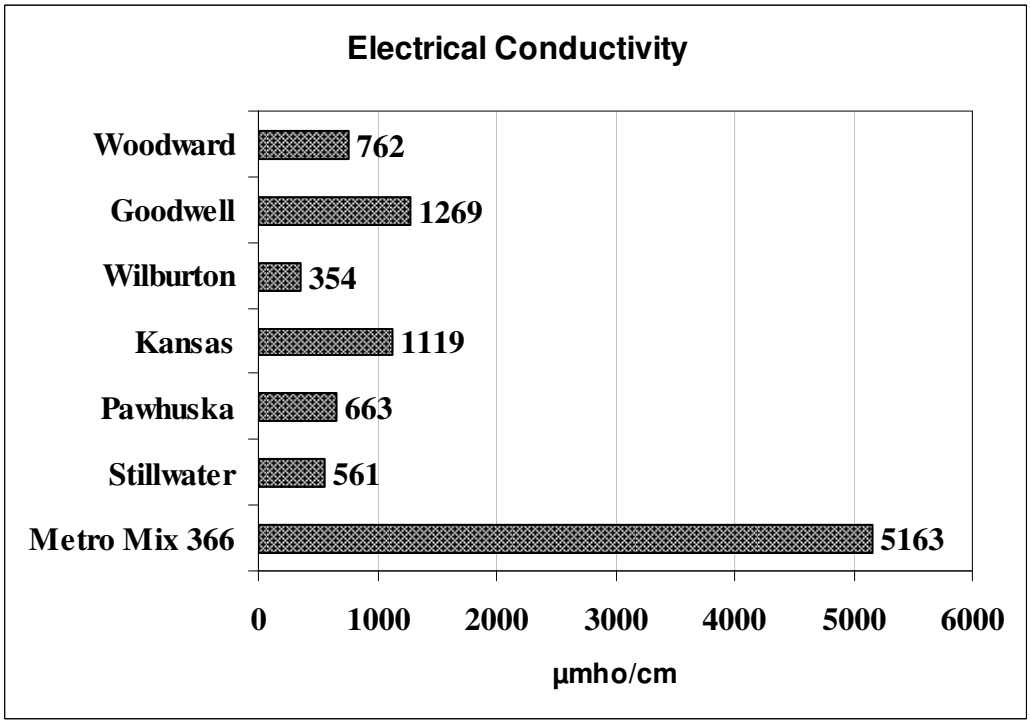

Figure 25. Electrical conductivity (EC) measurements of soil and soilless medium samples as reported by SWFAL.

Overall, the selected soils and growing medium were quite satisfactory at providing the broad diversity in physical and chemical properties desirable for this research. Large differences between the soils are evident with respect to many of the measured factors. The Wilburton (WLB), Kansas (KNS), and MetroMix® 366 (MM366) samples were significantly acidic, while the remaining samples were near neutral pH. Available nitrogen was substantially higher in the KNS and MM366 soils. Woodward (WDW), Goodwell (GDW) and MM366 samples had high amounts of plant-available phosphorous and potassium, but WLB was substantially deficient in phosphorous. MM366 contained significantly higher quantities of sulfur, calcium, and magnesium as compared to the natural soils. Iron and zinc were found in above-average quantity in the KNS soil. GDW and Stillwater (STW) soils contained significantly higher amounts of boron. Sodium content was especially high in the GDW and MM366 samples. As compared to the natural soils, MM366 was quite saline, due to the high concentrations of available plant nutrients, resulting in a high total soluble salt measurement along with a correspondingly high electrical conductivity.

Additionally, the textoral analysis indicated marked differences in texture between several of the natural soils. As a true loam, STW soil was most moderate in overall texture. GDW, WLB, and PAW were classified as clay loams, likely to be water-retaining and less aerated, while WDW represented a well-drained and aerated loamy sand (70.0% sand). KNS soil was classified as a stony silt loam (62.5% silt), making it likely to be fairly well-drained and aerated due to the secondary structure provided by the small stones.

Identity of Unknown Bacteria Surviving Surface Disinfection

BLAST® results for the forward and reverse sequences of the 16S rDNA inserts of the UNK sample set were examined and evaluated according to the rules described previously (see Table 6) to provide identification of the unknown organisms that had survived the surface disinfection procedure applied to *M. truncatula* roots. Table A3.8, located in the Appendix, provides data for the forward and reverse sequences found on the two contigs and the final identification assigned to each of the UNK insert sequences. Figures A4.15 and A4.16, also found in the Appendix, are cladograms of the forward and reverse sequences, respectively. The cladograms were generated from multiple sequence alignments performed with ClustalW2 (Larkin et al., 2007). All sequences were edited prior to alignment in order to remove extraneous 5' flanking pGEM®-T Easy vector sequence.

Identification	Number	Percent
No Hits	9	5.06
Unknown	17	9.55
Bacillus sp.	37	20.79
Bacillus cereus	47	26.40
Bacillus megaterium	32	17.98
Bacillus sphaericus	27	15.17
Bacillus subtilis	5	2.81
Lysinibacillus sphaericus	1	0.56
Niastella jeongjuensis	1	0.56
Paenibacillus polymyxa	1	0.56
Sinorhizobium meliloti	1	0.56
Total	**178**	

Table 7: Summarized identities of 16S rDNA inserts amplified from the culture of unknown bacteria surviving the surface disinfection treatment (UNK). Note that "No Data" insets are not included in this summary.

Table 7, above, provides a summary of the UNK 16S rDNA inserts. Taken together, known endospore-forming bacteria (*Bacillus* sp., *B. cereus, B. megaterium, B.*

sphaericus, B. subtilis, L. sphaericus, and *P. polymyxa*) constituted 84.3% of the sequenced inserts. However, examination of the two cladograms presented above revealed that almost all of the 16S rDNA inserts in the UNK series labeled as "No Hits" or "Unknown" aligned closely with sequences from these same bacteria. If all of the "No Hits" and "Unknown" sequences were assumed to represent endospore-forming bacteria as well, then the percentage of endospore-forming species in the UNK series would increase to 98.9%. In either case, it was highly likely that at least *some* (if not all) of the "No Hits" and "Unknown" sequences did in fact represent endospore-forming bacteria.

Efficacy of the Surface Disinfection Protocol

These results confirmed the hypothesis that the organisms surviving the root surface disinfection procedure were primarily endospore-forming species of bacteria. Bacteria from the order *Bacillales* comprised at minimum 84.3% of the UNK samples, and perhaps as much as 98.9% if the assumption is made that the "Unknown" and "No Hits" 16S rDNA inserts are also representative of organisms from this order. An insufficient ability to destroy bacterial endospores was a known limitation of the disinfection protocol from conception. However, as discussed previously, it was felt that attempting to devise a protocol that would completely sterilize the surfaces of root tissues without destroying the tissue and endophytic bacteria in the process would have been an ultimately unachievable goal (Lodewyckx et al., 2002).

One *Sinorhizobium meliloti* insert was identified among the 178 successfully sequenced UNK 16S rDNA inserts. This organism was detected at very high populations relative to the other putative endophytes in all sample series except WLB and KNS. The surface-disinfected roots giving rise to the UNK sample series were taken from heavily-

nodulated plants grown in Stillwater soil, and *S. meliloti* constituted approximately 55% of the endophytic 16S rDNA inserts identified in the STW sample series. Thus, because of the sheer population of this organism, it is not surprising that a small number of rhizosphere *S. meliloti* cells might survive surface disinfection. Additionally, exudates from root nodules disturbed during surface disinfection would likely have contained *S. meliloti* and could have lead to contamination of the rinsate surrounding a successfully disinfected root. In either case, as a well-known symbiotic nitrogen-fixing bacterium, *Sinorhizobium meliloti*'s status as an endophyte is without question.

One *Niastella yeongjuensis* (*N. jeongjuensis*) insert was identified among the 178 successfully sequenced UNK 16S rDNA inserts. *N. yeongjuensis* is a Gram-negative filamentous aerobe of the *Flexibacteraceae* family, originally isolated from soil cultivated with ginseng in the Yeongju region of Korea (Weon et al., 2006). It is not known to be endospore-forming and to this author's knowledge not been previously found as a putative endophyte. *N. yeongjuensis* was detected far less frequently than *S. meliloti* in the experimental samples, with only small numbers of 16S rDNA inserts appearing in the STW, GDW, and MM366 series. If truly endophytic, the presence of *N. yeongjuensis* in the UNK sample could possibly be explained by seepage of root exudates into the rinsate surrounding the surface disinfected root tissue. However, because this organism has not been previously reported as an endophyte, and because it was detected in the UNK sample series, classification of *N. yeongjuensis* as an endophyte of *M. truncatula* should be viewed as putative at best.

Identity of *M. truncatula* Root Bacterial Endophytes

As with the UNK clonal series, BLAST® results from the forward and reverse 16S rDNA insert sequences from the Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), Goodwell (GDW) and MetroMix® 366 (MM366) series clones were evaluated according to the rules described previously. Tables found in Appendix 3 provide identity data for the forward and reverse sequences located on the contigs and the final identification assigned to each of the 16S rDNA insert sequences. Additionally, cladograms of the forward and reverse sequences generated from multiple sequence alignments performed with ClustalW2 are provided in Appendix 4. Note that all of the sequences were edited prior to alignment in order to remove extraneous 5' flanking pGEM®-T Easy vector sequence. Summaries of final 16S rDNA insert identities, frequencies of occurrence, and representation by percentage within each soil are presented below in Tables 8 through 14.

WLB Inserts

Insert	Number	Percent
No Hits	24	22.02
Unknown	21	19.27
Phylum *Actinobacteria*	2	1.83
Phylum *Bacteroidetes*	4	3.67
Class *Flavobacteria*	5	4.59
Class *Alphaproteobacterium*	3	2.75
Family *Flexibacteraceae*	1	0.92
Agrobacterium rhizogenes	1	0.92
Agrobacterium tumefaciens	2	1.83
Bosea minatitlanensis	1	0.92
Bradyrhizobium sp.	3	2.75
Bradyrhizobium japonicum	1	0.92
Niastella jeongjuensis	12	11.01
Pantoea agglomerans	1	0.92
Phenylobacterium lituiforme	1	0.92
Pseudomonas fluorescens	1	0.92
Rhizobium sp.	1	0.92
Rhizobium leguminosarum	1	0.92
Rhizobium tropici	6	5.50
Shinella yambaruensis	2	1.83
Sinorhizobium sp.	1	0.92
Sinorhizobium meliloti	8	7.34
Sphingomonas sp.	1	0.92
Stenotrophomonas sp.	1	0.92
Streptomyces sp.	4	3.67
Streptomyces hygroscopicus	1	0.92
Total	109	

Table 8: Summary of WLB 16S rDNA insert sequence identification results. Representation of each insert type by count and percentage of total is shown. Note that *M. truncatula* chloroplast and "No Data" inserts were not included as part of this summary.

A high degree of diversity was observed in the root bacterial endophyte population of *M. truncatula* grown in the Wilburton soil. Identifiable putative endophytes encompassed 13 genera within 3 phyla. *Niastella, Rhizobium,* and *Sinorhizobium* were dominant endophytic genera, with *Agrobacterium, Bradythizobium,* and *Streptomyces* spp. appearing less frequently. However, unknown and "no hit" sequences dominated this population, accounting for over 41% of the 16S rDNA inserts.

Rare genera included *Bosea, Pantoea, Phenylobacterium, Pseudomonas, Shinella, Sphingomonas,* and *Stenotrophomonas*.

Bosea, Shinella, Stenotrophomonas, Phenylobacterium, and *Agrobacterium* were unique to the Wilburton soil. *B. minatitlanensis* is a member of the *Bradyrhizobiaceae* family discovered in anaerobic digester sludge. Species of *Bosea* have also been isolated from agricultural soils including rice paddies (Aboubakar et al., 2003) as well as from within leguminous plant root nodules (Zakhia et al., 2006).

S. yambaruensis, a member of the *Rhizobiaceae* family, was first isolated in 2006 from soil in Okinawa by Matsui et al. To this author's knowledge, this is the first time *S. yambaruensis* has been identified as a putative endophyte.

One insert was identified as *Stenotrophomonas* sp. A member of the *Xanthomonadaceae* family, nitrogen-fixing species of *Stenotrophomonas* have been isolated from dune grasses (Dalton et al., 2004), root nodules of several leguminous plant species including soybean (Kan et al., 2007; Kuklinsky-Sobral et al., 2005), potato (Garbeva et al, 2001), cucumber (Mahaffee and Kloepper, 1997), and rice roots (Sun et al., 2007; Mano et al., 2007).

Most *Phenylobacterium* species are reported as isolated from aquatic sources. In fact, *P. lituiforme* was originally isolated from a subsurface aquifer (Kanso and Patel, 2004). However, a novel species, *P. composti* was recently reported as an isolate from composted cotton waste (Weon et al., 2008). To this author's knowledge, this is the first report of any *Phenylobacterium* sp. identified as a putative endophyte.

Finally, *Agrobacterium rhizogenes* and *A. tumefaciens* 16S rDNA sequences were both identified in the WLB clonal library. Neither of these bacteria should be considered

endophytic as both are well-known tumor-inducing plant pathogens. *A. rhizogenes* is responsible for hairy root tumors, while *A. tumefaciens* is responsible for crown gall (White et al., 1982).

WDW Inserts

Insert	Number	Percent
No Hits	18	12.68
Unknown	9	6.34
Class *Alphaproteobacterium*	2	1.41
Class *Betaproteobacterium*	3	2.11
Bacillus sp.	1	0.70
Bradyrhizobium japonicum	2	1.41
Caulobacter sp.	1	0.70
Chitinophaga ginsengisoli	1	0.70
Frateuria aurantia	1	0.70
Matsuebacter chitosanotabidus	1	0.70
Pseudomonas sp.	1	0.70
Pseudomonas fluorescens	1	0.70
Sinorhizobium meliloti	101	71.13
Total	142	

Table 9: Summary of WDW 16S rDNA insert sequence identification results. Representation of each insert type by count and percentage of total is shown. Note that *M. truncatula* chloroplast and "No Data" inserts were not included as part of this summary.

A moderate degree of diversity was observed in the root bacterial endophyte population of *M. truncatula* grown in the Woodward soil. Identifiable putative endophytes encompassed 8 genera within 3 phyla. *Sinorhizobium* was by far the dominant genus, representing over 71% of the 16S rDNA inserts. Unknown and "no hit" sequences were less common than in other soils, accounting for slightly over 19% of the 16S rDNA inserts. Rare genera included *Pseudomonas, Bradyrhizobium, Bacillus, Caulobacter, Chitinophaga, Frateuria,* and *Matsuebacter*.

Caulobacter and *Chitinophaga* were unique to the Woodward soil. Endophytic species of *Caulobacter*, primarily *C. crescentus* have previously been reported in rice (Mano et al., 2007; Sun et al., 2007) and potato (Garbeva et al., 2001).

One *Chitinophaga ginsengisoli* 16S rDNA insert was identified in the WDW series. A member of the *Sphingobacteriales* order, this species was originally isolated from soil in a ginseng field in South Korea (Lee et al., 2007). Several other *Chitinophaga* species have been isolated from soil as well (Kim and Jung, 2007; Lee et al., 2007; An et al., 2007; Pankratov et al., 2006). To this author's knowledge, this is the first time *Chitinophaga ginsengisoli* has been identified as a putative endophyte.

KNS Inserts

Insert	Number	Percent
No Hits	51	40.48
Unknown	19	15.08
Phylum *Actinobacteria*	1	0.79
Class *Sphingobacteria*	1	0.79
Class *Alphaproteobacterium*	4	3.17
Class *Gammaproteobacterium*	3	2.38
Family *Hyphomicrobiaceae*	1	0.79
Bacillus sp.	4	3.17
Bacillus cereus	1	0.79
Bradyrhizobium sp.	1	0.79
Bradyrhizobium japonicum	3	2.38
Burkholderia sp.	1	0.79
Burkholderia cepacia	1	0.79
Dyella marensis	1	0.79
Escherichia coli	1	0.79
Frateuria aurantia	1	0.79
Labrys winsconsinensis	1	0.79
Mesorhizobium mediterraneum	1	0.79
Ochrobactrum sp.	1	0.79
Pantoea agglomerans	1	0.79
Pseudomonas corrugata	1	0.79
Pseudomonas kilonensis	1	0.79
Ralstonia sp.	2	1.59
Rhizobium sp.	2	1.59
Rhizobium mongolense	1	0.79
Rhizobium tropici	19	15.08
Sphingomonas pruni	1	0.79
Streptomyces sp.	1	0.79
Total	126	

Table 10: Summary of KNS 16S rDNA insert sequence identification results. Representation of each insert type by count and percentage of total is shown. Note that *M. truncatula* chloroplast and "No Data" inserts were not included as part of this summary.

A high degree of diversity was observed in the root bacterial endophyte population of *M. truncatula* grown in the Kansas soil. Identifiable putative endophytes encompassed 15 genera within 4 phyla. *Rhizobium* was the dominant genera, with *Bacillus* and *Bradythizobium* appearing less frequently. However, unknown and "no hit" sequences were also dominant in this population, accounting for over 55% of the 16S

rDNA inserts. Rare genera included *Burkholderia, Dyella, Escherichia, Frateuria, Labrys, Mesorhizobium, Ochrobactrum, Pantoea, Pseudomonas, Ralstonia, Sphingomonas,* and *Streptomyces*.

Dyella, Labrys, Ochrobactrum, Ralstonia, and *Escherichia* were unique to the Kansas soil. Species of *Dyella* have been previously isolated from soil and commercial growing media (Xie and Yokota, 2005; Kim et al., 2006), but to this author's knowledge, no *Dyella* species has been previously reported as endophytic.

Labrys species have been previously isolated from soil and sediments (Carvalho et al., 2008; Islam et al., 2007; Miller et al., 2005), and one species, *L. neptuniae,* has been isolated from root nodules of *Neptunia oleracea*, an aquatic legume (Chou et al, 2007). To this author's knowledge, this is the first report of *L. wisconsinensis* as an endophyte.

Several nitrogen-fixing *Ochrobactrum* species have previously been recognized as endophytes with isolations made from rice and root nodules of several leguminous plant species (Kang et al., 2007; Rosenblueth and Martinez-Romero, 2006; Ngom et al., 2004; Zakhia et al., 2006).

Two KNS 16S rDNA inserts were identified as *Ralstonia* sp. While some species of *Ralstonia* are known nodulating nitrogen-fixing bacteria endosymbiotic with leguminous plants (Muresu et al., 2008), others are known pathogens (Mercado-Blanco and Bakker, 2007). Species of *Ralstonia* have been identified as endophytes in a wide range of plants including soybean (Kuklinsky-Sobral et al., 2004; Kuklinsky-Sobral et al., 2005), pepper (Kang et al., 2007), wild legumes (Muresu et al., 2008), and mimosa (Chen et al., 2001).

Finally, one KNS 16S rDNA insert was identified as *Escherichia coli*. Cooley et al. (2003) found that *E. coli* could colonize *Arabidopsis thaliana* seeds. However, this author is not aware of any reports of *E. coli* isolated as a naturally-occurring endophyte. Additionally, in this author's experience, negative control PCR reactions using "universal" bacterial primers for amplification of 16 rRNA genes occasionally yield amplification products due to *E. coli* genomic DNA present in the *Taq* DNA polymerase stock solutions. Thus, it is also possible that the presence of an *E. coli* 16S rDNA insert can be attributed to a PCR artifact, rather than amplification of an endophyte gene.

STW Inserts

Insert	Number	Percent
No Hits	28	24.35
Unknown	5	4.35
Class *Alphaproteobacterium*	1	0.87
Class *Betaproteobacterium*	4	3.48
Class *Gammaproteobacterium*	1	0.87
Mesorhizobium amorphae	1	0.87
Niastella jeongjuensis	1	0.87
Niastella koreensis	1	0.87
Pseudomonas sp.	1	0.87
Rhizobium etli	1	0.87
Rhizobium leguminosarum	1	0.87
Sinorhizobium sp.	6	5.22
Sinorhizobium meliloti	63	54.78
Streptomyces sp.	1	0.87
Total	115	

Table 11: Summary of STW 16S rDNA insert sequence identification results. Representation of each insert type by count and percentage of total is shown. Note that *M. truncatula* chloroplast and "No Data" inserts were not included as part of this summary.

A moderate degree of diversity was observed in the root bacterial endophyte population of *M. truncatula* grown in the Stillwater soil. Identifiable putative endophytes encompassed 6 genera within 3 phyla. *Sinohizobium* was by far the dominant genera, representing 60% of the 16S rDNA inserts. Unknown and "no hit" sequences accounted

for 28.7% of the 16S rDNA inserts. Rare genera included *Mesorhizobium, Niastella, Pseudomonas, Rhizobium,* and *Streptomyces*. However, no identifiable genera were unique to this soil.

PAW Inserts

Insert	Number	Percent
No Hits	12	9.09
Unknown	2	1.52
Phylum *Bacteroidetes*	1	0.76
Class *Alphaproteobacterium*	4	3.03
Burkholderia sp.	2	1.52
Matsuebacter chitosanotabidus	1	0.76
Sinorhizobium sp.	6	4.55
Sinorhizobium meliloti	104	78.79
Total	132	

Table 12: Summary of PAW 16S rDNA insert sequence identification results. Representation of each insert type by count and percentage of total is shown. Note that *M. truncatula* chloroplast and "No Data" inserts were not included as part of this summary.

The lowest degree of diversity was observed in the root bacterial endophyte population of *M. truncatula* grown in the Pawhuska soil. Identifiable putative endophytes encompassed only 3 genera within 2 phyla. *Sinohizobium* was by far the dominant genera, representing over 83% of the 16S rDNA inserts. Unknown and "not hit" sequences accounted for 10.6% of the 16S rDNA inserts. Rare genera included *Matsuebacter* and *Burkholderia*. However, no identifiable genera were unique to this soil.

GDW Inserts

Insert	Number	Percent
No Hits	15	10.42
Unknown	14	9.72
Phylum *Bacteroidetes*	2	1.39
Class *Alphaproteobacterium*	2	1.39
Class *Betaproteobacterium*	1	0.69
Burkholderia phytofirmans	1	0.69
Dokdonella sp.	1	0.69
Glaucimonas multicolorus	1	0.69
Lactobacillus mobilis	1	0.69
Niastella jeongjuensis	1	0.69
Pseudomonas fluorescens	1	0.69
Pseudomonas saccharophila	1	0.69
Pseudoxanthomonas mexicana	1	0.69
Sinorhizobium sp.	5	3.47
Sinorhizobium fredii	1	0.69
Sinorhizobium meliloti	96	66.67
Total	144	

Table 13: Summary of GDW 16S rDNA insert sequence identification results. Representation of each insert type by count and percentage of total is shown. Note that *M. truncatula* chloroplast and "No Data" inserts were not included as part of this summary.

A moderate degree of diversity was observed in the root bacterial endophyte population of *M. truncatula* grown in the Goodwell soil. Identifiable putative endophytes encompassed 8 genera within 3 phyla. *Sinohizobium* was again the dominant genera, representing over 70% of the 16S rDNA inserts. Unknown and "no hit" sequences accounted for slightly more than 20% of the 16S rDNA inserts. Rare genera included *Burkholderia*, *Dokdonella, Glaucimonas, Lactobacillus, Niastella, Pseudomonas,* and *Pseudoxanthomonas*.

Dokdonella, Pseudoxanthomonas, and *Lactobacillus* were unique to the Goodwell soil. A member of the *Xanthomonadaceae* family, species of *Dokdonella* including *D. fugitiva* and *D. koreensis* have been isolated from soil and commercial growing media

(Yoon et al., 2006; Cunha et al., 2006). However, to this author's knowledge, no reports of endophytic *Dokdonella* sp. have been made previously. Additionally, a 16S rDNA insert identified as *Glaucimonas multicolorus* was also present in the GDW sample series. However, the UniProtKB Taxonomy database considers *G. multicolorus* to be synonymous with *D. fugitiva* (UniProt Consortium, 2008).

Pseudoxanthomonas mexicana was originally isolated from human urine as well as the sludge from an anaerobic reactor treating cheese factory wastewater (Thierry et al., 2004). However, Santiago-Mora et al. (2005) later isolated *P. mexicana* from olive field soil. Several other *Pseudoxanthomonas* species have also been isolated from soil (Yang et al., 2005; Young et al., 2007; Yoo et al., 2007). This author is not aware of any *Pseudoxanthomonas* species previously identified as putatively endophytic, however, *Pseudoxanthomonas suwonensis* was isolated from cotton waste compost (Weon et al., 2006), and *Pseudoxanthomonas* sp. were isolated from pulp and paper mill samples (Suihko et al., 2004; Desjardins and Bealieu, 2003), which might suggest endophytic activity.

One *Lactobacillus mobilis* insert was identified in the GDW series. Species of *Lactobacillus* have been previously reported in lemon (Gardner et al., 1982) and sugar beet (Jacobs et al., 1985).

MM366 Inserts

Insert	Number	Percent
No Hits	20	12.05
Unknown	9	5.42
Phylum Bacteroidetes	2	1.20
Phylum Planctomycetes	1	0.60
Class Alphaproteobacterium	2	1.20
Class Betaproteobacterium	6	3.61
Class Gammaproteobacterium	1	0.60
Family Xanthomonadaceae	1	0.60
Acidovorax sp.	3	1.81
Asticcacaulis sp.	1	0.60
Asticcacaulis taihuensis	1	0.60
Bacillus cereus	1	0.60
Bacillus megaterium	1	0.60
Burkholderia phytofirmans	1	0.60
Dyadobacter fermentans	1	0.60
Frateuria sp.	1	0.60
Herbaspirillum seropedicae	20	12.05
Hyphomicrobium facile	1	0.60
Lactobacillus mobilis	1	0.60
Mesorhizobium plurifarium	1	0.60
Niastella jeongjuensis	1	0.60
Niastella koreensis	3	1.81
Novosphingobium pentaromativorans	1	0.60
Pantoea sp.	2	1.20
Pantoea agglomerans	1	0.60
Pseudomonas fluorescens	1	0.60
Rhizobium leguminosarum	1	0.60
Sinorhizobium sp.	1	0.60
Sinorhizobium meliloti	79	47.59
Thermomonas fusca	1	0.60
Total	166	

Table 14: Summary of MM366 16S rDNA insert sequence identification results. Representation of each insert type by count and percentage of total is shown. Note that *M. truncatula* chloroplast and "No Data" inserts were not included as part of this summary.

The highest degree of diversity was observed in the root bacterial endophyte population of *M. truncatula* grown in the MetroMix® 366 soilless medium. Identifiable putative endophytes encompassed 17 genera within 4 phyla. *Sinohizobium* and *Herbaspirillum* were dominant genera, representing 48% and 12% of the 16S rDNA

inserts, respectively. *Niastella* appeared less frequently. Unknown and "no hit" sequences accounted for approximately 17.5% of the 16S rDNA inserts. Rare genera included *Acidovorax, Asticcacaulis, Bacillus, Burkholderia, Dyadobacter, Frateuria, Hyphomicrobium, Lactobacillus, Mesorhizobium, Novosphingobium, Pantoea, Pseudomonas, Rhizobium,* and *Thermomonas.*

Acidovorax, Asticcacaulis, Dyadobacter, Herbaspirillum, Hyphomicrobium, Novosphingobium, and *Thermomonas* were unique to plants grown in MetroMix® 366. *Acidovorax* spp. are members of the Burkholderiales order and have been previously reported as endophytes of pepper plants (Kang et al., 2007), red clover (Sturz et al., 1998) and rice seed (Mano et al., 2007).

Two 16S rDNA inserts were identified as species of *Asticcacaulis,* with one specifically identified as *A. taihuensis* in the MM366 series. *Asticcacaulis* species have been previously isolated from soil (Vasilyeva et al., 2006; Lester et al., 2007), but to this author's knowledge, no references to *Asticcacaulis* as an endophyte have been made previously.

One MM366 16S rDNA insert was identified as *Dyadobacter fermentans*. While several *Dyadobacter* species have been reported as rhizosphere bacteria (Dong et al., 2007; Liu et al., 2006; Reddy and Garcia-Pichel, 2000), the only previous report of an endophytic *Dyadobacter* sp. was made by Chelius and Triplett (2000) who isolated *D. fermentans* from maize stems.

Species of *Herbaspirillum,* including *H. seropedicae,* have been commonly identified as endophytes in a broad diversity of plants including rice, sugarcane, maize, sorghum, banana, and soybean (Rosenblueth and Martinea-Romero, 2006; Mano et al.,

2007; Kuklinsky-Sobral et al., 2005; Elbeltagy et al., 2001). A GUS (β-glucuronidase) - marked strain of *H. seropedicae* was used to study root colonization by endophytes in rice seedlings (James et al., 2002).

Several *Hyphomicrobium* species have been identified as nitrogen-fixing and denitrifying bacteria in soil (Fesefeldt et al., 1998). *Hyphomicrobium* sp. were previously identified as endophytes when isolated from rice plants by Mano et al., 2007. Isolated species included *H. facilis* and *H. sulfonivorans*.

One 16S rDNA insert identified as *Novosphingobium pentaromativorans* was found in the MM366 sample series. Mano et al. (2007) reported another *Novosphingobium* species, *N. subarcticum* as an endophyte of rice leaves. Additionally, a novel species named *N. nitrogenifigens* was found in a bioreactor used for treating pulp and paper-mill effluent in New Zealand (Addison et al., 2007), which suggests a potential for an endophytic relationship with the trees used for paper production.

One *Thermomonas fusca* 16S rDNA insert was identified in the MM366 sample series. *Thermomonas* species have been isolated previously from soil (Kim et al., 2006), however to this author's knowledge, this is the first report of a putatively endophytic *Thermomonas* species.

Genera of Putative Endophytic Bacteria Identified in Multiple Soils

Sphingomonas pruni was identified in the KNS series and one *Sphingomonas* sp. 16S rDNA insert was detected in the WLB series. *Sphingomonas* sp. have been previously identified as bacterial endophytes in maize kernels (Rijavec et al., 2007), papaya shoot tips (Thomas et al, 2007), pepper plants (Kang et al., 2007), rice plants

(Elbeltagy et al., 2007; Engelhard et al., 2000), potato plants (Garbeva et al., 2001), and leguminous plant root nodules (Zakhia et al., 2006).

One 16S rDNA insert identified specifically as *Streptomyces hygroscopicus* was detected in the WLB series. Other species of *Streptomyces* were also identified in the KNS and STW series. *Streptomyces* sp., including *S. hygroscopicus* are well-known rhizosphere bacteria which produce a wide diversity of antibiotic compounds. Several *Actinobacteria* including many *Streptomyces* sp. are well-documented as endophytes, with several *Streptomyces* sp. known to fix atmospheric nitrogen (Knapp and Jurtshuk, 1988). Cooms and Franco (2003a; 2003b) detected endophytic *Streptomyces* sp. in wheat roots and seeds. Mano et al., 2007 reported *Streptomyces lateritius/venezuelae* in rice leaves. Tokala et al. (2002) reported colonization of pea nodules by *Streptomyces lydicus*.

One 16S rDNA insert identified as *Frateuria* sp. was found in the MM366 series, while 16S rDNA inserts identified as *F. aurantia* were found in the WDW and KNS sample series. *F. aurantia* has been previously reported as an endophyte in potato stems and sweet pepper plants (Reiter et al., 2002; Rasche et al., 2006).

The PAW and WDW sample series each yielded 16S rDNA inserts identified as *Matsuebacter chitosanotabidus*. *M. chitosanotabidus* was first isolated from soil in Matsue, Japan, and possesses anti-fungal activity via chitosanase A (choA) production (Park et al., 1999; Shimono et al., 2001). To this author's knowledge, this is the first report of *M. chitosanotabidus* as a putative endophyte.

Two species of *Niastella*, *N. yeongjuensis (N. jeongjuensis)* and *N. koreensis* were identified in several sample series, including WLB, STW, GDW, and MM366. As

discussed previously, these bacteria are Gram-negative filamentous aerobes of the *Flexibacteraceae* family, originally isolated from soil cultivated with ginseng in the Yeongju region of Korea (Weon et al., 2006). To this author's knowledge, neither species has been previously reported as putatively endophytic.

Species of *Pantoea* were identified in the WLB, KNS, and MM366 sample series. All three sample series contained 16S rDNA inserts identified as *Pantoea agglomerans*, while the MM366 also contained 16S rDNA inserts identifiable only as *Pantoea* sp. *Pantoea* species, most commonly *P. agglomerans* and *P. ananatis*, have previously been often identified as endophytes of a broad spectrum of plants including red clover, grapevine, rice, pepper, maize, papaya, soybean, citrus, sweet potato, pea, and wild legumes (Mano et al., 2007; Rosenblueth and Martinez-Romero, 2006; Muresu et al., 2008; Elvira-Recuenco and van Vuurde, 2000; Kuklinsky-Sobral et al., 2004; Thomas et al., 2007; Rijavec et al., 2007; Kang et al., 2007; Lodewyckx et al., 2002).

Species of *Pseudomonas* were identified in every sample series. Species identified in this study included *P. corrugata, P. fluorescens, P. kilonensis,* and *P. saccharophila*. *Pseudomonas* spp. are commonly found in soil. Other studies have found *Pseudomonads* to be common endophytes with isolates including *P. agglomerans, P. chlororaphis, P. fluorescens, P. putida, P. citronellolis, P. synxantha, P. tolaasii, P. paucimbilis P. alcaligenes, P. oryzihabitans, P. aureofaciens, P. viridiflava, P. aeruginosa, P. savastoni, P. syringae, P. brassicacearum, P. straminea* and *P. rhenobacensis.* Host plant species are diverse and include marigold, carrot, soybean, Scots pine, potato, pepper, pea, poplar trees, wild rye, *M. truncatula, Hedysarum carnosum*, strawberry, red clover, lemon, cotton, alfalfa, corn, cucumber, sugar beet,

grapevine, tomato, rice, and many more (Mercado-Blanco and Bakker, 2007; Ryan et al., 2008; Zakhia et al., 2006; Muresu et al., 2008; Kang et al., 2007; Kuklinsky-Sobral et al., 2004; Kuklinsky-Sobral et al, 2005; Reiter et al., 2002; Garbeva et al., 2001; Rosenblueth and Martinez-Romero, 2006; Lodewyckx et al., 2002).

Burkholderia species including *B. cepacia* and *B. phytofirmans* were found in the KNS, PAW, GDW, and MM366 sample series. *B. cepacia* was identified only in the KNS series, while *B. phytofirmans* 16S rDNA inserts were identified in both GDW and MM366. *Burkholderia* species are known nitrogen-fixing endosymbiotic bacteria (Muresu et al., 2008) which have been isolated from many plant species including mimosa (Chen et al., 2005; Elliott et al., 2007), soybean (Kuklinsky-Sobral et al., 2005), maize, yellow lupine, citrus plants, banana, pineapple, rice (Rosenblueth and Martinez-Romero, 2006), cotton, and cucumber (Lodewyckx et al., 2002), among others.

Various well-known species of nitrogen fixing endosymbiotic bacteria were identified in all sample series. *Bradyrhizobium japonicum* was identified in the WLB, WDW, and KNS series. *Mesorhizobium amorphae* was found in the STW series, *M. mediterraneum* in KNS, and *M. plurifarium* in MM366. *Rhizobium etli* was identified in STW, *R. leguminosarum* in WLB, STW, and MM366, *R. mongolense* in KNS, and *R. tropici* in WLB and KNS. *Sinorhizobium meliloti* was nearly ubiquitous, found in all sample series except for KNS. One *S. fredii* insert was detected in the GDW series.

Several *Bacillus* species were identified in the WDW, KNS, and MM366 series, including *B. cereus, B. megaterium, B. sphaericus,* and *B. subtilis*. Reports of endophytic *Bacillus* sp. are widespread and include isolation from a broad diversity of plants including potato, citrus, maize, carrots, papaya, rice, sweet pepper, lemon, cotton,

cucumber, sugar beet, canola, soybean, pea, and several other legumes (Reiter et al., 2002; Rosenblueth and Martinez-Romero, 2006; Thomas et al., 2007; Mano et al., 2007; Rasche et al., 2006; Bai et al., 2003; Muresu et al., 2008; Elvira-Recuenco and van Vuurde, 2000; Lodewyckx et al., 2002). However, it should be noted that *Bacillus* species identified in this study cannot be classified as endophytic with a high degree of confidence due to the inability of the surface disinfection protocol to destroy endospores.

Soil-Dependant Variation in *M. truncatula* Root Endophyte Diversity

Large differences in *M. truncatula* root bacterial endophyte diversity are apparent when comparisons are made between roots taken from plants grown in different soils. These results show variation not only in the number of different identifiable endophytes, but also in the number of endophytes unique to a given soil. Additionally, differences in percent composition become apparent, not only at the species level, but throughout the higher taxa as well.

Table 15, below provides a comparison of the total number of different (with respect to BLAST® identification) identifiable 16S rDNA inserts found in each sample series as compared to the number of identifiable 16S rDNA inserts found to be unique (with respect to BLAST® identification) for a given sample series.

	Number of:	
Sample Series	**Different Inserts**	**Unique Inserts**
WLB	24	10
WDW	11	2
KNS	26	13
STW	12	2
PAW	6	0
GDW	14	5
MM366	28	14

Table 15: Comparison of identifiable 16S rDNA insert diversity among the soils used to grow *M. truncatula.* Note that inserts identified as "Unknown", "No Hits", or *M. truncatula* chloroplast are not included.

Examination of Table 15 shows that the number of 16S rDNA inserts unique to a given soil is proportional to the total number of different 16S rDNA inserts identified in that soil, thus soils yielding greater overall endophyte diversity also tended to yield more unique endophytic species. The WLB, KNS, and MM366 series yeilded far more overall endophyte diversity (13, 15, and 17 different genera, respectively), including greater numbers of unique endophytic genera (5, 5, and 7, respectively) as compared to the other soils. Additionally, the highest percentages of "no hit" and unknown 16S rDNA inserts occurred in the WLB and KNS series (approximately 41% and 55%). The most obvious commonality between these three soils is their forest background. The Wilburton soil originated from a mixed pine/hardwood forest in southeastern Oklahoma, the Kansas soil sample was collected along the boundary between a cleared pasture and the original mixed hardwood forest in northeastern Oklahoma, while MetroMix® 366 is a peat-lite growing medium containing 15-25% ground pine bark. Not surprisingly (given their forest background), WLB, WDW, and KNS soils were also found to be significantly acidic, with pH measurements of 5.1, 5.4, and 5.3, respectively. All other soils had pH values ranging from 6.4 to 7.6.

Taxonomic Composition of *M. truncatula* Endophyte Populations

Large differences can be seen when the taxonomic compositions of the *M. truncatula* root endophyte populations are compared between the different soils used for plant growth. Table 16, below, summarizes the taxonomic relationships between the BLAST®-identifiable 16S rDNA inserts. All of the taxonomic information presented in the table was obtained from the Taxonomic Outline of the Prokaryotes (Release 5.0) from Bergey's Manual of Systematic Bacteriology, 2nd ed. (Garrity, Bell, and Lilburn, 2004)

with the exception of the following genera which were not found in the Outline: *Shinella, Niastella, Matsuebacter, Lysinibacillus, Glaucimonas, Dokdonella,* and *Dyella*. Taxonomic information presented for these genera was obtained from the UniProt taxonomic database (UniProt Consortium, 2008).

Phylum	Class	Order	Family	Genus	Species
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Asticcacaulis	taihuensis
				Caulobacter	
				Phenylobacterium	lituiforme
		Rhizobiales	Brucellaceae	Ochrobactrum	
			Bradyrhizobiaceae	Bosea	minatitlanensis
				Bradyrhizobium	japonicum
			Hyphomicrobiaceae	Hyphomicrobium	facile
				Labrys	wisconsinensis
			Phyllobacteriaceae	Mesorhizobium	amorphae
					mediterraneum
					plurifarium
			Rhizobiaceae	Agrobacterium	rhizogenes
					tumefaciens
				Rhizobium	etli
					leguminosarum
					mongolense
					tropici
				Shinella	yambaruensis
				Sinorhizobium	fredii
					meliloti
		Sphingomonadales	Sphingomonadaceae	Novosphingobium	pentaromativorans
				Sphingomonas	pruni
	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	cepacia
					phytofirmans
				Ralstonia	
			Comamonadaceae	Acidovorax	
			Oxalobacteraceae	Herbaspirillum	seropedicae
			Unclassified	Matsuebacter	chitosanotabidus
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia	coli
				Pantoea	agglomerans
		Pseudomonadales	Pseudomonadaceae	Pseudomonas	corrugata
					fluorescens
					kilonensis
					saccharophila
		Xanthomonadales	Xanthomonadaceae	Dokdonella	
				Dyella	marensis
				Frateuria	aurantia
				Glaucimonas	multicolorus
				Pseudoxanthomonas	mexicana
				Stenotrophomonas	
				Thermomonas	fusca
Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	cereus
					megaterium
					sphaericus
					subtilis
				Lysinibacillus	sphaericus
			Paenibacillaceae	Paenibacillus	polymyxa
		Lactobacillales	Lactobacillaceae	Lactobacillus	mobilis
Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Streptomyces	hygroscopicus
Planctomycetes					
Bacteroidetes	Flavobacteria				
	Sphingobacteria	Sphingobacteriales	Crenotrichaceae	Chitinophaga	ginsengisoli
			Flexibacteraceae	Dyadobacter	fermentans
				Niastella	jeongjuensis
					koreensis

Table 16: Taxonomic relationships between BLAST®-identifiable 16S rDNA inserts as described by Bergey's Manual of Systematic Bacteriology, 2nd ed. (Garrity, Bell, and Lilburn, 2004) and the UniProt taxonomic database (UniProt Consortium, 2008). Shaded cells indicate taxonomic levels not identifiable by BLAST® results from any insert.

Figures 26 through 30 and Tables 17 through 21 provide a global perspective on compositional differences between the *M. truncatula* root endophyte populations at different taxonomic levels by comparing the percentages of total bacterial 16S rDNA inserts (including "No Hits" and "Unknown" inserts) with like classifications at a given taxonomic level across the different soils used for *M. truncatula* growth.

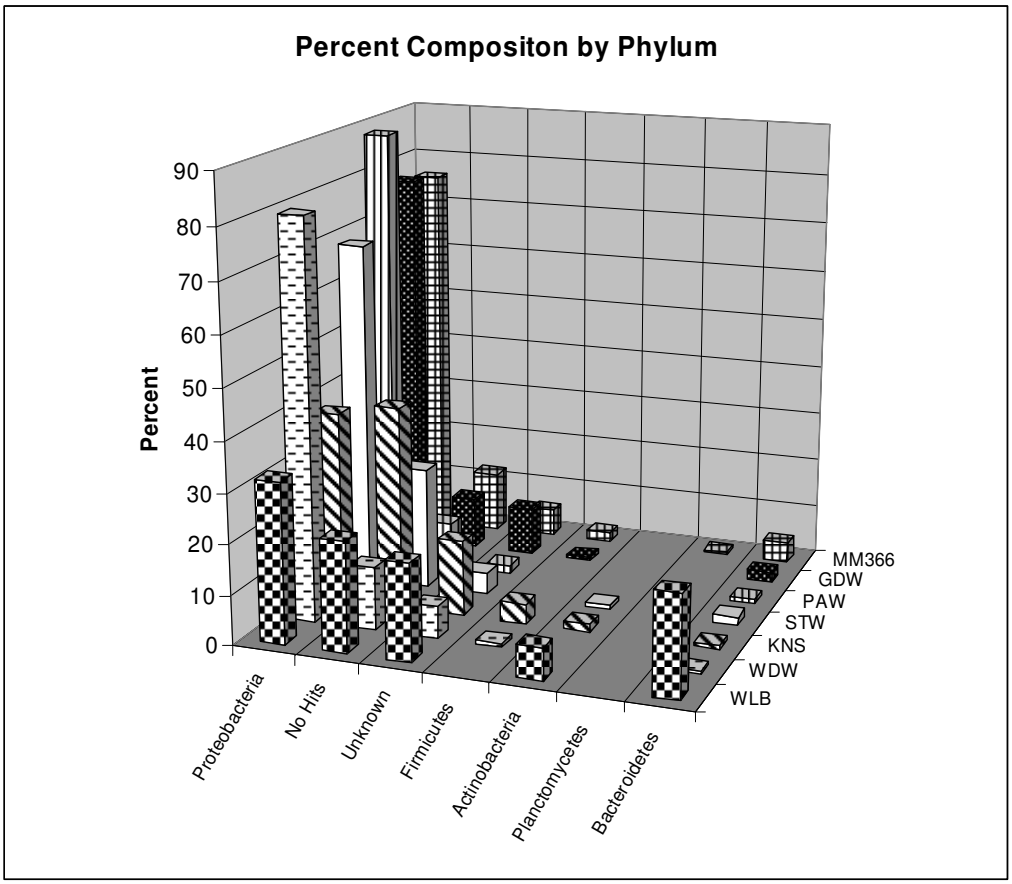

Figure 26: Percent composition of 16S rDNA inserts by phylum.

	WLB	WDW	KNS	STW	PAW	GDW	MM366
Proteobacteria	32.11	79.58	38.10	68.70	88.64	77.08	75.90
No Hits	22.02	12.68	40.48	24.35	9.09	10.42	12.05
Unknown	19.27	6.34	15.08	4.35	1.52	9.72	5.42
Firmicutes	0.00	0.70	3.97	0.00	0.00	0.69	1.81
Actinobacteria	6.42	0.00	1.59	0.87	0.00	0.00	0.00
Planctomycetes	0.00	0.00	0.00	0.00	0.00	0.00	0.60
Bacteroidetes	20.18	0.70	0.79	1.74	0.76	2.08	4.22

Table 17: Percent composition of 16S rDNA inserts by phylum.

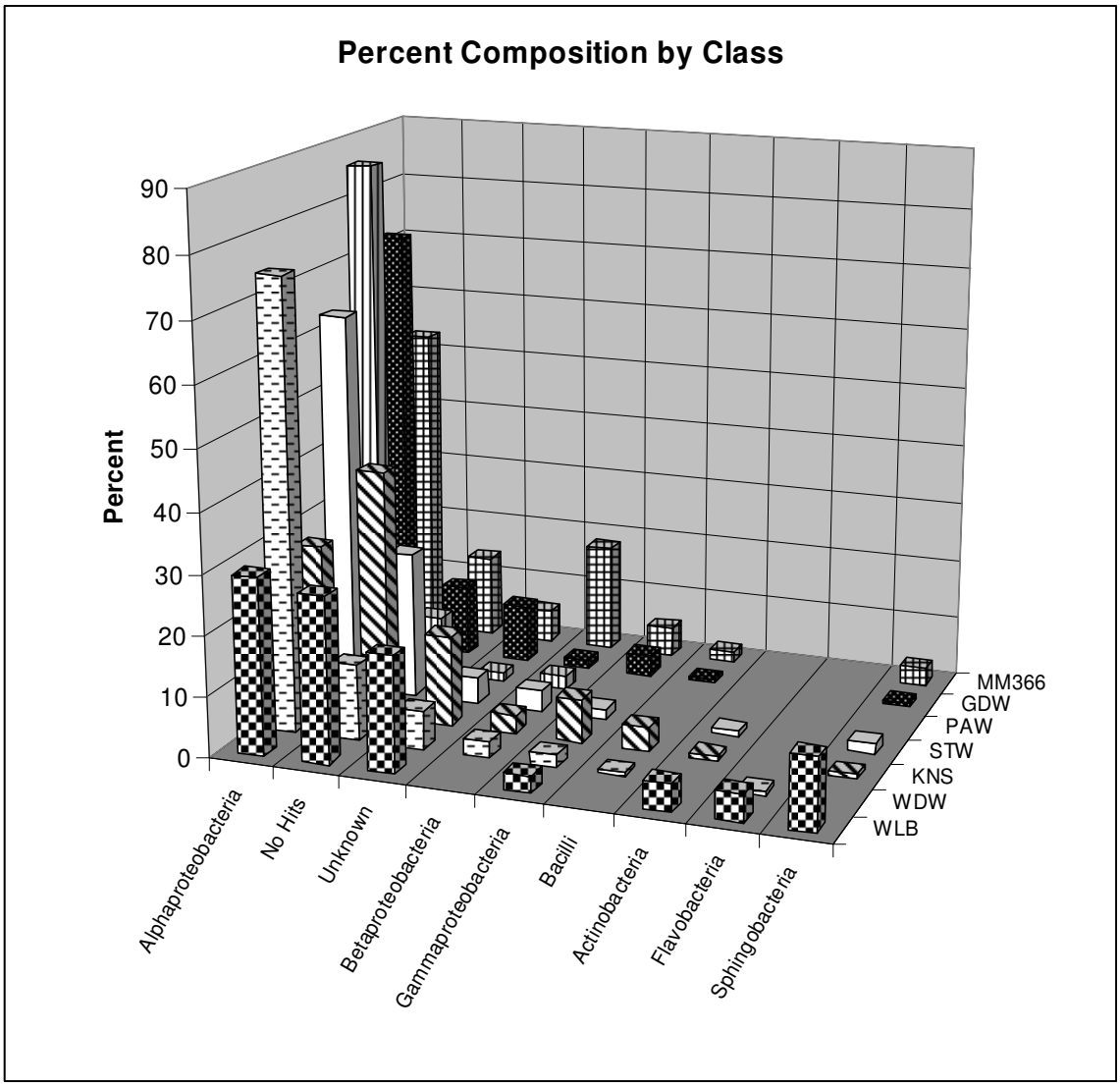

Figure 27: Percent composition of 16S rDNA inserts by class.

	WLB	WDW	KNS	STW	PAW	GDW	MM366
Alphaproteobacteria	29.36	74.65	27.78	63.48	86.36	72.22	53.01
No Hits	27.52	12.68	41.27	24.35	9.85	11.81	13.86
Unknown	19.27	6.34	15.08	4.35	1.52	9.72	5.42
Betaproteobacteria	0.00	2.82	3.17	3.48	2.27	1.39	18.07
Gammaproteobacteria	2.75	2.11	7.14	1.74	0.00	3.47	4.82
Bacilli	0.00	0.70	3.97	0.00	0.00	0.69	1.81
Actinobacteria	4.59	0.00	0.79	0.87	0.00	0.00	0.00
Flavobacteria	4.59	0.70	0.00	0.00	0.00	0.00	0.00
Sphingobacteria	11.93	0.00	0.79	1.74	0.00	0.69	3.01

Table 18: Percent composition of 16S rDNA inserts by class.

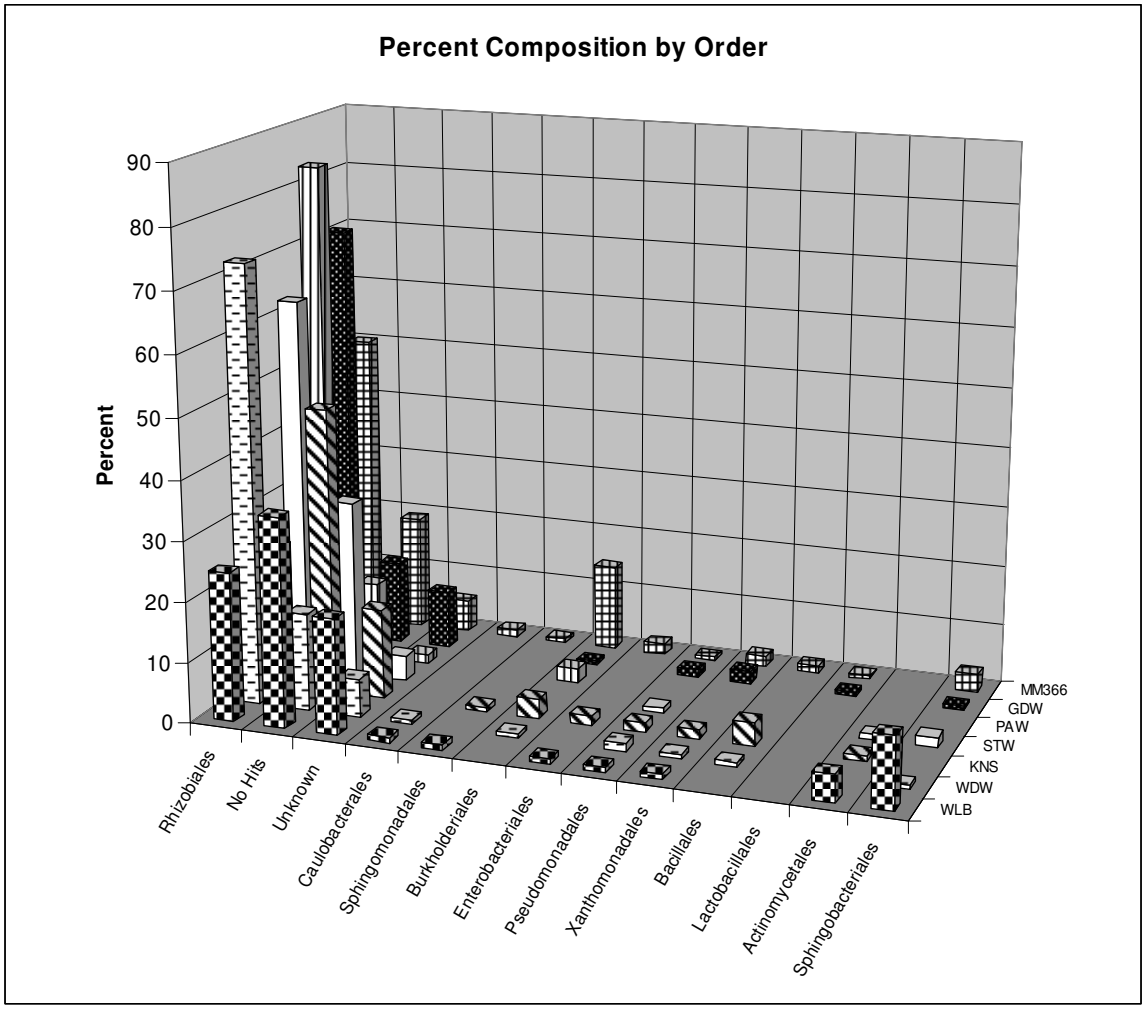

Figure 28: Percent composition of 16S rDNA inserts by order.

	WLB	WDW	KNS	STW	PAW	GDW	MM366
Rhizobiales	24.77	72.54	23.81	62.61	83.33	70.83	50.00
No Hits	34.86	16.20	47.62	29.57	12.88	13.89	19.28
Unknown	19.27	6.34	15.08	4.35	1.52	9.72	5.42
Caulobacterales	0.92	0.70	0.00	0.00	0.00	0.00	1.20
Sphingomonadales	0.92	0.00	0.79	0.00	0.00	0.00	0.60
Burkholderiales	0.00	0.70	3.17	0.00	2.27	0.69	14.46
Enterobacteriales	0.92	0.00	1.59	0.00	0.00	0.00	1.81
Pseudomonadales	0.92	1.41	1.59	0.87	0.00	1.39	0.60
Xanthomonadales	0.92	0.70	1.59	0.00	0.00	2.08	1.81
Bacillales	0.00	0.70	3.97	0.00	0.00	0.00	1.20
Lactobacillales	0.00	0.00	0.00	0.00	0.00	0.69	0.60
Actinomycetales	4.59	0.00	0.79	0.87	0.00	0.00	0.00
Sphingobacteriales	11.93	0.70	0.00	1.74	0.00	0.69	3.01

Table 19: Percent composition of 16S rDNA inserts by order.

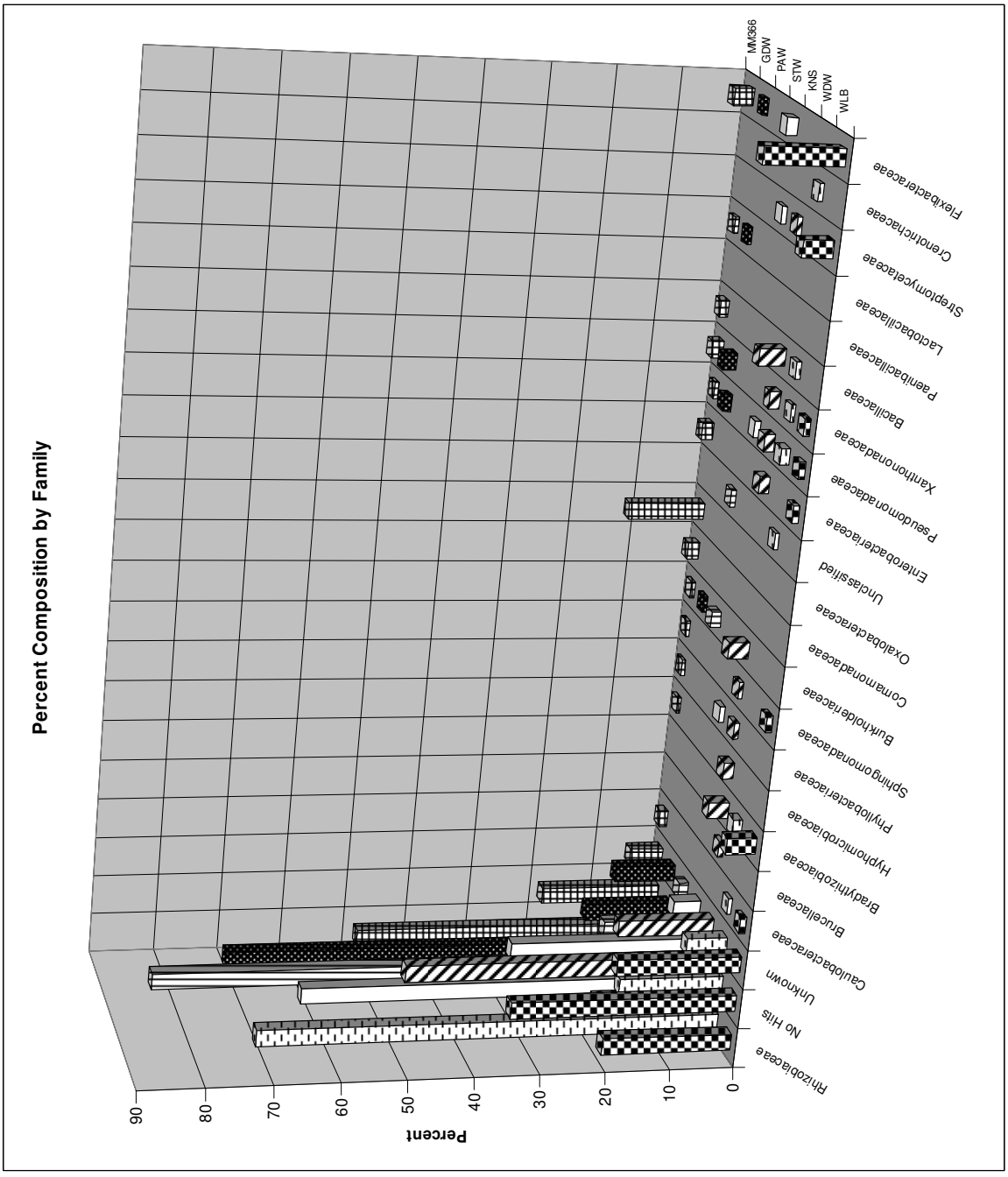

Figure 29: Percent composition of 16S rDNA inserts by family.

	WLB	WDW	KNS	STW	PAW	GDW	MM366
Rhizobiaceae	20.18	71.13	17.46	61.74	83.33	70.83	48.80
No Hits	34.86	16.20	47.62	29.57	12.88	13.89	19.28
Unknown	19.27	6.34	15.08	4.35	1.52	9.72	5.42
Caulobacteraceae	0.92	0.70	0.00	0.00	0.00	0.00	1.20
Brucellaceae	0.00	0.00	0.79	0.00	0.00	0.00	0.00
Bradythizobiaceae	4.59	1.41	3.17	0.00	0.00	0.00	0.00
Hyphomicrobiaceae	0.00	0.00	1.59	0.00	0.00	0.00	0.60
Phyllobacteriaceae	0.00	0.00	0.79	0.87	0.00	0.00	0.60
Sphingomonadaceae	0.92	0.00	0.79	0.00	0.00	0.00	0.60
Burkholderiaceae	0.00	0.00	3.17	0.00	1.52	0.69	0.60
Comamonadaceae	0.00	0.00	0.00	0.00	0.00	0.00	1.81
Oxalobacteraceae	0.00	0.00	0.00	0.00	0.00	0.00	12.05
Unclassified	0.00	0.70	0.00	0.00	0.76	0.00	0.00
Enterobacteriaceae	0.92	0.00	1.59	0.00	0.00	0.00	1.81
Pseudomonadaceae	0.92	1.41	1.59	0.87	0.00	1.39	0.60
Xanthononadaceae	0.92	0.70	1.59	0.00	0.00	2.08	1.81
Bacillaceae	0.00	0.70	3.97	0.00	0.00	0.00	1.20
Paenibacillaceae	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lactobacillaceae	0.00	0.00	0.00	0.00	0.00	0.69	0.60
Streptomycetaceae	4.59	0.00	0.79	0.87	0.00	0.00	0.00
Crenotrichaceae	0.00	0.70	0.00	0.00	0.00	0.00	0.00
Flexibacteraceae	11.93	0.00	0.00	1.74	0.00	0.69	3.01

Table 20: Percent composition of 16S rDNA inserts by family.

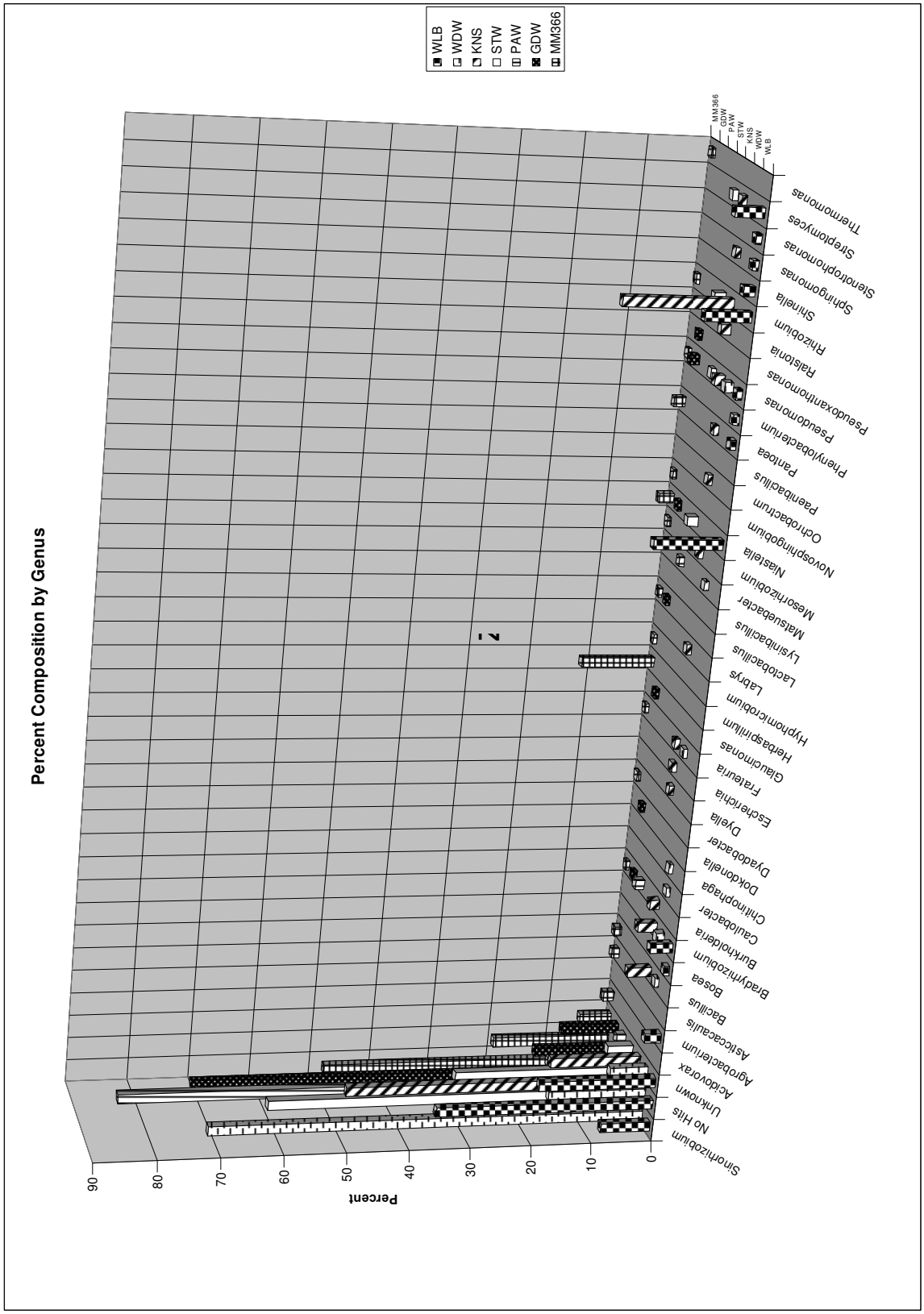

Figure 30: Percent composition of 16S rDNA inserts by genus.

	WLB	WDW	KNS	STW	PAW	GDW	MM366
Sinorhizobium	8.26	71.13	0.00	60.00	83.33	70.83	48.19
No Hits	35.78	16.20	48.41	29.57	12.88	13.89	19.88
Unknown	19.27	6.34	15.08	4.35	1.52	9.72	5.42
Acidovorax	0.00	0.00	0.00	0.00	0.00	0.00	1.81
Agrobacterium	2.75	0.00	0.00	0.00	0.00	0.00	0.00
Asticcacaulis	0.00	0.00	0.00	0.00	0.00	0.00	1.20
Bacillus	0.00	0.70	3.97	0.00	0.00	0.00	1.20
Bosea	0.92	0.00	0.00	0.00	0.00	0.00	0.00
Bradyrhizobium	3.67	1.41	3.17	0.00	0.00	0.00	0.00
Burkholderia	0.00	0.00	1.59	0.00	1.52	0.69	0.60
Caulobacter	0.00	0.70	0.00	0.00	0.00	0.00	0.00
Chitinophaga	0.00	0.70	0.00	0.00	0.00	0.00	0.00
Dokdonella	0.00	0.00	0.00	0.00	0.00	0.69	0.00
Dyadobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.60
Dyella	0.00	0.00	0.79	0.00	0.00	0.00	0.00
Escherichia	0.00	0.00	0.79	0.00	0.00	0.00	0.00
Frateuria	0.00	0.70	0.79	0.00	0.00	0.00	0.60
Glaucimonas	0.00	0.00	0.00	0.00	0.00	0.69	0.00
Herbaspirillum	0.00	0.00	0.00	0.00	0.00	0.00	12.05
Hyphomicrobium	0.00	0.00	0.00	0.00	0.00	0.00	0.60
Labrys	0.00	0.00	0.79	0.00	0.00	0.00	0.00
Lactobacillus	0.00	0.00	0.00	0.00	0.00	0.69	0.60
Lysinibacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Matsuebacter	0.00	0.70	0.00	0.00	0.76	0.00	0.00
Mesorhizobium	0.00	0.00	0.79	0.87	0.00	0.00	0.60
Niastella	11.01	0.00	0.00	1.74	0.00	0.69	2.41
Novosphingobium	0.00	0.00	0.00	0.00	0.00	0.00	0.60
Ochrobactrum	0.00	0.00	0.79	0.00	0.00	0.00	0.00
Paenibacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pantoea	0.92	0.00	0.79	0.00	0.00	0.00	1.81
Phenylobacterium	0.92	0.00	0.00	0.00	0.00	0.00	0.00
Pseudomonas	0.92	1.41	1.59	0.87	0.00	1.39	0.60
Pseudoxanthomonas	0.00	0.00	0.00	0.00	0.00	0.69	0.00
Ralstonia	0.00	0.00	1.59	0.00	0.00	0.00	0.00
Rhizobium	7.34	0.00	17.46	1.74	0.00	0.00	0.60
Shinella	1.83	0.00	0.00	0.00	0.00	0.00	0.00
Sphingomonas	0.92	0.00	0.79	0.00	0.00	0.00	0.00
Stenotrophomonas	0.92	0.00	0.00	0.00	0.00	0.00	0.00
Streptomyces	4.59	0.00	0.79	0.87	0.00	0.00	0.00
Thermomonas	0.00	0.00	0.00	0.00	0.00	0.00	0.60

Table 21: Percent composition of 16S rDNA inserts by genus.

The taxonomic distributions presented above in Figures 26 through 30 and Tables 17 through 21 can be condensed into the single chart presented below as Figure 31, which illustrates the total number of identifiable taxonomic groups per sample series at each taxonomic level.

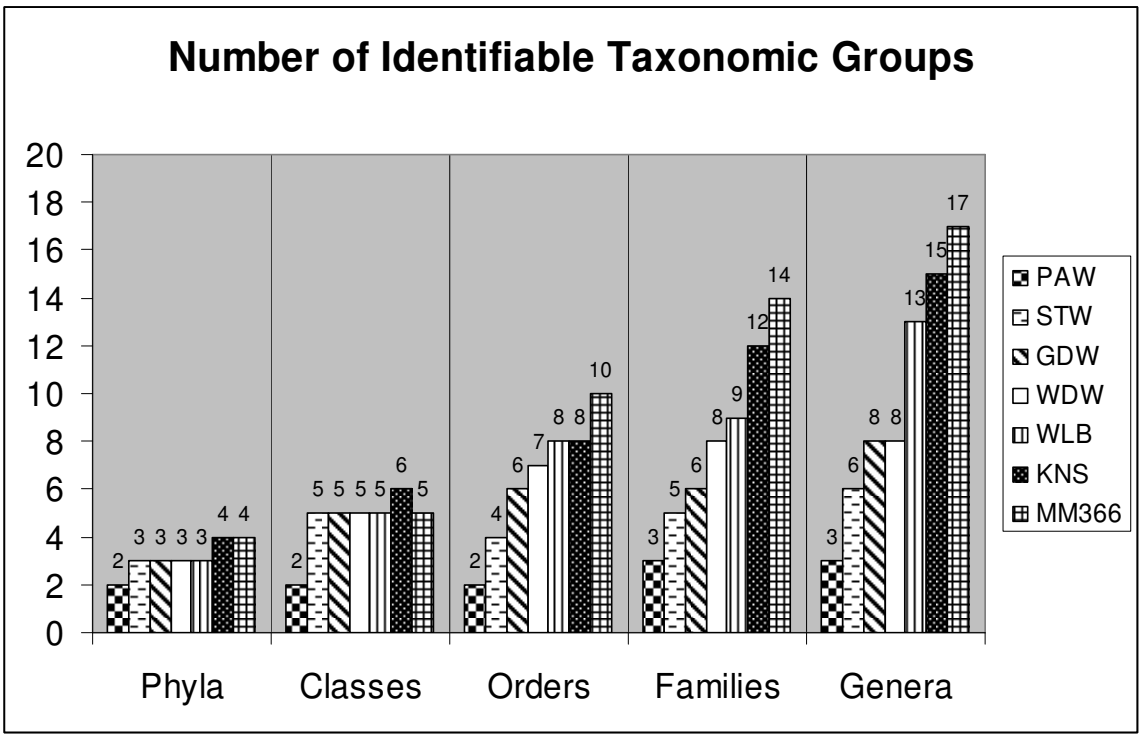

Figure 31: Number of identifiable taxonomic groups within each sample series at decreasing taxonomic levels.

These results clearly demonstrate large differences in *M. truncatula* root endophyte diversity between the sample series, which become increasingly pronounced at progressively lower taxa. Diversity at the level of phylum was comparable across the sample series, with the Tallgrass Prairie Preserve soil from Pawhuska (PAW) yielding the least number of phyla (two), while the Kansas (KNS) and MetroMix® 366 (MM366) soils yielded the greatest (four). However, at progressively lower taxonomic levels,

differences in diversity become increasingly pronounced, with three distinct groups emerging at the genus level.

Group 1 consists of MM366, KNS, and WLB. These soils (and commercial growing medium) yielded the most endophyte diversity at lower taxonomic levels, with 17, 15, and 13 different genera represented, respectively. Numbers of unique genera were also greatest among these soils (7, 5, and 5, respectively), with the WLB and KNS series also containing the highest percentages of total inserts classified as unknown or "no hits" (approximately 42% and 55%, respectively). As discussed previously, the commonalities between these soils are low pH and a forest background. The Wilburton soil originated from a mixed pine/hardwood forest in southeastern Oklahoma, the Kansas soil from the boundary between a cleared pasture and the original mixed hardwood forest in northeastern Oklahoma, while MetroMix® 366 is a peat-lite growing medium containing 15-25% ground pine bark.

Group 2 consists of WDW, GDW, and STW. These soils yielded a moderate level of endophyte diversity at the lower taxonomic levels, with 8, 8, and 6 different genera represented, respectively. Numbers of unique genera were low among these soils (2, 3, and 0, respectively) with only moderate percentages of the total inserts classified as "no hits" or unknown (approximately 19%, 20.14%, and 28.7%, respectively). The common linkage between these soils would seem to be agricultural management. The Stillwater soil is managed for alfalfa production, the Goodwell soil for alfalfa and winter wheat production, and the Woodward soil intensively managed as improved rangeland for beef cattle production. All of these soils were utilized for essentially monocrop agriculture, and would have been subjected to tillage, planting, harvesting, and

applications of agricultural fertilizers and pesticides. The Stillwater and Goodwell soils were also irrigated during production cycles.

Group 3 contains only the PAW sample series. The Tallgrass Prairie Preserve soil yielded very low endophyte diversity at both higher and lower taxonomic levels, with only 3 identifiable genera. None of the identifiable putative endophytic genera in were unique to this soil, and only 10.6% of the inserts were classified as "no hits" or unknown. From an ecological perspective, this soil is unique in that it supports an undisturbed native prairie ecosystem grazed primarily by bison, and is not intensively managed for agricultural production.

These findings are somewhat similar to those of Roesch et al. (2007) who used pyrosequencing to examine bacterial and archaeal diversity in one gram samples of four soils. Their results found far more diversity at the level of phylum in a Canadian boreal forest soil as compared to phylum diversity in three agricultural soils from Illinois, Florida, and Brazil. These results would tend to agree with the findings of this study, at least with respect to the differences in diversity observed when the MM366, KNS, and WLB (Group 1) sample series are compared to the WDW, GDW and STW (Group 2) samples.

Roesch et al. (2007) hypothesized that forest soils have a higher degree of soil bacterial diversity than agricultural soils due to the higher biodiversity of flora and fauna in the undisturbed forest ecosystem. However, this hypothesis is not supported by the findings of this work, as the Group 2 agricultural soils (WDW, GDW, and STW) yielded more endophyte diversity than the undisturbed PAW soil from the Tallgrass Prairie Preserve.

This hypothesis was also unsupported by the work of Fierer and Jackson (2006), who compared the bacterial diversity of 98 different soils and found that soil bacterial diversity actually decreased as plant biodiversity increased. This finding is supportive of the results of this study insofar as the agricultural soils yielded higher endophyte biodiversity than did the undisturbed prairie soil. However, it does not correlate with the finding of highest endophyte biodiversity in *M. truncatula* plants grown in the Group 1 soils, or with the results of the Roesch et al. (2007) study.

Fierer and Jackson (2006) suggested that the most important environmental factor influencing soil bacterial diversity was pH. Their work strongly correlated lower soil pH with reduced bacterial diversity. However, this observation does not seem to be in agreement with the findings of this study, nor was it fully supported by the findings of Roesch et al. (2007).

Perhaps most importantly, the two studies described above were examinations of rhizosphere bacterial populations, not endophyte populations. While the endophyte population is certainly a subset of the rhizosphere population, it is not necessarily true that endophyte diversity must be directly proportional to the rhizosphere diversity since the host plant itself certainly has an influence on the endophyte population by either active or passive selection of bacterial species allowed to internally colonize the plant. While a high degree of rhizosphere biodiversity would certainly seem to provide greater opportunities for the existence of species capable of colonization and, therefore, more endophyte diversity, it is also possible that a soil with less overall rhizosphere diversity might possess a high percentage of species suitable for the colonization of a given host plant due to natural selection by the same or similar flora already present in the

ecosystem. Further study is needed in this area (perhaps involving the identification of endophytes from a diverse collection of plant species grown in the same soil) to provide a better understanding of host-dependant variation in endophyte populations.

Thus, while this study has shown that the endophytic bacterial population of a plant is certainly strongly influenced by the soil in which the plant is grown, the specific environmental factors responsible for such differences remain to be elucidated by future research.

Finally, in light of these results, it is advisable that the selection of soil or growing medium be considered carefully in future studies of endophytic bacterial populations. Certainly, no single medium or soil could be selected that would provide all possible endophytes of a given plant species. However, the results of this study do suggest that a broader diversity of root endophytes might be observed if plants are grown in an artificial growing medium containing bark as opposed to an unamended natural soil.

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EVALUATION OF ROOT BACTERIAL ENDOPHYTE DIVERSITY IN *MEDICAGO TRUNCATULA* GROWN IN DIVERSE SOILS BY TEMPERATURE GRADIENT GEL ELECTROPHORESIS (TGGE)

ABSTRACT

The endophytic bacterial populations of surface disinfected *M. truncatula* root tissues from plants grown in diverse soils were evaluated for diversity and differences in composition using temperature gradient gel electrophoresis (TGGE) as a comparison of technique to the "shotgun" cloning method described in Chapter 2.

A larger version of the TGGE apparatus described by Wartell et al. (1990) was constructed in-house, tested for performance, and used for this study. Additionally, a novel pair of GC-clamped primers based on the "universal" bacterial 16S rDNA primers described by Engelen et al. (1998) were designed to incorporate an improved GC-clamp onto the 3' end of ±400 bp PCR-amplified 16S rDNA gene fragments containing the V6 through V8 hypervariable regions as opposed to the 5' GC-clamp placement of the original primer pair which corresponded to the predicted highest melting domain.

The in-house manufactured TGGE apparatus failed to adequately resolve the complex endophytic bacterial 16S rDNA fragments with respect to the level of diversity revealed by the "shotgun" cloning method. Additionally, 16S rDNA fragment bands on the silver-stained TGGE gel were unable to be sequenced or cloned into plasmid vectors for identification.

As a result, TGGE was felt to be an inferior technique for analysis of complex bacterial populations as compared to the "shotgun" cloning method.

INTRODUCTION

Excellent results were achieved with respect to evaluation of the *M. truncatula* root endophyte population using the "shotgun" cloning approach detailed in Chapter 2. However, the method does suffer from a disadvantage in that a great deal of extra expense and labor are involved due to the redundant cloning and sequencing of identical inserts from the amplified pool of 16S rDNA fragments. This redundancy is not always undesirable because it allows for estimation of abundance of specific endophytes with respect to the population as a whole. However, if the goal of a study is to simply produce a list of endophytic species or genera present in a sample without regard to frequency of occurrence, then the redundancy inherent in the "shotgun" cloning technique adds unnecessary labor and expense to the research.

Therefore, a second technique which would, in principle, involve less redundancy and expense with respect to cloning and sequencing of 16S rDNA amplification products was evaluated for the study of the same *M. truncatula* root endophytic bacterial populations, so that the efficacy of both approaches could be compared. This alternative technique was temperature gradient gel electrophoresis (TGGE).

In 1976, Gross et al. found that single-stranded nucleic acids (mRNAs) of near-identical molecular weights normally unresolvable by traditional polyacrylamide and agarose gel electrophoresis could be electrophoretically separated if a concentration gradient of a chemical denaturant such as urea were included in a polyacrylamide gel matrix. Separation was achievable because conformational changes in the secondary structures of the molecules caused by the increasing denaturant concentration had a far greater influence on electrophoretic mobility than did molecular weight. Since nucleic

acid sequence directly influences secondary structure, differences in sequence resulted in altered electrophoretic mobility as the DNA migrated through the denaturing urea gradient.

Gross et al. (1976) also found that the denaturing effects of increasing chemical concentration and increasing temperature were interchangeable, thus suggesting that similarly-sized nucleic acids could be electrophoretically separated not only by maintaining a constant temperature across a gel with an increasing chemical denaturant concentration, but also by the inverse approach: applying a temperature gradient to a gel having a constant concentration of chemical denaturant. These two electrophoretic techniques later became widely known as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE).

In 1979, Fischer and Lerman applied the DGGE technique to double-stranded DNA molecules in two-dimensional electrophoresis of *Eco*R I –digested *E. coli* genomic DNA. The first electrophoretic dimension was a standard non-denaturing separation based on molecular weight (fragment length). However, the second dimension utilized a urea-formamide denaturing gradient which allowed separation of similarly-sized fragments unresolvable in the first dimension on the basis of sequence differences.

In 1983, Fischer and Lerman demonstrated that the DGGE technique had the capability to resolve DNA fragments of identical length with only single base-pair substitutions. However, they also found that not all single base substitutions lead to detectable differences in migration distances. Myers et al. (1985) found that this problem was caused by the mechanism by which strand dissociation occurs during migration of the double-stranded molecule through the denaturing gradient.

As the molecule migrates electrophoretically into a region of higher denaturant concentration, strand dissociation does not occur at once along the entire length of the molecule. Instead, small regions of the molecule (melting domains) dissociate individually. As migration proceeds into increasingly higher denaturing conditions, more and more melting domains undergo strand dissociation with the order of domain dissociation determined by differences in the respective domain melting temperatures (T_M). The T_M of any given melting domain is determined by the nucleotide sequence within the domain. GC-rich domains have higher melting temperatures than AT-rich domains due to the increased number of hydrogen bonds between the complementary strands. As each domain dissociates, migration rate is altered, resulting in visible differences in the overall migration distance over time due directly to differences in nucleotide sequence (Myers et al., 1985).

When the final, most stable domain having the highest T_M melts, the double-stranded DNA (dsDNA) molecule becomes completely dissociated into two complementary single-stranded DNA (ssDNA) molecules with effectively no migration relative to other dsDNA molecules (in reality, migration of ssDNA molecules does continue, albeit at a greatly reduced rate). Because a single-base substitution does not yield a substantial difference in domain T_M, molecules with single-base substitutions occurring in the final melting domain cease migration at distances so similar that they are unresolvable when the gel is viewed (Myers et al., 1985). It should be emphasized that this problem applies only with respect to molecules that are identical in sequence with exception of the final melting domain. Molecules with substitutions outside of the

highest melting domain would have already been resolved due to dissociation of other domains prior to final dissociation.

The solution to this problem was the GC-clamp. Developed by Myers et al. (1985), the GC-clamp is a GC-rich sequence (at minimum 80% G+C) attached to the end of the dsDNA molecule with the highest T_M. Added by PCR using appropriate primers, the GC-clamp prevents complete dissociation of the molecule into ssDNA when the final domain melts, thereby allowing resolution of base-pair substitutions in the most stable melting domain.

Muyzer et al. (1983) was the first to apply this technology toward the analysis of complex unknown environmental microbial populations. A mixture of PCR-amplified bacterial 16s rDNA gene fragments from sediments and biofilms were separated by DGGE into ten distinct bands which were assumed to represent at least an equal number of bacterial species. Since that time, denaturing gradient electrophoretic separation of PCR-amplified 16S rDNA has became a popular technique for generating genetic "fingerprints" of complex environmental microbial populations, including rhizosphere and endophytic bacteria. DGGE and TGGE are considered to be interchangeable techniques, yielding comparable "fingerprints" with banding patterns interpreted as being representative of the major constituents of the evaluated communities (Heuer and Smalla, 1997).

The objective of this study was to compare the effectiveness of TGGE to that of the "shotgun" cloning technique described in Chapter 2 for the evaluation of variation with respect to composition and diversity of *M. truncatula* root endophyte populations from plants grown in diverse soils collected from across the state of Oklahoma.

MATERIALS AND METHODS

Construction of the TGGE Apparatus

The TGGE apparatus utilized for this research was constructed in-house due to financial limitations preventing the purchase of a commercially-sold device. The apparatus was designed and constructed as a much larger version of the device described by Wartell et al. (1990). A substantial increase in size was desirable in order to allow for longer migration distances and a lower Δ temperature:migration distance ratio, thus providing a higher resolution potential.

Components of the primary base unit (consisting of upper and lower buffer reservoirs separated by supporting columns with an attached backplate), the movable cassette stand in the lower reservoir, and the gel casting stand were constructed from sheets of ¼" extruded acrylic (aka "plexiglass") and joined with Amazing Goop® contact adhesive and sealant (Eclectic Products Inc., Eugene, OR). Platinum wire was used for the electrodes in the upper and lower buffer reservoirs. A ¼"-thick rubber gasket was used to seal the union between the notched glass plate described below and an equivalent notch cut into the front wall of the upper buffer reservoir. These notched openings in the upper buffer reservoir and the adjoining glass plate were necessary to allow for direct electrical contact between the gel and the upper reservoir buffer solution. Electrical contact between the gel and the lower buffer solution was provided by immersion of the lower portion of the gel within the lower buffer reservoir itself.

The cassette containing the gel itself consisted of several components. The polyacrylamide gel was cast between two 1/8" tempered glass plates. The glass plates were 14 ½" in height by 8 ½" width. One plate had a 1" deep notch cut into the upper

edge to allow for direct electrical contact between the Casting combs and spacers were cut from sheets of 1/16" Teflon® PTFE (poly(tetrafluoroethylene), DuPont Chemical Co., Wilmington, DE).

When the cassette was completely assembled, these glass plates were "sandwiched" between two thermal plates used to create the temperature gradient. These thermal plates were constructed of ½" aluminum sheet and bar stock. Heating of the plates was accomplished by circulating water through horizontal channels milled into the upper and lower edges of plates. Cooler water was circulated through the upper channel, and warmer water through the lower. A vertical temperature gradient was thus established across the plates, and thereby across the gel sandwiched between them.

All surfaces of the thermal plates not in direct contact with the glass plates containing the gel were covered with ½" Rboard® insulation (Atlas Roofing Corp., Atlanta, GA). This outer shell of insulation was covered with aluminum-backed tape in order to hold the insulation segments together, as well as to prevent entry of splashed liquids into the insulation.

The entire assembled cassette "sandwich" was held together by three pairs of outer steel straps (½" wide and ¼" thick) positioned horizontally, with each strap pair joined at both ends using a segment of threaded rod and wing nuts.

Temperature-regulated water was supplied to the thermal blocks via two circulating water baths. The direction of water flow in one thermal block was always maintained opposite to the direction of flow in the second thermal block. This was done to ensure that a horizontal temperature gradient did not become established in the gel due

to minor cooling of the water as it passed through the thermal block and transferred heat to the gel.

Buffer solution was recirculated between the upper and lower buffer reservoirs at all times during electrophoresis using a peristaltic pump. This was required to ensure that the concentrations of salts in the upper and lower buffer solutions remained equal and constant during electrophoresis. Without recirculation, anionic salts would migrate and accumulate at the platinum anode in the lower buffer reservoir, and cations would likewise accumulate at the platinum cathode in the upper reservoir.

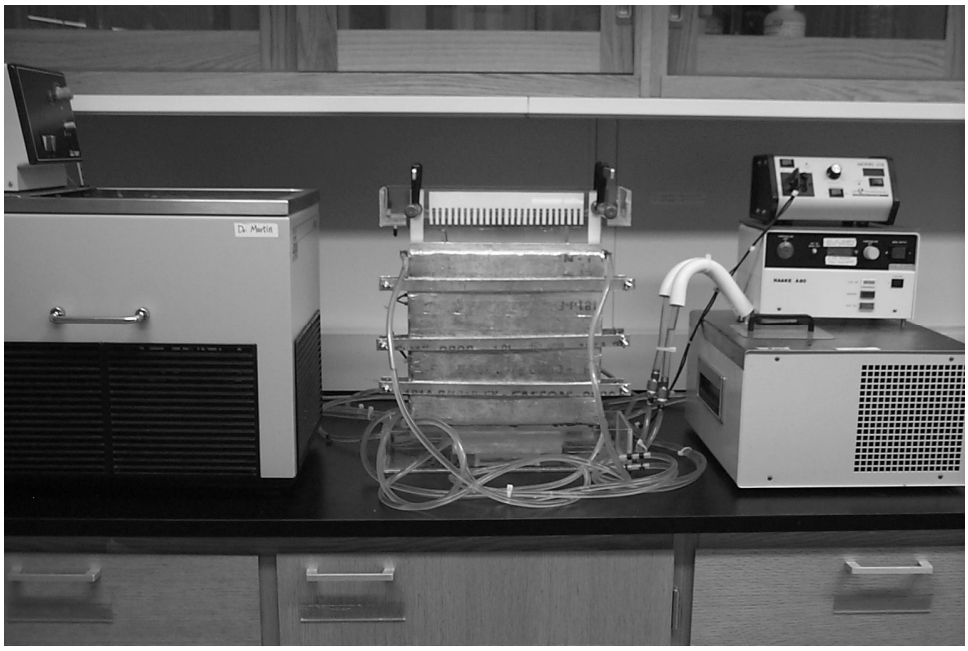

Figure 32: The complete TGGE apparatus prepared for electrophoresis. The two circulating water baths used to establish the vertical temperature gradient can be seen to either side of the apparatus.

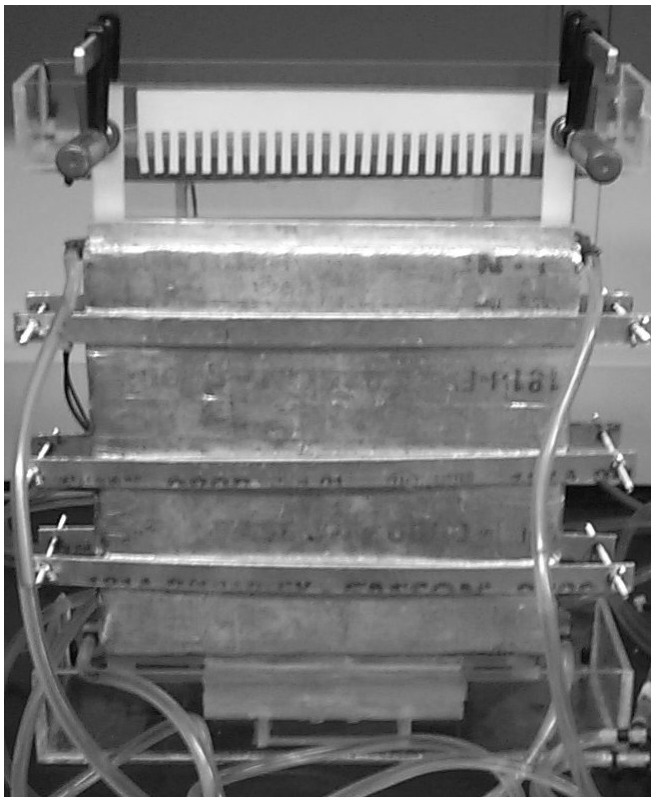

Figure 33: A closer view of the TGGE apparatus during electrophoresis. The Teflon® comb can be seen between two woodworking clamps used to hold the notched glass plate firmly against the rubber gasket surrounding the corresponding notch on the upper buffer reservoir. The three steel strap pairs holding the cassette assembly "sandwich" together are also clearly visible.

Figures 32 and 33, above show the completed TGGE apparatus prepared for operation. Schematic diagrams and drawings of the assembly process of the various components of the TGGE apparatus can be found in Appendix 5.

Verification of the Temperature Gradient Achieved by the TGGE Apparatus

Stability and linearity of the vertical temperature gradient produced in the denaturing polyacrylamide TGGE gel were verified by temperature measurements taken via using a thermocouple connected to an Extech multimeter (Extech Instruments Corp., Waltham, MA) inserted into the gel during a mock electrophoresis procedure. The

multimeter was factory-calibrated for use with the thermocouple and performed automatic *in silica* conversion of thermocouple resistance to temperature.

To accommodate the thermocouple, the gel cast for this experiment was required to be ¼" thick, instead of the 1/16"-thick gel normally used for TGGE. However, this difference in gel thickness was not of particular concern, as a stable temperature gradient would actually be more difficult to achieve in the thicker test gel than in the thinner gels used for TGGE.

Temperature measurements were taken at intervals over a 1200 min (20 hour) period with the apparatus operating normally in a mock electrophoresis procedure. Measurements were made at 2.5" depth intervals within the gel, spanning the entire region of the gel covered by the thermal plates. The data collected is presented Table 22 and Figure 34, below. Note that the measurement depths presented below are relative to the upper edge of the thermal plates.

The gel utilized for this experiment was a 40% polyacrylamide (37.5:1, acrylamide:bis-acrylamide), 7 M urea (electrophoresis-grade), 1.25 X TAE pH 8.0 gel. Mock electrophoresis was performed using 1.25 X TAE pH 8.0 running buffer, with a constant circulating flow of buffer solution between the upper and lower reservoirs provided by a peristaltic pump. Twin circulating water baths were used to provide a constant flow of temperature-regulated water to the aluminum blocks of the gel cassette. The low-temperature water bath providing flow to the upper thermal block channels was set to maintain 50°C, and the high temperature bath providing flow to the lower thermal block channels to 80°C. The water bath temperatures were set using the same

thermocouple used to measure gel temperature. The electrophoresis power supply was set to provide 12 W of constant power.

	Time (min)					
Inches	**0**	**60**	**120**	**240**	**420**	**1200**
1.25	53	53	53	53	53	54
3.75	58	60	60	59	60	60
6.00	63	64	64	64	65	65
8.25	69	70	70	70	70	70
10.75	75	76	76	75	76	76
cold bath	50	50	50	50	50	50
hot bath	80	80	80	80	80	80

Table 22: TGGE gel temperature measurements taken at specific depths at intervals during 1200 min of mock electrophoresis. Temperatures are reported in °C.

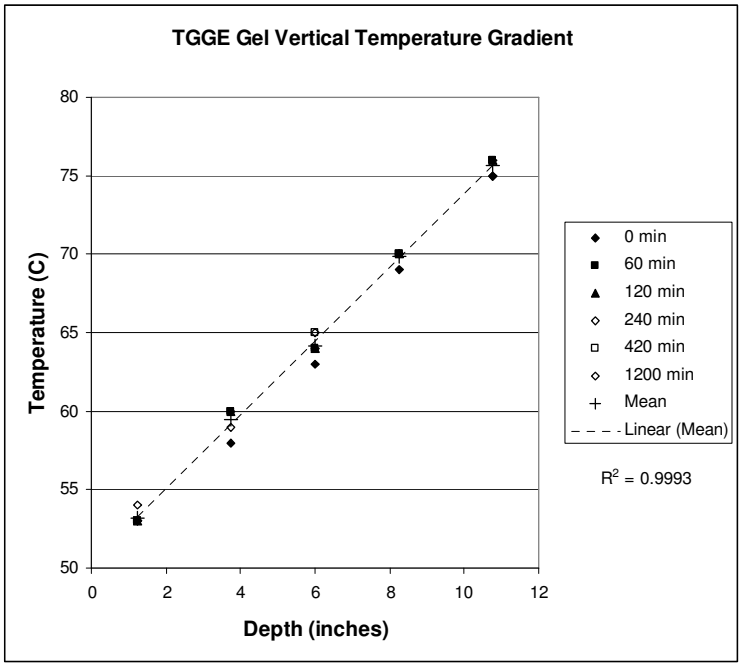

Figure 34: TGGE gel temperature measurements taken at specific depths at intervals during 1200 min of mock electrophoresis. Mean temperature at each depth was calculated and used for linear regression to create the trendline.

The temperature gradient produced by the TGGE apparatus appeared to be very linear with only minor temperature fluctuations over time. These minor temperature fluctuations are likely due to differences in heat accumulation rates within the gel as a

result of electrical current flow. Current flow generates heat, and varies somewhat over time due to changing electrolytic conditions in the running buffer as salts bind to the electrodes. Some minor temperature fluctuations in measured temperatures could also be attributed to small differences in thermocouple insertion depth during temperature measurement, as view of the thermocouple itself was obstructed by the aluminum thermal plates covering the gel and insertion depth had to be measured using marks on the thermocouple wire extending out of the gel. However, in the final analysis, the overall temperature gradient was considered to be quite linear and sufficiently stable for TGGE purposes.

Design of PCR Primers for TGGE Analysis

During initial experimentation with the TGGE apparatus, many different pairs of "universal" 16S rDNA primers were evaluated both *in silica* as well as in actual practice. The primers eventually selected for this work were based on the "universal" bacterial F-968-GC / R-1401 primer pair designed for TGGE described by Engelen et al. (1998). This primer pair amplifies a fragment of the 16S rRNA gene approximately 400 bp in length containing the V6, V7, and V8 hypervariable regions.

Initially, these primers were intended to be used as originally described. However, when synthesis of the GC-clamped primer was requested by Integrated DNA Technologies (IDT), Inc. (Coralville, IA), concern was expressed by the synthesis laboratory with respect to the GC-clamp. The original 40 bp GC-clamp sequence specified by Engelen et al. (1998) was 5'-CGCCCGGGGCGCGCCCCGGGCGGGG CGGGGGCACGGGGGG-3'. It is known that series of four or more guanines may produce a cruciform structure known as a guanine tetraplex during synthesis. This self-

complementary structure is very stable and results in pre-mature termination of the synthesis reaction (Poon and Macgregor, 1998). This GC-clamp contained four such regions, indicated by underlining in the sequence presented above. Therefore, while some synthesis was possible, the total yield was expected to be exceptionally low. However, as it was desirable to perform amplification using known primers supported by prior peer-review publication, synthesis was performed despite the concern raised. The result was as predicted by IDT. The synthesis yield was exceptionally low, resulting in an excessively high cost per PCR reaction.

In order to increase synthesis yield and reduce PCR cost, the 40 bp GC-clamp was redesigned to eliminate the guanine tetraplexes. Redesign of the GC-clamp was performed *in silica* using IDT's OligoAnalyzer 3.0 algorithm (Integrated DNA Technologies, Inc., 2003) with an oligonucleotide concentration of 0.25 μM and a 50 mM salt concentration specified (default settings). Redesigned clamps were evaluated by considering T_M as well ΔG values for the most likely hairpin, self-dimer, and hetero-dimer.

During the process of *in silica* primer analysis, an additional potential problem was uncovered affecting the F-968-GC primer. For optimum TGGE performance, the GC-clamp should be applied to the end of the amplification product already possessing the higher melting domain. If not, the heat-stable GC-clamp will interfere with successful denaturation of the lower melting domain at the appropriate temperature during migration through the temperature gradient (Chang BioScience Inc., 2002a). PrimoMelt 3.4 (Chang BioScience Inc., 2002b) was used to evaluate the normal melting

domains of the target 16S rDNA fragment with no GC-clamp. Figure 35, below presents the result of this analysis.

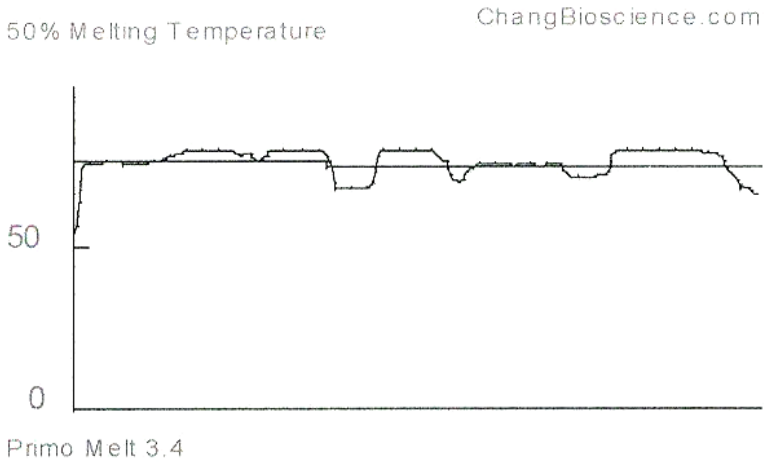

Figure 35: PrimoMelt 3.4 (Chang BioScience Inc., 2002a) *in silica* thermal denaturation profile predicted for the 16S rDNA fragment amplified by the TGGE primer pair. Note the lowest melting domain located at the 5' end (bases 1 through 125).

The lowest melting domain occurs on the 5' end of the product from bases 1-125, which would possess the GC-clamp if the original F-968-GC primer were used. Using the original primer pair, the 5' end of the amplification product would possess the GC-clamp. Therefore, in addition to redesigning the GC-clamp itself, it was also necessary to move the GC-clamp from the 5' forward primer to the 3' reverse primer, thus placing the GC-clamp on the 3' end of the amplification product and away from the lowest melting domain.

Finally, it was desirable to extend the annealing length of the primer bearing the GC-clamp in order to reduce the potential for mispriming due to annealing of the clamp itself to non-target DNA. Therefore, in addition to the redesigned GC-clamp an added to

the 5' end of the reverse primer, an additional 7 bp annealing length was added to the 3' end.

Sequences for the final primer pair used to amplify GC-clamped 16S rDNA for TGGE analysis (F968 and R1401/1378GC) are provided below in Table 23. Primer R1401/1378 is identical to primer R1401/1378GC except that it lacks the GC-clamp. This primer was needed for re-amplification of non-clamped products from 16S rDNA fragments separated by TGGE to provide template for sequencing.

F968	Sequence	5'-AACGCGAAGAACCTTAC-3'
	Length (bp)	17
	T_M (C)	50.1
	Hairpin ΔG (kcal/mol)	0.61
	Self-Dimer ΔG (kcal/mol)	-10.36
R1401/1378	Sequence	5'-CGGTGTGTACAAGGCCCGGGAACG-3'
	Length (bp)	24
	T_M (C)	67.3
	Hairpin ΔG (kcal/mol)	-1.02
	Self-Dimer ΔG (kcal/mol)	-15.89
	F968-Dimer ΔG (kcal/mol)	-6.61
R1401/1378GC	Sequence	5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCG CGGCCGCCGGTGTGTACAAGGCCCGGGAACG-3'
	Length (bp)	64
	T_M (C)	93.5
	Hairpin ΔG (kcal/mol)	-11.72
	Self-Dimer ΔG (kcal/mol)	-22.78
	F968-Dimer ΔG (kcal/mol)	-10.36

Table 23: Sequence and design data for TGGE primers. T_M and ΔG values were predicted *in silica* using IDT's OligoAnalyzer 3.0 (Integrated DNA Technologies, Inc., 2003).

These primers were synthesized by IDT and utilized for the TGGE experimentation described below. Figure 36, below shows the annealing sites of the primers relative to the full-length *E. coli* 16S rRNA gene. GC-clamped and unclamped amplification products produced by these primer pairs were approximately 474 bp and 434 bp in length, respectively and included the V6, V7, and V8 hypervariable regions.

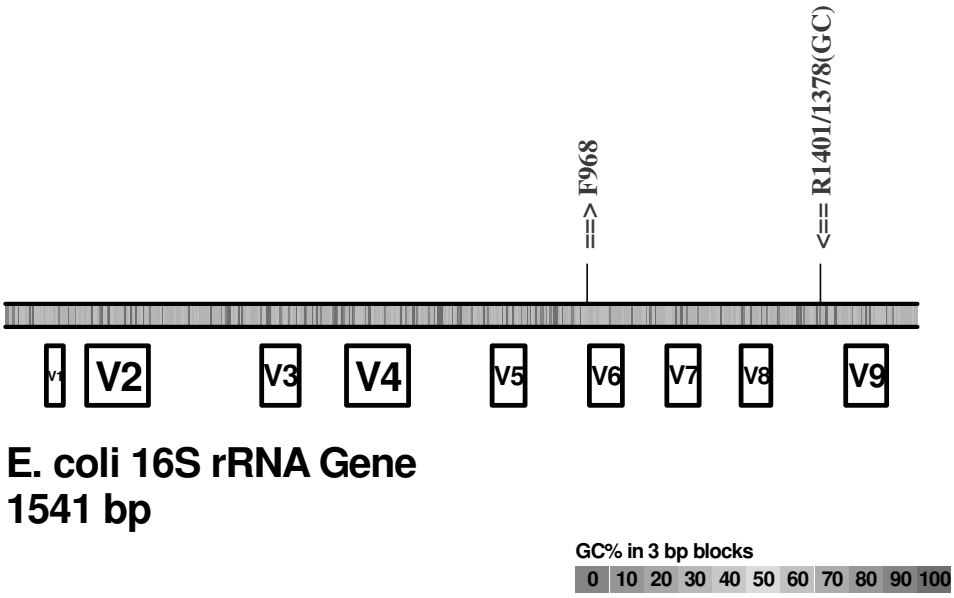

Figure 36: Annealing sites of the TGGE primers with respect to the full-length *E. coli* 16S rRNA gene sequence reported by Ehresmann et al. (1972). The approximate locations of the nine hypervariable regions valuable for identification and phylogenetic purposes are also indicated (Neefs et al., 1990; Chakravorty et al., 2007). Illustration created using pDraw32 (Acaclone Software, 2007).

Amplification and Gel Elution of GC-Clamped 16S rDNA Fragments

The pooled total root DNA extracts from surface disinfected *Medicago truncatula* roots (see Chapter 2) were used as template for PCR amplification of a GC-clamped fragment of the bacterial 16S rRNA gene. Each 100 µL PCR reaction mixture contained the following: 10 µL of Qiagen® 10X PCR buffer, 20 µL of Qiagen® 5X Q Solution, 10 µL of 25 mM $MgCl_2$, 2 µL of dNTP mix (10 mM each), 1 µL of 10 µM primer F968, 1 µL of 10 µM primer R1401/1378GC, 0.5 µL of Qiagen® *Taq* DNA polymerase, 5 µL of pooled total root DNA extract, and sterile QH_2O to volume. A series of positive control reactions were included in the PCR which substituted equal volumes of genomic DNA purifications from *Bacillus megaterium, Escherichia coli* XL-10 Gold, *Agrobacterium*

rhizogenes ATCC 15834, and *Agrobacterium tumefaciens*, respectively, for the 5 µL of pooled total root DNA extract template. Additionally, a negative control reaction was included which substituted an equal volume of sterile Q H_2O for the 5 µL of template. Reactions were performed using an MJ Research® PTC-200 thermal cycler programmed for an initial denaturation step of 94°C for 3 min, followed by 45 amplification cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min. To increase specificity, a simulated "hot start" was performed as described previously (see Chapter 2). PCR products were evaluated by electrophoresis.

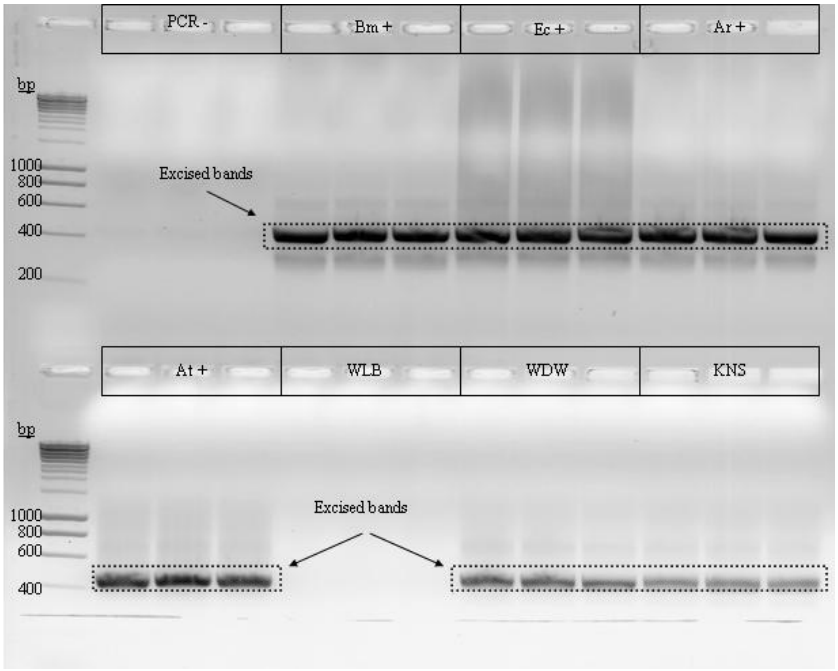

Figure 37a

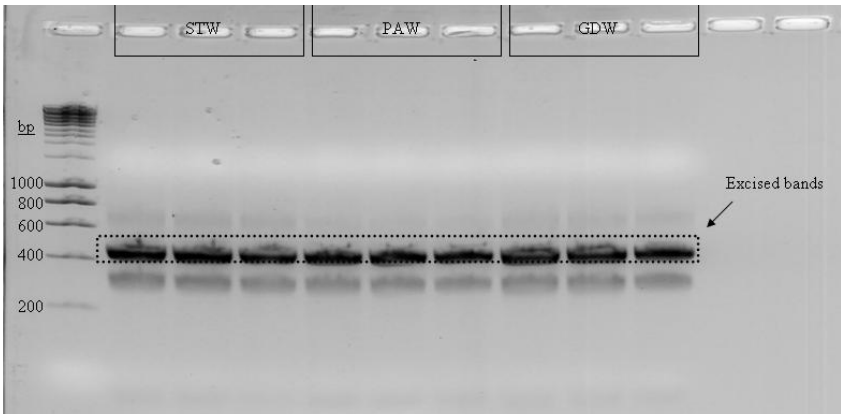

Figure 37b

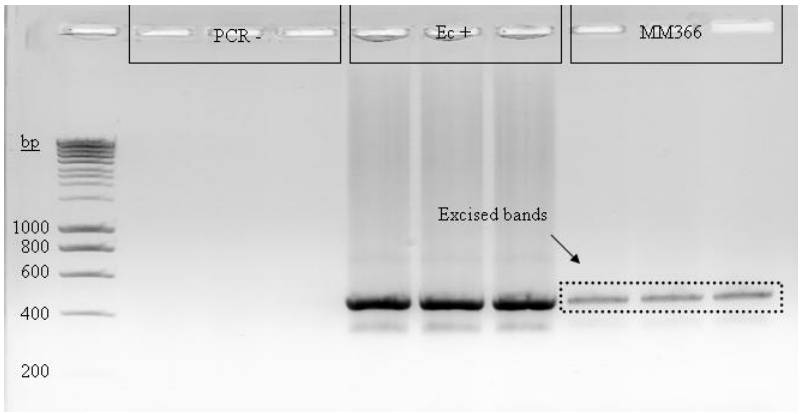

Figure 37c

Figures 37a, b, & c: Agarose gel electrophoresis of products from PCR amplification using pooled total root DNA as template. Each 100 µL product volume was divided into three consecutive lanes. The major product bands (indicated by dotted lines) were excised and the DNA eluted from the gel as a product purification step. The PCR negative control reaction containing no template is labeled as PCR -. PCR positive control reactions utilizing known genomic DNA templates are labeled as Bm + (*Bacillus megaterium*), Ec + (*Escherichia coli*), Ar + (*Agrobacterium rhizogenes*), and At + (*Agrobacterium tumefaciens*). Abbreviations used for soils and the growing medium are as follows: Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), Goodwell (GDW), and MetroMix® 366 (MM366). Standard lanes contained GeneChoice® DNA Ladder I. Electrophoresis was performed at 200 V for 30 min using 1.2% agarose, 1X sodium borate (pH 8.5) gels containing 0.5 µg/mL EtBr.

Note that in Figure 37a, PCR utilizing the total root DNA extracted from plants grown in unamended soil from Wilburton, OK (WLB samples) failed to produce any amplification products. Because amplification was achieved with all other templates, a reaction failure was thought to be highly unlikely. A second attempt at amplification using the same template failed to produce a product as well. Because sampling, surface disinfection, homogenization, and extraction of total DNA from the roots of these plants had been performed simultaneously and in parallel with the other experimental groups (and amplification of DNA was excellent from the other experimental groups was successful) it is also unlikely that amplification failure was due to a fault that occurred during these procedures. Furthermore, amplification of 16S rDNA from the same WLB extract using the full length 16S rDNA primer pair described in Chapter 2 for "shotgun" cloning was very successful. Regardless of the reason, all of the *M. truncatula* plants growing in unamended Wilburton soil had by now been harvested and their roots processed for extraction of total DNA, thus there was no possibility of repeating the DNA extraction procedure to supply new template for PCR. Nor would doing so have been advisable, as sampling error would have been introduced into the experiment due to potential differences in growth conditions, collection dates, pest influences, and myriad other factors. For this reason, the WLB sample was unable to be included as part of the TGGE analysis described below.

A PCR reaction equivalent to that described above was also used to amplify the 16S rRNA gene fragment from the total bacterial DNA extracted from the unknown bacteria which survived surface disinfection (see Chapter 2).

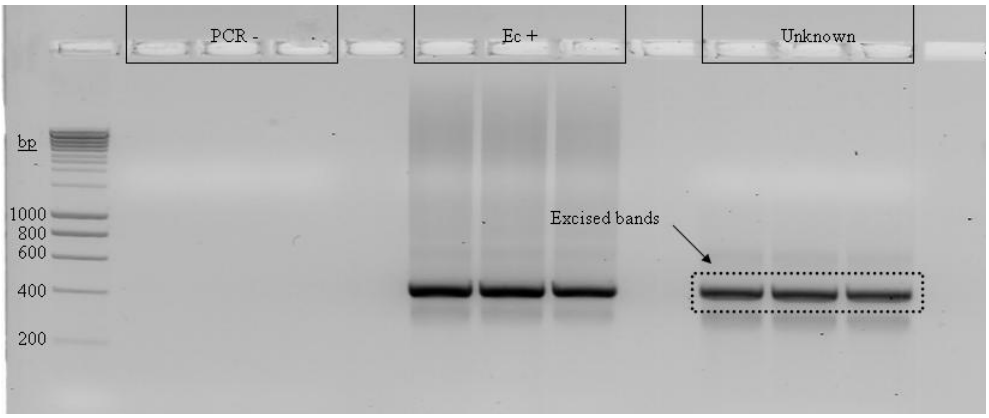

Figure 38: Agarose gel electrophoresis of products from PCR amplification using pooled DNA extracted from unknown bacteria surviving the surface disinfection protocol. Each 100 µL product volume was divided into three consecutive lanes. The major product bands resulting from amplification of this DNA (lanes titled "Unknown") were excised and the DNA extracted from the gel as a product purification step. The PCR negative control reaction containing no template is labeled as PCR -. The PCR positive control reactions utilizing a purified *E. coli* genomic DNA template is labeled as "Ec +". The standard lane contained GeneChoice® DNA Ladder I. Electrophoresis was performed at 200 V for 30 min using a 1.2% agarose, 1X sodium borate (pH 8.5) gel stained containing 0.5 µg/mL EtBr.

Because PCR amplification using the GC-clamped primer pair typically produced doublets as a result of primer-primer interaction and non-specific binding, it was necessary to purify the desired major product from each sample. This was accomplished by excising the major product band from each lane of the gel, followed by elution of the DNA using a QBioGene® GeneClean® *Turbo* kit according to the manufacturer's instructions. Excised bands are indicated by dotted lines in Figures 37a, b, & c, as well as in Figure 38, above.

Before the eluted 16S rDNA products could be utilized for TGGE analysis, it was necessary to determine the concentration and quantity of DNA available for each sample. Previous experimentation with the TGGE apparatus had determined that a minimum of

200 ng of DNA would need to be loaded per lane in order to produce visible bands on the gel. Therefore, DNA concentration in each elution product was measured by comparison to a known standard following agarose gel electrophoresis.

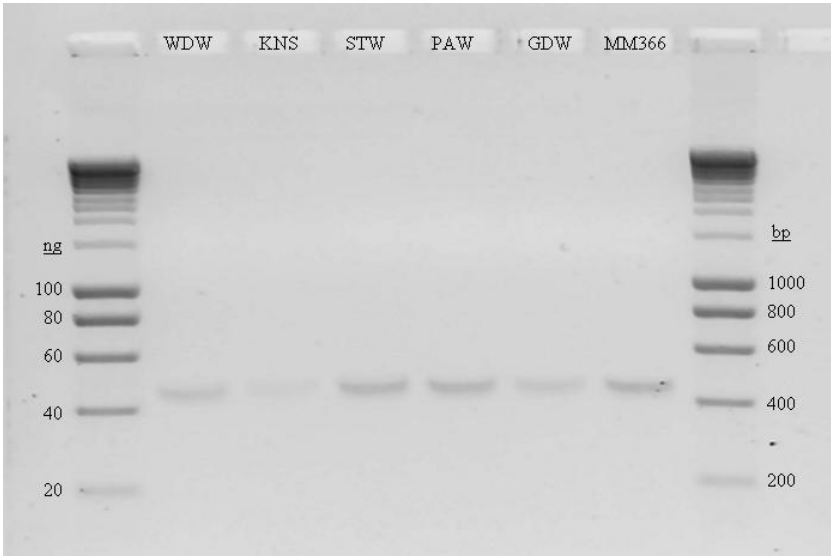

Figure 39: Electrophoresis of 3 µL volumes of gel elution products performed to estimate quantity and concentration of DNA in each sample. Sample name abbreviations used are as follows: Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), Goodwell (GDW), and MetroMix® 366 (MM366). Standard lanes contained GeneChoice® DNA Ladder I. Electrophoresis was performed at 200 V for 20 min using a 1.2% agarose, 1X sodium borate (pH 8.5) gel, containing 0.5 µg/mL EtBr.

Sample	DNA Quantity (ng/band)	[DNA] (ng/uL)
WDW	20	6.7
KNS	10	3.3
STW	30	10
PAW	30	10
GDW	20	6.7
MM366	30	10

Table 24: Estimated quantity and concentration of DNA in gel elution products as determined by comparison to the GeneChoice DNA Ladder I standard. Sample name abbreviations used are as follows: Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), Goodwell (GDW), and MetroMix® 366 (MM366). DNA concentration ([DNA]) was determined mathematically (ng per band / 3 µL per band).

DNA concentrations of each sample were estimated based on comparison to the GeneChoice® DNA Ladder I standard. These results are summarized above in Table 24. Because samples with estimated DNA concentrations less than 10 ng/µL lacked sufficient DNA to supply the 200 ng needed for TGGE, it was necessary to repeat the PCR amplification, band excision, and gel extraction protocols for these samples in order to produce amplification products in sufficient quantity. Thus, these procedures were performed several times for all samples in the same manner as described above until sufficient DNA was obtained by pooling together like purified end products. This process was likewise applied to the unknown surface disinfection-resistant bacterial DNA, as well as to purified genomic DNA from four different bacterial species in order to provide known samples as positive controls for TGGE. Final DNA concentrations of all samples used for TGGE are shown below in Table 25. All final gel-eluted amplification products were stored at -20°C until used for TGGE.

Sample	[DNA] (ng/uL)
B. megaterium	30
E. coli	40
A. rhizogenes	30
A. tumefaciens	20
WDW	10
KNS	10
STW	10
PAW	6.7
GDW	6.7
MM366	6.7
UNK	20

Table 25: Estimated concentrations of gel-eluted PCR amplification products in samples used for TGGE as determined by comparison to the GeneChoice DNA Ladder I standard. Abbreviations used are as follows: Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), Goodwell (GDW), MetroMix® 366 (MM366), and unknown surface disinfection-resistant bacteria (UNK). Amplification products from the genomic DNA of known bacterial species were included for use as positive controls during TGGE analysis.

TGGE of 16S rRNA Gene Fragment PCR Products

Aliquots of the gel-eluted PCR amplification products were prepared for TGGE by diluting as needed with sterile Q H_2O and TGGE loading buffer to provide equal sample volumes, each containing 200 ng of DNA. Products were loaded onto a a 40% polyacrylamide (37.5:1, acrylamide:bis-acrylamide), 7 M urea (electrophoresis-grade), 1.25 X TAE pH 8.0 gel with a vertical temperature gradient set at 69°C to 73°C. Electrophoresis was performed using 1.25 X TAE pH 8.0 running buffer, continually recirculated between the upper and lower reservoirs with the power supply set to provide 24 W of constant power over a total run time of 24 hours.

Following electrophoresis, the gel was silver-stained using a Bioneer® Silverstar® staining kit (Bioneer, Inc., Alameda, CA) according to the manufacturer's instructions. After staining, the gel was mounted for documentation and preservation by transfer to a clean sheet of transparent 1/8" acrylic. The mounted gel was temporarily preserved by covering with transparent plastic wrap to prevent desiccation and documented by scanning with a desktop flatbed scanner prior to storage at 4°C.

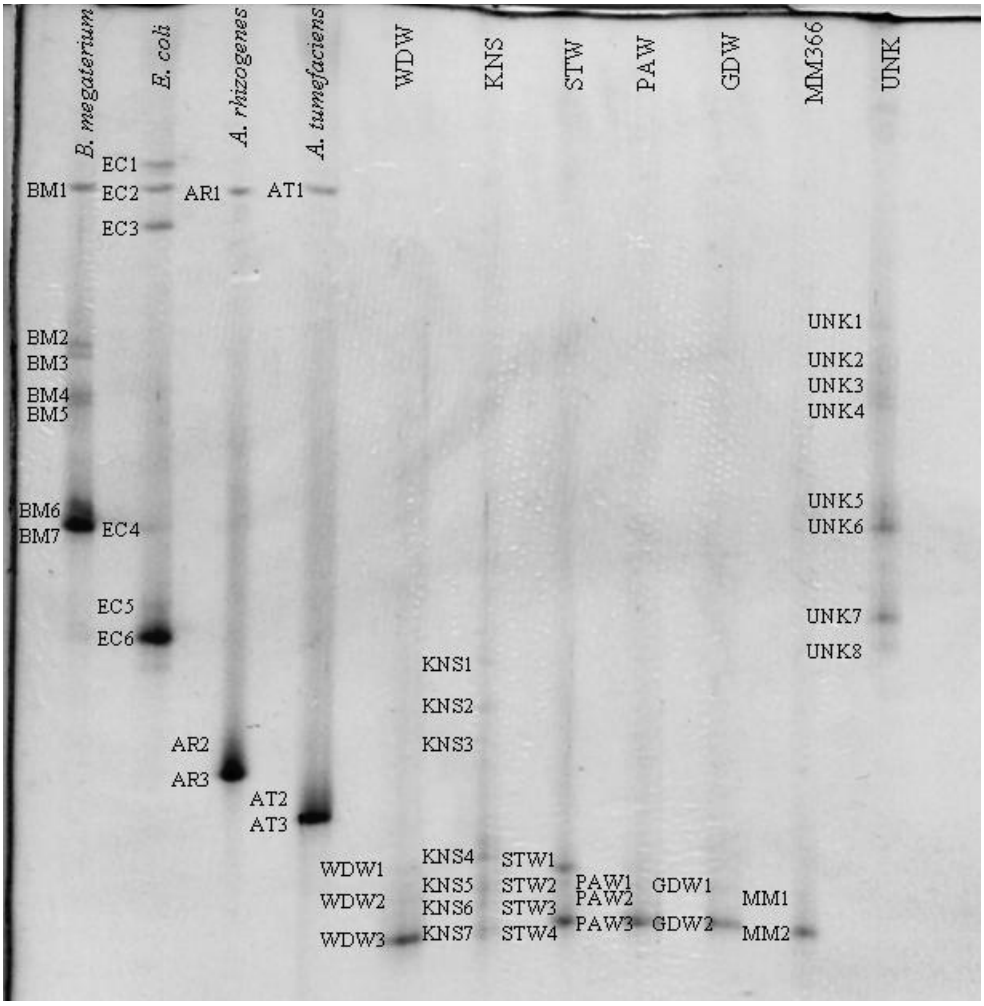

Figure 40: TGGE of 16S rRNA gene fragments. Numbered bands were selected for further amplification using the unclamped primer pair to provide DNA for cloning. The four leftmost lanes contained PCR-amplified 16S rRNA gene fragments from known bacterial stock cultures (*B. megaterium, E. coli, A. rhizogenes,* and *A. tumefaciens*). The rightmost lane (UNK) contained the amplification product from the unknown bacteria surviving the surface disinfection protocol. Abbreviations used for *M. truncatula* root samples are: Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), Goodwell (GDW), and MetroMix® 366 (MM366).

PCR Amplification of TGGE Bands

In order to provide DNA for cloning purposes, each discreet band appearing on the TGGE gel was subjected to PCR amplification using the unclamped primer pair. The scanned image of the TGGE gel presented above serves as a key for the identifying numbers assigned to each band on the gel.

PCR amplification of TGGE bands using the unclamped primer pair was performed using two 100 µL reactions per band. Each 100 µL PCR reaction volume contained the following: 10 µL of Qiagen® 10X PCR buffer, 20 µL of Qiagen® 5X Q Solution, 12 µL of 25 mM $MgCl_2$, 8 µL of dNTP mix (2.5 mM each), 1 µL of 10 µM primer F968, 1 µL of 10 µM primer R1401/1378, 0.5 µL of Qiagen® *Taq* DNA polymerase, and sterile QH_2O to volume.

DNA templates from the TGGE bands were obtained by stabbing with a pipettor tip under a laminar flow hood. Each band was stabbed by hand with a fresh sterile 20 µL pipettor tip approximately ten times. The tip was then placed into the 100 µL volume of PCR reaction mixture and swirled briefly. A fresh tip was used to stab the same band in the fashion described above to provide template for the second 100 µL reaction volume created for each sample.

A positive control reaction was included in the PCR which included 1µL of purified *Bacillus megaterium* genomic DNA from a stock solution. A PCR negative control reaction was also included, which consisted of reaction mixture without any added template. An additional band stab negative control reaction was created with template provided by stabbing the TGGE gel as described above in an area well removed from the sample lanes. This control was included to ensure that DNA from any

environmental microbes that might be present on the surface of the stabbed gel would not be amplified.

Reactions were performed using an MJ Research® PTC-200 thermal cycler programmed for an initial denaturation step of 94°C for 3 min, followed by 35 amplification cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min. To increase specificity, a simulated "hot start" was performed as described previously (see Chapter 2).

Purification of PCR-Amplified TGGE Bands by Gel Elution

In order to produce DNA of the highest possible quality for cloning purposes, it was desirable to clean the PCR products by gel excision. Therefore, amplification products were electrophoresed and major product bands excised and eluted using a QBioGene® GeneClean® *Turbo* kit according to the manufacturer's instructions. These gels can be seen in Appendix 6. Excised bands are denoted by dotted lines in the gel images. Like elution products were pooled and stored at -20°C.

Midipreparation of the pGEM®-5Zf (+) Cloning Vector

The pGEM®-5Zf(+) plasmid (Promega Corp, Madison, WI) construct was used as the cloning vector for this experiment. A 20 µL volume of *E. coli* XL-10 Gold® pGEM®-5Zf(+) was taken from a -20°C stock culture and inoculated into 2 mL of sterile LB+amp200 (Luria-Bertani media containing 200 µg/mL ampicillin) broth and incubated overnight at 37°C with mild agitation on a platform incubator/shaker. The following day, a loopful of the overnight culture was quadrant streaked to a Petri dish of LB+amp200 then incubated overnight at 37°C. The following day, a well isolated colony was

identified and inoculated to a culture flask containing 50 mL of LB+amp200 broth. The 50 mL culture was then incubated overnight at 37°C with mild agitation.

Four 10 mL aliquots were removed from the overnight culture and processed in parallel for recovery of plasmid DNA using the alkaline lysis midipreparation protocol described by Sambrook and Russell (2001). Midipreparation products were pooled to ensure uniformity, then divided into 100 µL aliquots in and stored at -80°C.

The plasmid DNA was checked for quality and concentration by agarose gel electrophoresis. A 2 µL aliquot of the midipreparation product was removed and used to create a 1:1, 1:10, 1:100 dilution series in Q H_2O, with a 1 µL aliquot from each dilution utilized for electrophoresis.

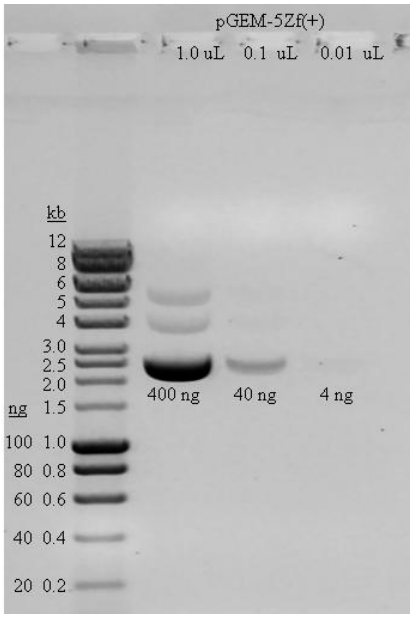

Figure 41: Electrophoresis of a pGEM®-5Zf(+) midipreparation product dilution series for determination of plasmid concentration. Lane labels indicate µL of midipreparation product. Plasmid concentration was estimated to be approximately 400 ng/µL based upon comparison to the GeneChoice® DNA Ladder I standard. Electrophoresis was performed at 200 V for 25 min using a 0.5% agarose, 1X sodium borate (pH 8.5) gel, containing 0.5 µg/mL EtBr.

Restriction Digestion of pGEM®-5Zf(+) with *Eco*R V

The pGEM®-5Zf(+) vector was cut with Invitrogen® *Eco*R V (Invitrogen Corp., Carlsbad, CA) to supply an insertion point for sequencing products into the vector's MCS (multiple cloning site).

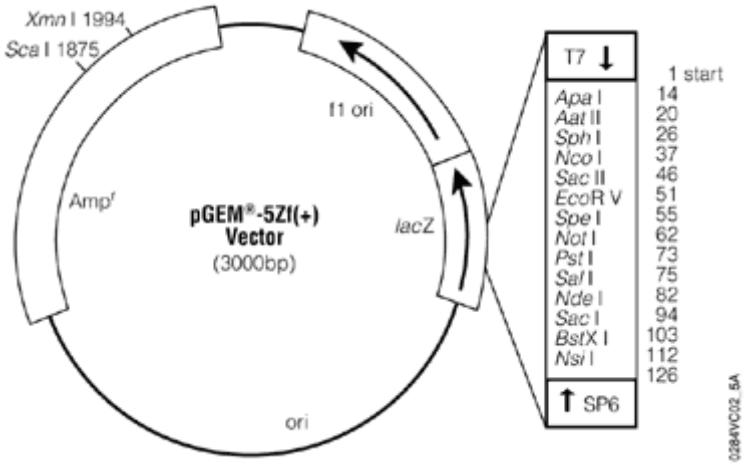

Figure 42: The Promega pGEM®-5Zf(+) cloning vector showing the *Eco*R V cleavage site at position 51. Image courtesy of Promega Corporation (Promega Corp., 2006).

Restriction digests of the pGEM®-5Zf(+) vector were performed using Invitrogen® *Eco*R V according to the manufacturer's instructions (Invitrogen, 2001). Final restriction products were pooled, then divided into aliquots and stored at -20°C. Successful cleavage of the vector was confirmed by agarose gel electrophoresis of a 1 µL aliquot containing 100 ng of the *Eco*R V-cleaved vector together with a 0.25 µL aliquot (100 ng) of the uncleaved midipreparation product for comparative purposes.

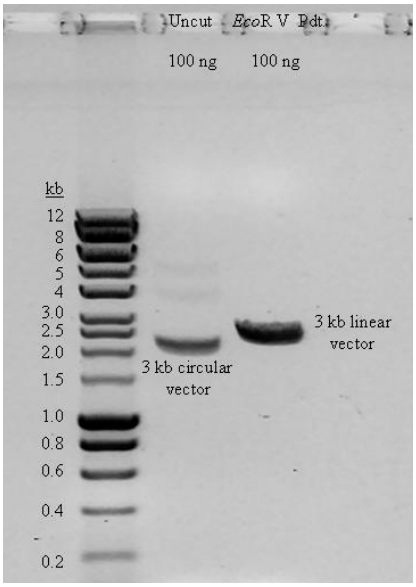

Figure 43: Agarose gel electrophoresis of uncut and *Eco*R V-cleaved pGEM®-5Zf(+) vector. The uncut circular plasmid migrates abnormally fast during electrophoresis, resulting in an easily detectable difference in migration distance as compared to the linear cleaved plasmid. The standard lane contained GeneChoice® DNA Ladder I. Electrophoresis was performed at 200 V for 25 min using a 0.5% agarose, 1X sodium borate (pH 8.5) gel, containing 0.5 µg/mL EtBr.

T-Tailing of the *Eco*R V-Restricted pGEM®-5Zf(+) Vector

Three T-tailing reactions were created and performed in parallel to provide sufficient vector for future usage. Each 20 µL T-tailing reaction mixture contained 1 µL of 50 mM $MgCl_2$, 2 µL of 20 mM dTTP, 2 µL of BioLine® 10X KCl reaction buffer, 1 µL of BioLine®*Taq* DNA polymerase (5 U/µL), 10 µL of *Eco*R V-restricted pGEM®-5Zf(+) (100 ng/µL), and sterile Q H_2O to volume. Reactions were incubated at 70°C for 2 hours, followed by pooling of the final products and storage at -20°C.

A-Tailing Protocol Trial Using *Bacillus megaterium* Band-Stab Products

While optimizing the protocol for PCR amplification of stabbed TGGE bands, several extra samples of gel-eluted PCR products from *B. megaterium* bands had been collected and stored at -20°C. Rather than consuming valuable experimental samples, the

A-tailing protocol was tested using these "spare" products. The DNA concentration in these products had been previously estimated by electrophoresis and comparison to a GeneChoice® DNA Ladder I standard.

Sample	[DNA] (ng/uL)
BM1	5
BM2	10
BM3	5
BM4	5
BM5	10
BM6	20
BM7	10

Table 26: Estimated concentration of gel-eluted TGGE band-stab PCR amplification products in samples used for testing of the A-tailing protocol.

A-tailing reactions were performed using each of these amplification products. Each 10 µL reaction contained 0.5 µL of 50 mM $MgCl_2$, 1 µL of 2.5 mM dATP, 1 µL of BioLine® *Taq* DNA polymerase (5U/µL), 1 µL of BioLine® 10X KCl reaction buffer, a volume of amplification product solution sufficient to provide 3 ng/µL in the final reaction mixture, and sterile Q H_2O to volume. Reactions were incubated for 30 minutes at 70°C, then stored at 4°C until used for the ligation performed shortly thereafter.

Ligation of T-Tailed pGEM®-5Zf(+) and A-Tailed Trial Inserts

Trial ligation reactions were performed using a 3:1 insert:vector ratio containing 50 ng of vector per reaction. NEB® (New England Biolabs, Inc., Ipswich, MA) T4 DNA ligase (200,000 CELU/µL) in 1X ligation buffer was utilized for the reaction, according to the instructions provided by the manufacturer (New England BioLabs, 2006). Reactions were incubated at 14°C for 4 hours then stored at 4°C until examination by agarose gel electrophoresis using 4 µL aliquots of the ligation products. For comparative

purposes, 0.5 µL aliquots of the uncut pGEM®-5Zf(+) midipreparation product and the *Eco*R V-cleaved vector were also included on the gel.

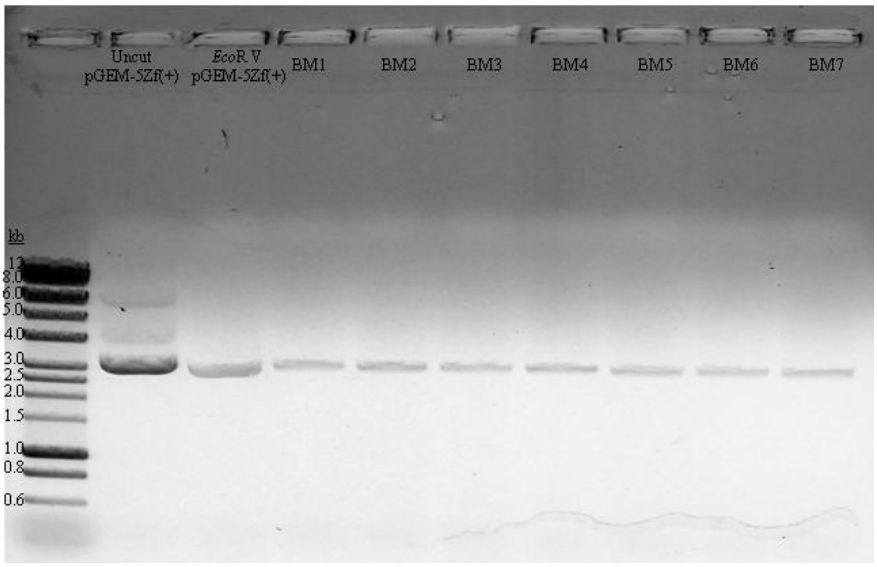

Figure 44: Electrophoresis of trial ligation products of T-tailed pGEM®-5Zf(+) – *Eco*R V and A-tailed *B. megaterium* (BM) TGGE band stab PCR products. Also included are samples of the pGEM®-5Zf(+) vector in native and *Eco*R V-restricted form. Electrophoresis was performed at 200 V for 25 min using a 0.5% agarose, 1X sodium borate (pH 8.5) gel, containing 0.5 µg/mL EtBr.

The migration distance of the ligation products differed than that of the linear vector, suggesting that ligation had been successful. However, due to the limited ability of the agarose gel to resolve an approximate 400 base difference between the ligation product and the native 3 kb vector lacking an insert, the success of the protocol was not entirely certain. However, it was decided to proceed with a trial transformation of *E. coli* XL-10 Gold® using these products to gain experience with the protocol.

First Trial Transformation of *E. coli* With pGEM®-5ZF(+) Constructs

The trial transformation was performed by the heat-shock method. For each transformation, a 50 µL aliquot of competent *E. coli* XL-10 Gold was thawed on ice and

mixed with a 5 µL aliquot of ligation product. Cells suspensions were incubated on ice for 60 min, then heat-shocked by transfer to a 42°C water bath for 90 sec, followed by a return to ice for 2 min. A 0.8 mL volume of SOC medium (2% tryptone, 0.5% yeast extract, 8.5 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose at pH 7) was then added to the suspension, followed by an incubation period at 37°C for 1 hour with gently agitation at 800 RPM using a Thermomixer R. Two control transformations were included in the transformation procedure. A no-insert control transformation was performed using 1 µL of the pGEM5®-Zf(+) midipreparation product, and a no-vector control transformation was created by omitting the addition of any plasmid to one competent cell culture.

A 100 µL aliquot was then removed from each cell suspension and spread to LB+tet50, cam20, amp200 (Luria-Bertani media containing 50 µg/mL tetracycline, 20 µg/mL chloramphenicol, and 200 µg/mL ampicillin) plates having surfaces treated with 2 µL of 20% IPTG and 100 µL of 2% X-Gal for blue/white screening. An additional 100 µL inoculation was made from each cell suspension to media identical to that described above, except lacking ampicillin. These plates were intended as controls to verify that the competent cells remained viable through the heat-shock protocol, regardless of transformation success. Inoculated plates were incubated at 37°C for 24 hours and examined for blue/white colonies.

All control plates appeared as appropriate. However, few-to-no white colonies were observed on the LB+tet50, cam20, amp200 plates inoculated with the cells transformed with the construct, indicating a failure of either the A- or T-tailing protocol, or a very low ligation efficiency.

Second Trial Transformation of *E. coli* With pGEM®-5ZF(+) Constructs

A second attempt was made to transform of *E. coli* XL-10 Gold with a pGEM®-5Zf(+) construct containing a PCR-amplified TGGE band insert. In this attempt, the A-tailing and T-tailing reactions were carried out in a manner identical to that described above. Ligation was attempted using an overnight incubation at 4°C rather than the 4 hour incubation at 14°C performed previously in an effort to increase efficiency. A 5 µL aliquot of each ligation product was examined by agarose gel electrophoresis along with a 1 µL aliquot of non-ligated T-tailed vector as a reference.

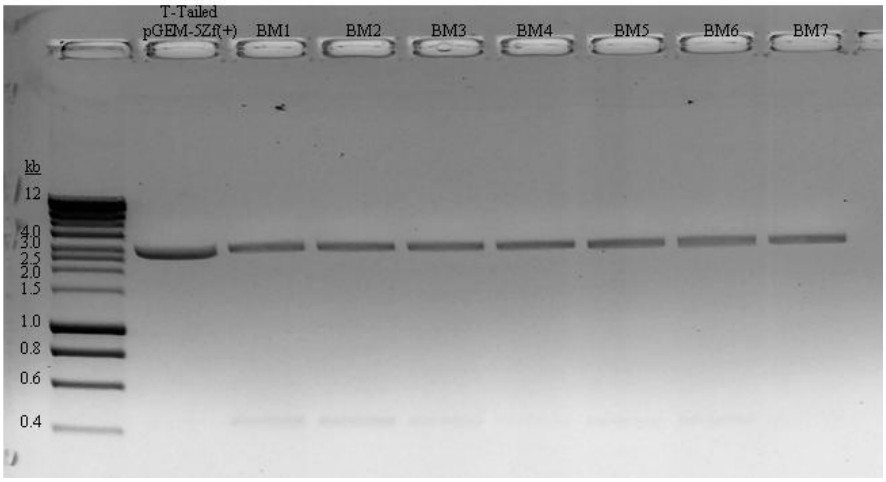

Figure 45: Electrophoresis of second trial ligation products. The standard lane contained GeneChoice DNA Ladder I. The second lane contains 1 µL of T-tailed pGEM®-5Zf(+) as a no-ligation reference. Subsequent lanes contain 5 µL of ligation products. Lane labels correspond to the identifications assigned to stabbed TGGE gel bands. Electrophoresis was performed at 200 V for 25 min using a 1% agarose, 1X sodium borate (pH 8.5) gel, containing 0.5 µg/mL EtBr.

Electrophoresis indicated that the results of this ligation were no different than the first trial. Additionally, electrophoresis of an *Eco*R V restriction digest performed on the ligation products yielded only a single band, thus indicating that no insert was present.

Final Trial Transformation of *E. coli* With pGEM®-5ZF(+) Constructs

A third and final attempt was made to successfully transform *E. coli* XL-10 Gold with a pGEM®-5Zf(+) construct containing a PCR-amplified TGGE band insert. Due to concern that the gel-excised TGGE band stab PCR product might have degraded during storage, these products were re-amplified by PCR to provide fresh samples for A-tailing. PCR using the unclamped primer pair was performed as described previously, but utilized 1 µL volumes of the original gel-eluted TGGE band stab PCR products as templates. A 5 µL aliquot of each amplification product was then examined by agarose gel electrophoresis.

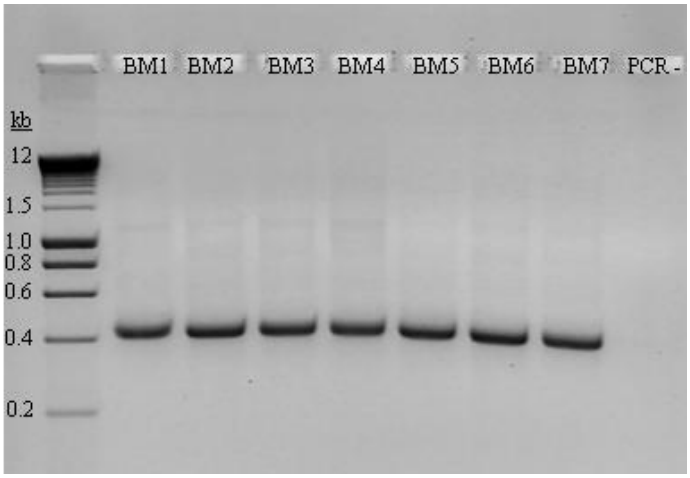

Figure 46: Re-amplification of trial *B. megaterium* TGGE band stab PCR products to provide fresh insert DNA for A-tailing. The right-most lane contained a PCR negative control reaction (PCR -) lacking any template. Lane labels correspond to the identifications assigned to stabbed TGGE gel bands. The standard lane contained GeneChoice® DNA Ladder I. Electrophoresis was performed at 200 V for 25 min using a 1.5% agarose, 1X sodium borate (pH 8.5) gel, containing 0.5 µg/mL EtBr.

Electrophoresis revealed amplification products that appeared to be of good quality. A 1 µL aliquot of each product was then analyzed for DNA purity and concentration using a NanoDrop® ND-1000 UV/Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA).

Sample	A260	A280	260/280	ng/uL
BM1	8.352	4.606	1.81	417.6
BM2	8.258	4.580	1.80	412.9
BM3	8.335	4.604	1.81	416.7
BM4	8.560	4.735	1.81	428.0
BM5	8.292	4.568	1.82	414.6
BM6	8.209	4.552	1.80	410.4
BM7	8.390	4.625	1.81	419.5

Table 27: Absorbance measurements and concentration estimates of re-amplified *B. megaterium* TGGE band stab PCR products used for trial A-tailing and ligation to the pGEM®-5Zf(+) cloning vector.

A-tailing and T-tailing reactions were again performed as described previously. Ligation was attempted under various conditions covering a broad spectrum of incubation times and insert:vector ratios, in an effort to identify a working combination. As this created a rather large experimental sample group, it was decided to use only the A-tailed re-amplification products from the BM-1 sample. The 20 µL ligation reaction volumes were formulated according to NEB instructions with appropriate adjustments made to the vector and insert solution volumes to provide reactions utilizing insert:vector ratios of 1:3, 1:1, 3:1, and 5:1. Three replicate series of these reaction volumes were created, with each series incubated under different conditions. The first series of reactions was incubated at 22°C for four hours, the second at 15°C for 18 hours, and the third at 4°C for 18 hours. After incubation, the ligation products were stored at -20°C until examination by agarose gel electrophoresis. The entire 20 µL volume of each ligation product was used for electrophoresis as well as prepared samples of T-tailed pGEM®-5Zf(+) vector (8

µL at 50 ng/µL), A-Tailed BM-1 re-amplified insert (4 µL at 20 ng/µL), unrestricted pGEM®-5Zf(+) (1µL of the midipreparation product at 400 ng/µL), *Eco*R V-cleaved pGEM®-5Zf(+) (1 µL at 100 ng/µL), and GeneChoice DNA Ladder I for comparative purposes

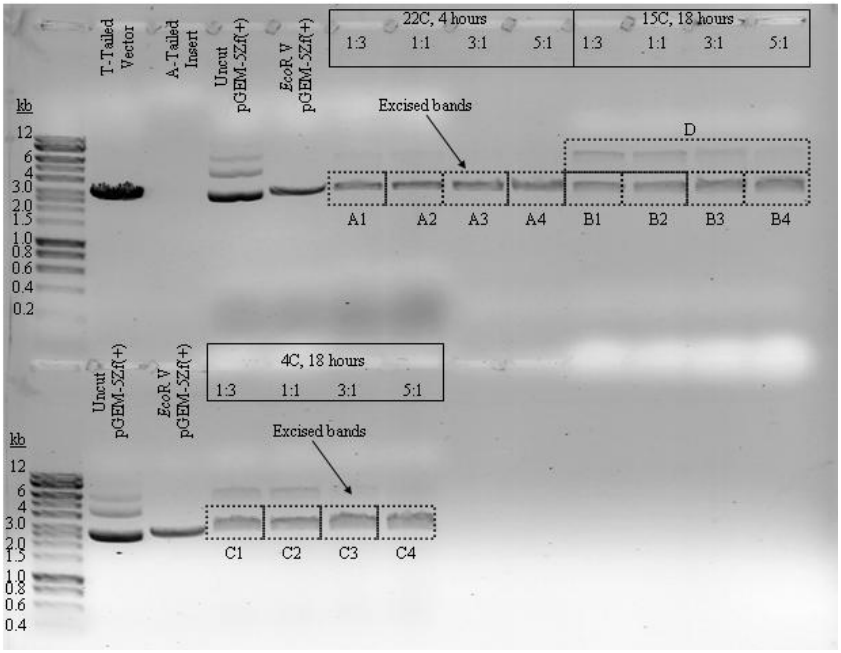

Figure 47: Agarose gel electrophoresis of ligation products. Ligation of the A-tailed *B. megaterium* TGGE band 1 insert with the T-tailed pGEM®-5Zf(+) vector was attempted at four different insert:vector ratios under three different incubation conditions. Bands indicated by dotted lines were excised and used as template for PCR to check for the presence of the insert. Excised bands were assigned the identifying labels indicated. Bands labeled as series A, B, and C were excised individually. The four bands identified as "D" were barely visible when viewed by the naked eye and had to be treated as a single band during excision. Electrophoresis was performed at 200 V for 25 min using a 0.5% agarose, 1X sodium borate (pH 8.5) gel, containing 0.5 µg/mL EtBr.

Additionally, PCR was used to test each ligation product for presence of an insert. Ligation product bands indicated by dotted lines and identified in Figure 47, above, were excised and the DNA eluted using the QBioGene® GeneClean® *Turbo* kit as per the manufacturer's instructions. Eluted DNA was used as template for PCR using the

FpGEM (5'-CGACTCACTATAGGGCGAATTG-3') / RpGEM (5'-CTCAAGCTAT GCATCCAACG-3') primer pair provided by Dr. Anderson. These primers anneal to sites flanking the MCS of the pGEM®-5Zf(+) vector (see Figure 48, below). Using this primer pair, ligation products lacking an insert would produce a 92 bp amplification product, while ligation products containing the BM-1 insert would produce an amplification product approximately 500 bp in length. Figure 64, below illustrates the annealing sites of the FpGEM / RpGEM primer pair to the pGEM®-5Zf(+) cloning vector.

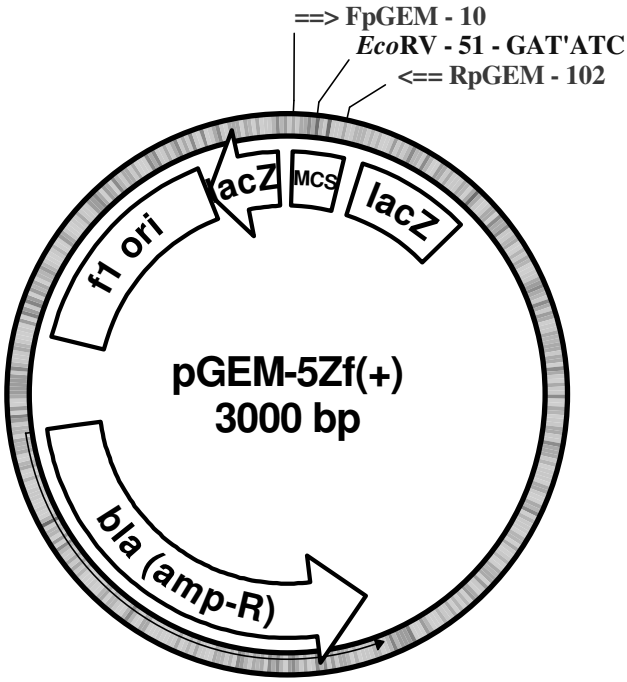

Figure 48: The pGEM®-5Zf(+) vector showing the location of the *Eco*R V recognition sequence and annealing sites of the FpGEM and RpGEM primers flanking the MCS. Image was created using pDraw32 (Acaclone Software, 2007).

Each 50 µL PCR reaction volume consisted of 5 µL of Qiagen® 10X PCR buffer, 10 µL of Qiagen® 5X Q Solution, 6 µL of 25 mM $MgCl_2$, 4 µL of dNTP mix (2.5 mM each), 2 µL of 2.5 µM primer FpGEM, 2 µL of 2.5 µM primer RpGEM, 0.5 µL of Qiagen® *Taq* DNA polymerase, 1 µL of the gel-eluted ligation product as template, and sterile QH_2O to volume. A no-insert control reaction was included which substituted 1 µL of the pGEM®-5Zf(+) midipreparation product as template. Finally, a PCR negative control reaction contained no template.

Reactions were performed using an MJ Research® PTC-200 thermal cycler programmed for an initial denaturation step of 94°C for 3 min, followed by 35 amplification cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min. To increase specificity, a simulated "hot start" was performed by starting the program with the thermal cycler unloaded. Reaction tubes were not loaded into the thermal cycler until the sample block had reached the initial denaturation temperature of 94°C. Following PCR, a 10 µL volume of each product was examined by agarose gel electrophoresis.

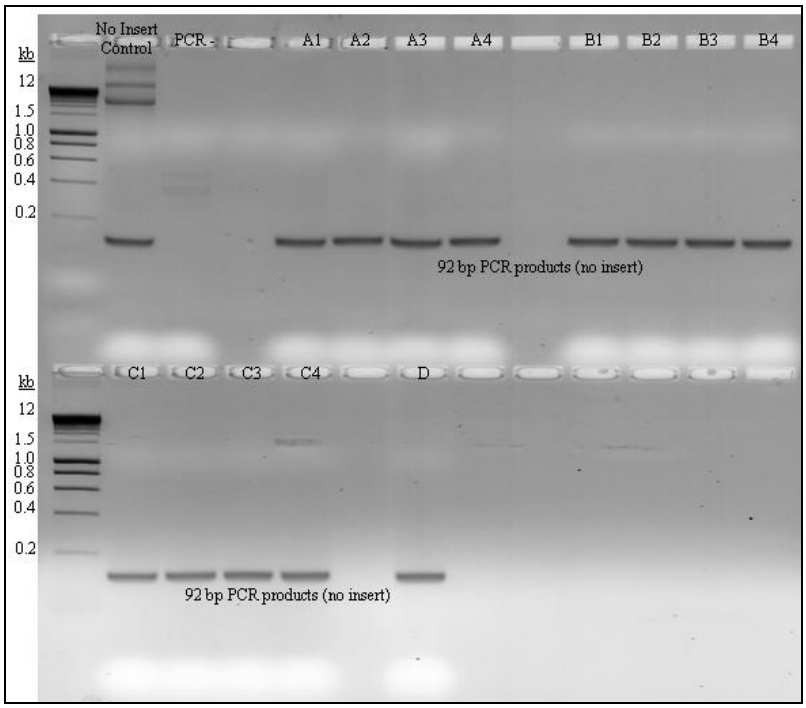

Figure 49: Electrophoresis of amplification products from PCR-based screening of gel-eluted ligation product bands for the presence of the BM-1 insert using the FpGEM / RpGEM primer pair. Each sample lane contained 10 µL of PCR product. The presence of an insert in any of the samples would have produced a band approximately 500 bp in length. All screened ligation products contain no insert DNA. Lane labels correspond to the excised bands shown above in Figure 47 used as templates for the PCR reactions. The "no insert control" reaction utilized pGEM®-5Zf(+) with no insert as template. The "PCR - " control reaction did not include any template. Standard lanes contained GeneChoice® DNA Ladder I. Electrophoresis was performed at 200 V for 25 min using a 1.5% agarose, 1X sodium borate (pH 8.5) gel, containing 0.5 µg/mL EtBr.

All of the samples produced amplification products less than 200 bp in length, indicating that the T-tailed vector had ligated back to itself without including any insert DNA under all of the ligation conditions tested. The reason for this failure remains unknown.

RESULTS AND DISCUSSION

Attempting to use TGGE for examination of *M. truncatula* root endophytes yielded unsatisfactory results from multiple standpoints.

Firstly, the primer pair designed for TGGE failed on two occasions to amplify any 16S rDNA fragments from the WLB treatment, while amplification of 16S rDNA fragments from the remaining six treatments was very successful, resulting in high yields of appropriately sized products (approximately 400 bp). However, the "shotgun" cloning technique yielded a tremendous diversity in endophytic 16S rDNA from the WLB treatment, therefore a lack of suitable template cannot explain amplification failure using the TGGE primer pair. At a minimum, one would expect *M. truncatula* chloroplast DNA to have been amplified.

Secondly, the resolution achieved by TGGE in this experiment was completely inadequate with respect to the number of distinguishable bands observed on the final gel versus the number of different endophytic species identified using the "shotgun" cloning approach described in Chapter 2. The TGGE gel presented previously in Figure 40 represents the highest resolution gel that was achievable with these samples using this apparatus. Alteration of the temperature gradient either reduced resolution or had no effect. Some positional similarities can be seen between bands appearing in the *Bacillus megaterium* control lane and bands in the UNK lane which are known to represent *Bacillus* species as a result of the shotgun cloning experiment. However, the shotgun cloning method also identified *Bacillus megaterium* in the MM366 extract, yet no *B. megaterium* control bands correspond to a defined band in the MM366 lane on the TGGE gel.

Thirdly, all attempts at cloning reamplified TGGE bands into the pGEM®-5Zf(+) vector for sequencing purposes failed. Several hypotheses were proposed to explain this failure, with the most likely being low-fidelity PCR amplification of the stabbed TGGE bands resulting from silver staining of the TGGE gel. Unfortunately, this problem prevented sequencing of the TGGE bands, thus their identities remain unknown and no meaningful results could be obtained from the technique.

While silver staining provides the highest sensitivity of all currently available methods for visualization of DNA on polyacrylamide gels, it has been documented to caused adverse effects on downstream manipulation of DNA including complete failures in reamplification by PCR and sequencing (Engelen et al., 1998; Lauretti et al., 2003; Peats, 1984) due to the mechanism of the stain, in which silver ions bind to either the phosphate backbones of nucleic acid chains or to nitrogen 7 of guanine or adenine (Lauretti et al., 2003), thereby causing potential interference with DNA polymerase activity.

Alternative staining methods known to allow downstream manipulation of DNA including SYBR Green I (Invitrogen Corp., Carlsbad, CA) and ethidium bromide were attempted, but both methods proved inadequate for visualizing minor TGGE bands as they were either not visible to the naked eye, or would did not remain visible for a sufficient time period for band stabbing when visualizing the gel using a UV transilluminator. Thus, silver staining was the only viable option for this work.

Direct sequencing of PCR amplification products from stabbed TGGE bands was attempted during trial experimentation with the TGGE apparatus, but was never successful. At the time, sequencing failure was ascribed to the likely presence of

multiple conflicting sequences within individual TGGE bands due to a lack of sufficient resolving capacity by our TGGE apparatus. Thus, the decision was made to clone the products into vectors prior to sequencing in order to resolve this problem. However, complete failure of both direct sequencing as well as ligation under so many tested conditions suggests some defect with the TGGE band stab amplification products themselves, again most likely due to low-fidelity PCR resulting from silver staining of the TGGE gel.

The TGGE results also raised concern with respect to the suitability of this technique for evaluation of this complex microbial population. The resolution of the gel was insufficient for adequate separation of amplified 16S rDNA fragments from the macerated root tissue yet multiple bands were produced in the control lanes containing bacterial 16S rDNA amplification products from single, known bacterial species. Review of literature suggests that this is not an uncommon result of TGGE and DGGE. In results presented by Heuer et al. (1997) multiple bands can be seen in many lanes containing 16S rDNA fragments amplified from individual known bacterial species. Heuer et al. (1997) ascribes this phenomenon to sequence differences arising from the presence of multiple *rrn* operons on the bacterial chromosome as well as artifact bands which result from single-stranded DNA not influenced differentially by the temperature gradient.

The TGGE technique was selected for this work as a method which could serve to condense endophytic bacterial 16S rDNA amplification products into a fingerprint with each band representing a unique sequence, thereby reducing the sequencing redundancy and associated expense encountered with “shotgun” cloning strategies. However, the

production of multiple bands by amplification products from a single bacterial species would seem to be contrary to this goal.

In conclusion, this author's experience with the TGGE technique proved to be an exceptionally time-consuming and frustrating experience. Each experimental TGGE gel required a minimum investment of two working days for casting, electrophoresis, and silver staining. Many "trial-and-error" electrophoreses were required to optimize parameters such as electrical conditions, sample loading concentration and volume, and the temperature gradient, representing months of labor investment to yield a final gel which contained 16S rDNA fingerprints that were overly-complex for known species, insufficiently resolved for complex samples, and unsuitable for downstream manipulation including PCR, cloning, and sequencing.

Without the capability to isolate, amplify, and sequence large numbers of bands from a TGGE gel, foreknowledge of expected organisms in the fingerprinted community is required so that lanes can be loaded with known DNA standards representing all possible community constituents for comparative purposes if any attempt at identification is to be made.

In conclusion, the "shotgun" cloning approach described in Chapter 2, while undoubtedly more expensive, proved to be far easier in execution, required much less time, and yielded meaningful results.

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APPENDIX

Appendix 1. These figures depict the electrophoresis of PCR products from surface disinfection efficacy verification tests. Each lane contained 10 µL of amplification product. A product band appearing in a sample lane is indicative of a sample that was not effectively surface disinfected and should be rejected from further analysis. PCR positive control reactions utilizing known genomic DNA templates are labeled as Bm + (*Bacillus megaterium*), Ec + (*Escherichia coli*), Ar + (*Agrobacterium rhizogenes*), and At + (*Agrobacterium tumefaciens*). The PCR negative control reaction containing no template is labeled as PCR -. Soils are abbreviated as follows: Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), and Goodwell (GDW). Ladder lanes contained GeneChoice® DNA Ladder I. Electrophoresis was performed at 200 V for 25 min using 1.2% agarose, 1X sodium borate (pH 8.5) gels containing 0.5 µg/mL EtBr.

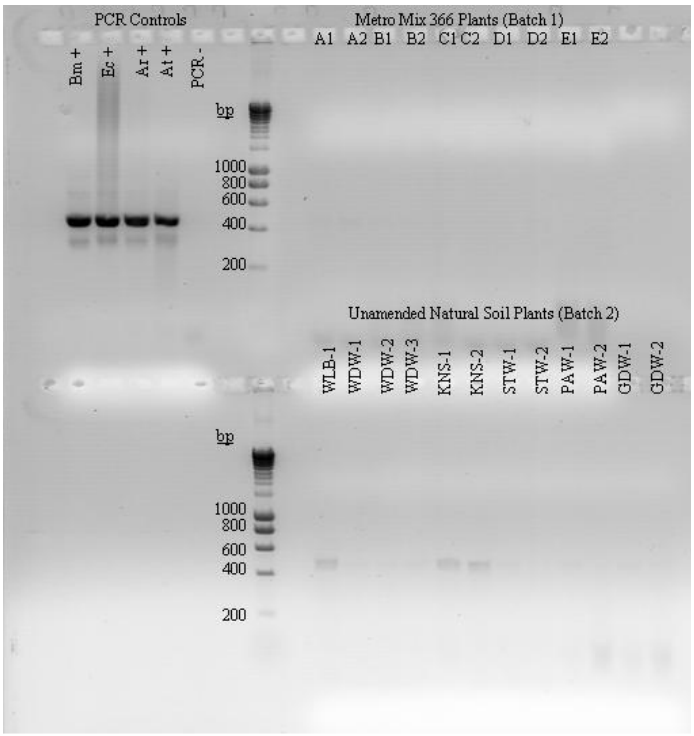

Figure A1.1: Electrophoresis of the amplification products from PCR-based verification of surface disinfection efficacy. This gel contained samples from tissue collection batches 1 and 2.

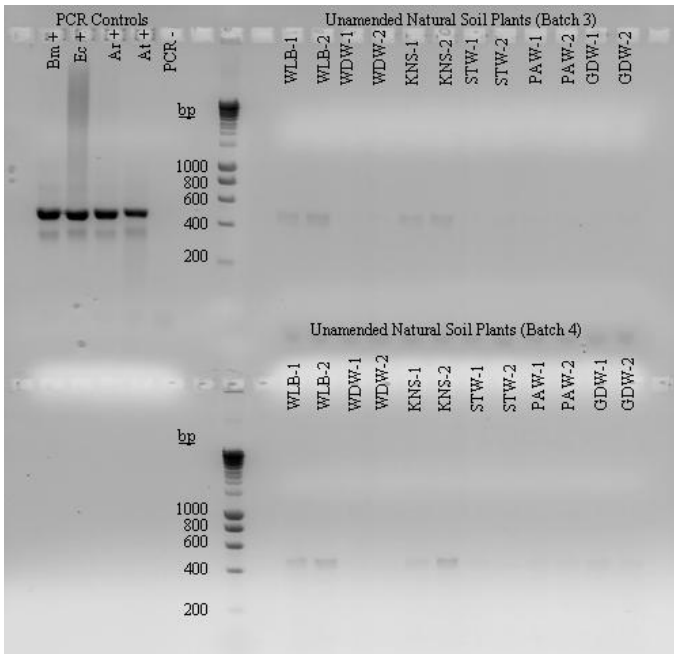

Figure A1.2: Electrophoresis of the amplification products from PCR-based verification of surface disinfection efficacy. This gel contained samples from tissue collection batches 3 and 4.

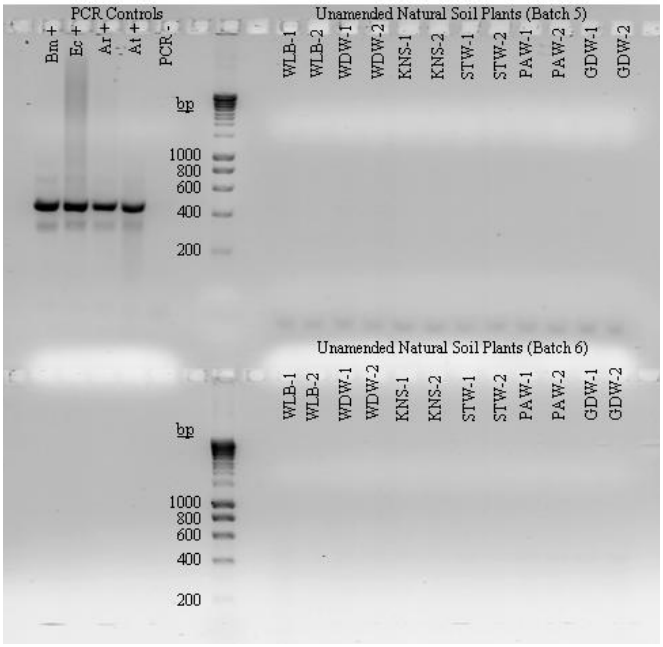

Figure A1.3: Electrophoresis of the amplification products from PCR-based verification of surface disinfection efficacy. This gel contained samples from tissue collection batches 5 and 6.

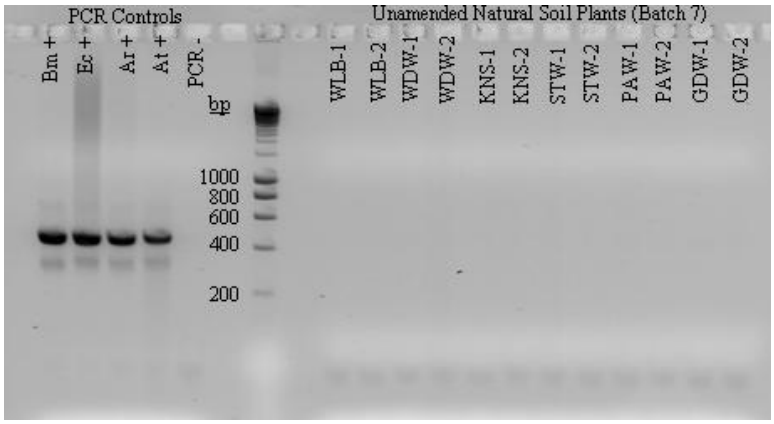

Figure A1.4: Electrophoresis of the amplification products from PCR-based verification of surface disinfection efficacy. This gel contained samples from tissue collection batch 7.

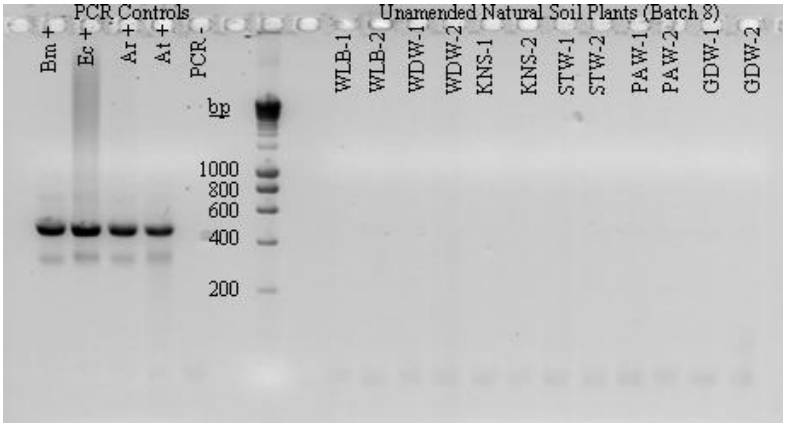

Figure A1.5: Electrophoresis of the amplification products from PCR-based verification of surface disinfection efficacy. This gel contained samples from tissue collection batch 8.

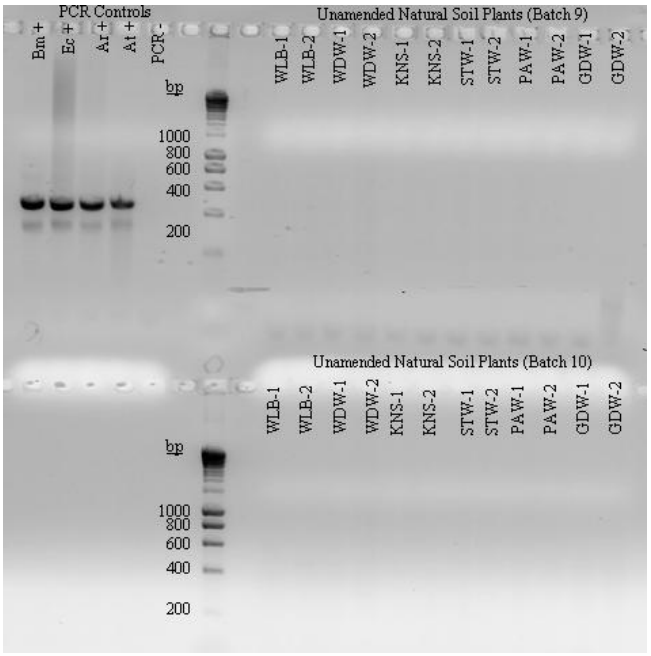

Figure A1.6: Electrophoresis of the amplification products from PCR-based verification of surface disinfection efficacy. This gel contained samples from tissue collection batches 9 and 10.

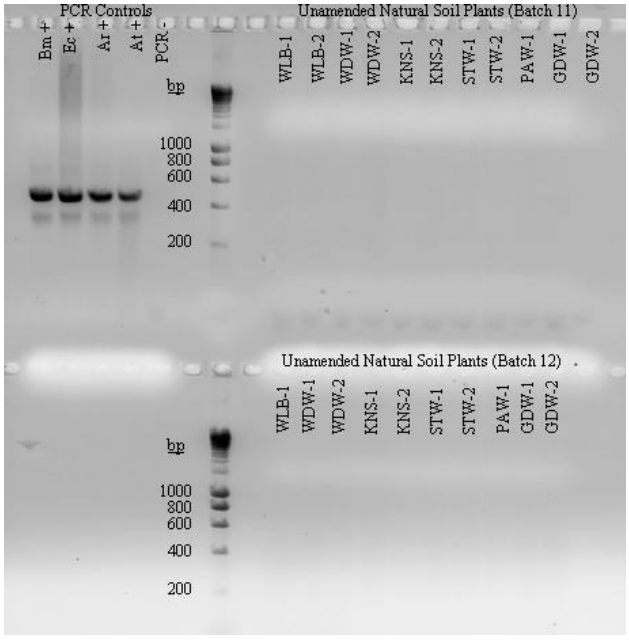

Figure A1.7: Electrophoresis of the amplification products from PCR-based verification of surface disinfection efficacy. This gel contained samples from tissue collection batches 11 and 12.

Appendix 2. The following tables are identification keys for the 384-well plate clonal library of *E. coli* XL-10 Gold (pGEM®-T Easy) containing the PCR amplified 16S rRNA genes from total DNA extracted from surface disinfected *M. truncatula* roots grow in different soils. Abbreviations used to identify clones identification labels indicate the soil used for *M. truncatula* growth: Wilburton (WLB), Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), Goodwell, (GDW), and MetroMix® 366 (MM366). Also included in the library were clones containing inserts from the amplification products of the unknown bacterium surviving surface disinfection (UNK), as well as from the following known bacterial species: *Bacillus megaterium* (BM), *Escherichia coli* XL-10 Gold (EC), *Agrobacterium rhizogenes* ATCC 15834 (AR), and *Agrobacterium tumefaciens* AGL-1 (AT).

A duplicate copy of each 384-well plate was created and preserved at -80°C. Plates MTE-1 (*Medicago truncatula* endophyte) through MTE-4 were sequenced by Dr. Bruce Roe of the University of Oklahoma Biochemistry Division. Plate MTE-5 was preserved, but not sequenced as it contained inserts from known organisms only.

MTE-1

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
B	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	
C	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
D	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	WLB
E	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
F	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	
G	145	146	147	148	149	150	151	152	153	154	155	156	169	170	171	172	173	174	175	176	177	178	179	180	
H	157	158	159	160	161	162	163	164	165	166	167	168	181	182	183	184	185	186	187	188	189	190	191	192	
I	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
J	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	
K	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
L	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	WDW
M	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
N	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	
O	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	
P	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	

Table A2.1: Identification key to 384-well clonal library plate MTE-1. This plate contained clones with vectors carrying 16S rRNA gene inserts from the Wilburton (WLB) and Woodward (WDW) sample series.

MTE-2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	23	24	25	
B	21	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	
C	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
D	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	**KNS**
E	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
F	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	
G	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	
H	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	
I	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
J	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	
K	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
L	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	**STW**
M	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
N	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	
O	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	
P	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	

Table A2.2: Identification key to 384-well clonal library plate MTE-2. This plate contained clones with vectors carrying 16S rRNA gene inserts from the Kansas (KNS) and Stillwater (STW) sample series.

MTE-3

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
B	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	
C	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
D	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	**PAW**
E	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
F	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	
G	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	
H	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	
I	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
J	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	
K	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
L	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	**GDW**
M	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
N	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	
O	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	
P	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	

Table A2.3: Identification key to 384-well clonal library plate MTE-3. This plate contained clones with vectors carrying 16S rRNA gene inserts from the Pawhuska (PAW) and Goodwell (GDW) sample series.

MTE-4

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
B	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	
C	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
D	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	MM
E	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	366
F	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	
G	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	165	164	166	167	168	
H	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	
I	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
J	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	
K	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
L	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	UNK
M	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
N	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	
O	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	
P	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	

Table A2.4: Identification key to 384-well clonal library plate MTE-4. This plate contained clones with vectors carrying 16S rRNA gene inserts from the MetroMix® 366 (MM366) and the unknown bacteria surviving surface disinfection (UNK) sample series.

MTE-5

	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	
A	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	
B	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10									
C																							BM (A1 - A10)		
D																							EC (A11-A20)		
E																							AR (A21-B6)		
F																							AT (B7-B16)		
G																									
H																									
I																									
J																									
K																									
L																									
M																									
N																									
O																									
P																									

Table A2.5: Identification key to 384-well clonal library plate MTE-5. This plate contained clones with vectors carrying 16S rRNA gene inserts from the following known bacterial species: *B. megaterium* (BM), *E. coli* (EC), *A. rhizogenes* (AR), and *A. tumefaciens* (AT).

Appendix 3. The following tables present the BLAST® results used for putative identification of 16S rDNA insert sequences from the WLB, WDW, KNS, STW, PAW, GDW, MM366, and UNK clonal libraries. For each insert, the strongest BLAST® hits with identities >97% for the forward and reverse sequences present in the twin contigs are shown, along with the final identification assigned to the insert according to the rules described in Chapter 2. Row and column identifications are with respect to the sample location on the 384-well clonal library microplate.

WLB

		Column					
		1	2	3	4	5	6
Row A	Contig 1	No Hits	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Pseudomonas* sp.	No Hits	No Hits
	Contig 2	No Data	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Pseudomonas fluorescens*	Unknown	No Hits
	Putative ID	No Hits	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Pseudomonas fluorescens*	Unknown	No Hits
Row B	Contig 1	Class Flavobacteria	*M. truncatula* chloroplast	No Hits	No Data	*M. truncatula* chloroplast	No Data
	Contig 2	No Data	No Hits	No Data	No Data	*M. truncatula* chloroplast	No Data
	Putative ID	Class Flavobacteria	*M. truncatula* chloroplast	No Hits	No Data	*M. truncatula* chloroplast	No Data
Row C	Contig 1	Unknown	Unknown	No Data	*Niastella jeongjuensis*	*M. truncatula* chloroplast	Unknown
	Contig 2	Phylum Actinobacteria	No Hits	No Data	No Data	*M. truncatula* chloroplast	Unknown
	Putative ID	Phylum Actinobacteria	Unknown	No Data	*Niastella jeongjuensis*	*M. truncatula* chloroplast	Unknown
Row D	Contig 1	Unknown	No Data	Unknown	No Data	No Data	No Data
	Contig 2	No Hits	No Data	No Data	No Data	No Data	No Data
	Putative ID	Unknown	No Data	Unknown	No Data	No Data	No Data
Row E	Contig 1	*Streptomyces hygroscopicus*	*Sphingomonas* sp.	No Data	*Niastella jeongjuensis*	No Hits	No Data
	Contig 2	*Streptomyces hygroscopicus*	*Hyphomicrobium denitrificans*	No Data	No Data	No Hits	No Data
	Putative ID	*Streptomyces hygroscopicus*	*Sphingomonas* sp.	No Data	*Niastella jeongjuensis*	No Hits	No Data
Row F	Contig 1	*M. truncatula* chloroplast	No Data	No Hits	No Hits	No Data	Family Flexibacteraceae
	Contig 2	No Data	No Data	No Data	No Data	No Data	No Data
	Putative ID	*M. truncatula* chloroplast	No Data	No Hits	No Hits	No Data	Family Flexibacteraceae
Row G	Contig 1	*Streptomyces acidiscabies*	No Data	*Rhizobium tropici*	*Niastella jeongjuensis*	Unknown	*M. truncatula* chloroplast
	Contig 2	*Streptomyces griseorubiginosus*	No Data	No Hits	Phylum Bacteroidetes	Unknown	*M. truncatula* chloroplast
	Putative ID	*Streptomyces* sp.	No Data	*Rhizobium tropici*	*Niastella jeongjuensis*	Unknown	*M. truncatula* chloroplast
Row H	Contig 1	*M. truncatula chloroplast*	No Hits	No Hits	Class Flavobacteria	No Data	No Data
	Contig 2	No Data	Unknown	No Data	No Data	No Data	No Data
	Putative ID	*M. truncatula chloroplast*	Unknown	No Hits	Class Flavobacteria	No Data	No Data

		Column					
		7	8	9	10	11	12
Row A	Contig 1	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Streptomyces* sp.	Unknown	*M. truncatula* chloroplast	Unknown
	Contig 2	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Streptomyces* sp.	Unknown	*M. truncatula* chloroplast	No Hits
	Putative ID	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Streptomyces* sp.	Unknown	*M. truncatula* chloroplast	Unknown
Row B	Contig 1	*Niastella jeongjuensis*	No Data	*M. truncatula* chloroplast	Phylum Bacteroidetes	No Hits	*M. truncatula* chloroplast
	Contig 2	*Niastella jeongjuensis*	No Data	*M. truncatula* chloroplast	Unknown	*Sinorhizobium melliloti*	*M. truncatula* chloroplast
	Putative ID	*Niastella jeongjuensis*	No Data	*M. truncatula* chloroplast	Phylum Bacteroidetes	*Sinorhizobium melliloti*	*M. truncatula* chloroplast
Row C	Contig 1	*M. truncatula* chloroplast	Phylum Bacteroidetes	No Hits	Phylum Bacteroidetes	No Hits	No Hits
	Contig 2	*M. truncatula* chloroplast	*Flavobacterium* sp.	Class Flavobacteria	No Data	No Data	*Niastella jeongjuensis*
	Putative ID	*M. truncatula* chloroplast	Phylum Bacteroidetes	Class Flavobacteria	Phylum Bacteroidetes	No Hits	*Niastella jeongjuensis*
Row D	Contig 1	No Hits	*M. truncatula* chloroplast	Unknown	No Hits	No Hits	No Data
	Contig 2	No Data	*M. truncatula* chloroplast	Unknown	Unknown	No Data	No Data
	Putative ID	No Hits	*M. truncatula* chloroplast	Unknown	Unknown	No Hits	No Data
Row E	Contig 1	Unknown	No Data	No Hits	Phylum Bacteroidetes	Unknown	Unknown
	Contig 2	No Data	No Data	No Data	No Hits	*Niastella jeongjuensis*	*Pantoea agglomerans*
	Putative ID	Unknown	No Data	No Hits	Phylum Bacteroidetes	*Niastella jeongjuensis*	*Pantoea agglomerans*
Row F	Contig 1	No Data	No Data	No Data	No Hits	No Data	No Data
	Contig 2	No Data	No Data	No Data	*Niastella jeongjuensis*	No Data	No Data
	Putative ID	No Data	No Data	No Data	*Niastella jeongjuensis*	No Data	No Data
Row G	Contig 1	Unknown	*M. truncatula* chloroplast	No Hits	No Hits	*M. truncatula* chloroplast	*Rhizobium* sp.
	Contig 2	No Hits	*M. truncatula* chloroplast	*Niastella jeongjuensis*	*Niastella jeongjuensis*	*M. truncatula* chloroplast	*Rhizobium tropici*
	Putative ID	Unknown	*M. truncatula chloroplast*	*Niastella jeongjuensis*	*Niastella jeongjuensis*	*M. truncatula chloroplast*	*Rhizobium tropici*
Row H	Contig 1	No Data	*M. truncatula chloroplast*	No Data	Unknown	No Hits	No Hits
	Contig 2	No Data	*M. truncatula chloroplast*	No Data	*Niastella jeongjuensis*	No Hits	*Sinorhizobium melliloti*
	Putative ID	No Data	*M. truncatula chloroplast*	No Data	*Niastella jeongjuensis*	No Hits	*Sinorhizobium melliloti*

		Column					
		13	14	15	16	17	18
Row A	Contig 1	*Rhizobium tropici*	*Bosea minatitlanensis*	No Data	No Data	No Hits	*M. truncatula* chloroplast
	Contig 2	*Rhizobium* sp.	Class Alphaproteobacterium	No Data	No Data	Unknown	*M. truncatula* chloroplast
	Putative ID	*Rhizobium tropici*	*Bosea minatitlanensis*	No Data	No Data	Unknown	*M. truncatula* chloroplast
Row B	Contig 1	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	*Bradyrhizobium* sp.	Class Alphaproteobacterium
	Contig 2	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	*Bradyrhizobium* sp.	*Rhizobium tropici*
	Putative ID	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	*Bradyrhizobium* sp.	*Rhizobium tropici*
Row C	Contig 1	*Agrobacterium* sp.	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	*M. truncatula* chloroplast	Unknown
	Contig 2	*Rhizobium leguminosarum*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	*M. truncatula* chloroplast	Class Alphaproteobacterium
	Putative ID	*Rhizobium leguminosarum*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	*M. truncatula* chloroplast	Class Alphaproteobacterium
Row D	Contig 1	No Hits	*Sinorhizobium meliloti*	*Rhizobium rhizogenes*	*Sinorhizobium* sp.	No Data	*Niastella jeongjuensis*
	Contig 2	No Data	No Data	*Rhizobium tropici*	No Data	No Data	*Niastella jeongjuensis*
	Putative ID	No Hits	*Sinorhizobium meliloti*	*Rhizobium* sp.	*Sinorhizobium* sp.	No Data	*Niastella jeongjuensis*
Row E	Contig 1	*Rhizobium tropici*	No Data	Unknown	Class Alphaproteobacterium	Unknown	*M. truncatula chloroplast*
	Contig 2	No Data	No Data	No Data	No Data	No Data	No Data
	Putative ID	*Rhizobium tropici*	No Data	Unknown	Class Alphaproteobacterium	Unknown	*M. truncatula chloroplast*
Row F	Contig 1	*Bradyrhizobium* sp.	No Data	*Agrobacterium tumefaciens*	No Data	No Hits	No Hits
	Contig 2	*Bradyrhizobium* sp.	No Data	No Data	No Data	No Data	No Data
	Putative ID	*Bradyrhizobium* sp.	No Data	*Agrobacterium tumefaciens*	No Data	No Hits	No Hits
Row G	Contig 1	Unknown	No Data	Unknown	No Data	No Hits	Class Flavobacteria
	Contig 2	No Data	No Data	*Phenylobacterium lituiforme*	No Data	Unknown	No Data
	Putative ID	Unknown	No Data	*Phenylobacterium lituiforme*	No Data	Unknown	Class Flavobacteria
Row H	Contig 1	No Hits	No Data	No Hits	*Agrobacterium tumefaciens*	*Sinorhizobium meliloti*	*Bradyrhizobium* sp.
	Contig 2	Unknown	No Data	No Data	No Data	No Data	No Data
	Putative ID	Unknown	No Data	No Hits	*Agrobacterium tumefaciens*	*Sinorhizobium meliloti*	*Bradyrhizobium* sp.

		Column					
		19	20	21	22	23	24
Row A	Contig 1	*M. truncatula* chloroplast	*Bradyrhizobium japonicum*	No Hits	*M. truncatula* chloroplast	*Niastella jeongjuensis*	*Sinorhizobium meliloti*
	Contig 2	*M. truncatula* chloroplast	*Bradyrhizobium* sp.	No Hits	*M. truncatula* chloroplast	*Flavobacterium* sp.	*Sinorhizobium meliloti*
	Putative ID	*M. truncatula* chloroplast	*Bradyrhizobium japonicum*	No Hits	*M. truncatula* chloroplast	*Niastella jeongjuensis*	*Sinorhizobium meliloti*
Row B	Contig 1	*Streptomyces ambifaciens*	Unknown	No Hits	*Stenotrophomonas* sp.	Class Alphaproteobacterium	No Data
	Contig 2	*Streptomyces flavidovirens*	Unknown	No Hits	No Hits	Unknown	No Data
	Putative ID	*Streptomyces* sp.	Unknown	No Hits	*Stenotrophomonas* sp.	Class Alphaproteobacterium	No Data
Row C	Contig 1	*Sinorhizobium meliloti*	*Shinella yambaruensis*	No Data	No Data	*M. truncatula* chloroplast	Unknown
	Contig 2	*Sinorhizobium meliloti*	No Hits	No Data	No Data	*Phenylobacterium lituiforme*	Unknown
	Putative ID	*Sinorhizobium meliloti*	*Shinella yambaruensis*	No Data	No Data	*M. truncatula* chloroplast	Unknown
Row D	Contig 1	No Data	No Data	*M. truncatula* chloroplast	Class Flavobacteria	No Data	No Hits
	Contig 2	No Data	No Data	No Data	No Data	No Data	No Hits
	Putative ID	No Data	No Data	*M. truncatula* chloroplast	Class Flavobacteria	No Data	No Hits
Row E	Contig 1	*M. truncatula chloroplast*	*Agrobacterium rhizogenes*	Phylum Actinobacteria	*M. truncatula chloroplast*	*Rhizobium tropici*	No Data
	Contig 2	No Data	No Data	No Data	No Data	No Data	No Data
	Putative ID	*M. truncatula chloroplast*	*Agrobacterium rhizogenes*	Phylum Actinobacteria	*M. truncatula chloroplast*	*Rhizobium tropici*	No Data
Row F	Contig 1	No Data	No Data	*M. truncatula chloroplast*	*M. truncatula chloroplast*	*Shinella yambaruensis*	No Hits
	Contig 2	No Data	No Data	No Data	*M. truncatula chloroplast*	No Hits	No Data
	Putative ID	No Data	No Data	*M. truncatula chloroplast*	*M. truncatula chloroplast*	*Shinella yambaruensis*	No Hits
Row G	Contig 1	No Hits	*M. truncatula* chloroplast	*Streptomyces corchorusii*	No Hits	*M. truncatula chloroplast*	No Hits
	Contig 2	No Data	No Data	*Streptomyces griseorubiginosus*	No Data	*M. truncatula chloroplast*	No Hits
	Putative ID	No Hits	*M. truncatula* chloroplast	*Streptomyces* sp.	No Hits	*M. truncatula chloroplast*	No Hits
Row H	Contig 1	No Data	*M. truncatula chloroplast*	No Data	*M. truncatula chloroplast*	*M. truncatula chloroplast*	No Data
	Contig 2	No Data	No Data	No Data	No Data	*M. truncatula chloroplast*	No Data
	Putative ID	No Data	*M. truncatula chloroplast*	No Data	*M. truncatula chloroplast*	*M. truncatula chloroplast*	No Data

Table A3.1: BLAST® results used for putative identification of 16S rDNA insert sequences from the WLB endophyte clonal library.

WDW

		Column					
		1	2	3	4	5	6
Row I	Contig 1	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row J	Contig 1	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	Class Alphaproteobacterium
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Data	No Hits
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	Class Alphaproteobacterium
Row K	Contig 1	No Data	No Hits	No Hits	No Hits	*Sinorhizobium meliloti*	No Data
	Contig 2	No Data	Unknown	No Data	No Data	No Data	No Data
	Putative ID	No Data	Unknown	No Hits	No Hits	*Sinorhizobium meliloti*	No Data
Row L	Contig 1	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data	No Data
	Putative ID	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
Row M	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Matsuebacter chitosanotabidus*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Hits	*Sinorhizobium meliloti*	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Matsuebacter chitosanotabidus*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row N	Contig 1	No Data	*Sinorhizobium meliloti*	No Data	No Hits	No Hits	No Data
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Hits	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Hits	No Hits	No Data
Row O	Contig 1	*Sinorhizobium meliloti*	Unknown	No Hits	*Pseudomonas fluorescens*	No Hits	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Pseudomonas fluorescens*	No Hits	*Sinorhizobium meliloti*
Row P	Contig 1	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Unknown
	Contig 2	No Data	*Sinorhizobium meliloti*	No Data	Class Alphaproteobacterium	*Sinorhizobium meliloti*	Unknown
	Putative ID	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Unknown

		Column					
		7	8	9	10	11	12
Row I	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Class Betaproteobacterium	No Data	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Class Betaproteobacterium	No Data	*Sinorhizobium meliloti*
Row J	Contig 1	*Sinorhizobium meliloti*	Unknown	Unknown	No Data	*Sinorhizobium meliloti*	*Chitinophaga ginsengisoli*
	Contig 2	No Data	No Data	*Frateuria aurantia*	No Data	No Data	*Chitinophaga ginsengisoli*
	Putative ID	*Sinorhizobium meliloti*	Unknown	*Frateuria aurantia*	No Data	*Sinorhizobium meliloti*	*Chitinophaga ginsengisoli*
Row K	Contig 1	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Putative ID	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
Row L	Contig 1	*Bradyrhizobium japonicum*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data
	Contig 2	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*
	Putative ID	*Bradyrhizobium japonicum*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row M	Contig 1	*Sinorhizobium meliloti*	No Data	No Hits	*Sinorhizobium meliloti*	No Hits	*M. truncatula* chloroplast
	Contig 2	*Sinorhizobium meliloti*	No Data	No Hits	*Sinorhizobium meliloti*	No Hits	*M. truncatula* chloroplast
	Putative ID	*Sinorhizobium meliloti*	No Data	No Hits	*Sinorhizobium meliloti*	No Hits	*M. truncatula* chloroplast
Row N	Contig 1	*Bacillus* sp.	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data
	Putative ID	*Bacillus* sp.	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row O	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row P	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data
	Contig 2	No Data	Class Alphaproteobacterium	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data

		Column					
		13	14	15	16	17	18
Row I	Contig 1	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*
Row J	Contig 1	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
	Contig 2	*Sinorhizobium meliloti*	No Data	No Data	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
Row K	Contig 1	*Sinorhizobium meliloti*	No Data	No Hits	No Hits	No Hits	*Bradyrhizobium japonicum*
	Contig 2	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	No Data	No Hits	*Sinorhizobium meliloti*	No Hits	*Bradyrhizobium japonicum*
Row L	Contig 1	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	Unknown	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	*M. truncatula* chloroplast	No Data	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	Unknown	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row M	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Class Alphaproteobacterium
	Contig 2	No Hits	No Data	No Data	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Class Alphaproteobacterium
Row N	Contig 1	*Sinorhizobium meliloti*	*Pseudomonas saccharophila*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	Class Betaproteobacterium	No Data	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	Class Betaproteobacterium	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row O	Contig 1	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	Unknown	*Sinorhizobium meliloti*	Unknown	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row P	Contig 1	No Data	*M. truncatula chloroplast*	*Sinorhizobium meliloti*	No Data	*Pseudomonas* sp.	No Hits
	Contig 2	No Data	*M. truncatula chloroplast*	*Sinorhizobium meliloti*	No Data	No Data	No Hits
	Putative ID	No Data	*M. truncatula chloroplast*	*Sinorhizobium meliloti*	No Data	*Pseudomonas* sp.	No Hits

		Column					
		19	20	21	22	23	24
Row I	Contig 1	No Data	No Data	*M. truncatula* chloroplast	No Hits	*Sinorhizobium meliloti*	No Hits
	Contig 2	No Data	No Data	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	No Data	No Data	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
Row J	Contig 1	*M. truncatula* chloroplast	No Data	No Hits	No Data	Unknown	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	No Data	*Caulobacter* sp.	*Asticcacaulis* sp.
	Putative ID	*M. truncatula* chloroplast	No Data	No Hits	No Data	*Caulobacter* sp.	*Sinorhizobium meliloti*
Row K	Contig 1	No Data	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	No Data	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row L	Contig 1	No Hits	Unknown	*Sinorhizobium meliloti*	No Hits	No Data	No Data
	Contig 2	No Data	No Data	No Data	No Data	No Data	No Data
	Putative ID	No Hits	Unknown	*Sinorhizobium meliloti*	No Hits	No Data	No Data
Row M	Contig 1	Unknown	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
	Contig 2	No Data	No Hits	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data
	Putative ID	Unknown	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
Row N	Contig 1	No Data	No Data	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Putative ID	No Data	No Data	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
Row O	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Data	No Hits
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Data	No Hits
Row P	Contig 1	Class Betaproteobacterium	No Data	No Data	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
	Contig 2	Unknown	No Data	No Data	No Data	No Data	No Data
	Putative ID	Class Betaproteobacterium	No Data	No Data	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*M. truncatula* chloroplast

Table A3.2: BLAST® results used for putative identification of 16S rDNA insert sequences from the WDW endophyte clonal library.

KNS

		Column					
		1	2	3	4	5	6
Row A	Contig 1	No Data	No Data	*Labrys wisconsinensis*	No Hits	*M. truncatula* chloroplast	*Burkholderia cepacia*
	Contig 2	No Data	No Data	No Data	No Hits	No Hits	No Hits
	Putative ID	No Data	No Data	*Labrys wisconsinensis*	No Hits	*M. truncatula* chloroplast	*Burkholderia cepacia*
Row B	Contig 1	No Data	No Data	No Data	No Hits	*Rhizobium tropici*	No Hits
	Contig 2	No Data	No Data	No Data	No Hits	*Rhizobium tropici*	No Hits
	Putative ID	No Data	No Data	No Data	No Hits	*Rhizobium tropici*	No Hits
Row C	Contig 1	No Data	No Hits	No Data	No Data	*Rhizobium tropici*	Unknown
	Contig 2	No Data	Unknown	No Data	No Data	*Rhizobium tropici*	Unknown
	Putative ID	No Data	Unknown	No Data	No Data	*Rhizobium tropici*	Unknown
Row D	Contig 1	No Data	No Hits	No Data	No Data	No Hits	*Rhizoobium tropici*
	Contig 2	No Data	No Hits	No Data	No Data	No Hits	No Data
	Putative ID	No Data	No Hits	No Data	No Data	No Hits	*Rhizobium tropici*
Row E	Contig 1	No Data	No Data	*Rhizobium tropici*	No Data	No Hits	No Hits
	Contig 2	No Data	No Data	*Pseudomonas carboxydohydrogena*	No Data	Class Alphaproteobacterium	No Data
	Putative ID	No Data	No Data	*Rhizobium tropici*	No Data	Class Alphaproteobacterium	No Hits
Row F	Contig 1	No Data	No Data	No Hits	No Data	*Rhizobium tropici*	No Hits
	Contig 2	No Data	No Data	No Hits	No Data	*Rhizobium* sp.	No Hits
	Putative ID	No Data	No Data	No Hits	No Data	*Rhizobium tropici*	No Hits
Row G	Contig 1	*M. truncatula* chloroplast	*Rhizobium mongolense*	*Rhizobium tropici*	No Hits	No Hits	No Hits
	Contig 2	No Hits	No Hits	No Data	No Data	No Hits	*Frateuria aurantia*
	Putative ID	*M. truncatula* chloroplast	*Rhizobium mongolense*	*Rhizobium tropici*	No Hits	No Hits	*Frateuria aurantia*
Row H	Contig 1	No Hits	No Hits	No Hits	No Hits	*Bacillus cereus*	*Mesorhizobium mediterraneum*
	Contig 2	No Hits	No Hits	No Hits	No Hits	No Hits	No Hits
	Putative ID	No Hits	No Hits	No Hits	No Hits	*Bacillus cereus*	*Mesorhizobium mediterraneum*

		Column					
		7	8	9	10	11	12
Row A	Contig 1	No Hits	No Hits	Class Alpha Proteobacterium	*M. truncatula* chloroplast	No Data	No Data
	Contig 2	No Hits	No Hits	Family Hyphomicrobiaceae	*M. truncatula* chloroplast	No Data	No Data
	Putative ID	No Hits	No Hits	Family Hyphomicrobiaceae	*M. truncatula* chloroplast	No Data	No Data
Row B	Contig 1	*M. truncatula* chloroplast	*Rhizobium tropici*	*M. truncatula* chloroplast	*Ochrobactrum* sp.	No Hits	*M. truncatula* chloroplast
	Contig 2	*M. truncatula* chloroplast	*Rhizobium tropici*	*M. truncatula* chloroplast	No Hits	No Hits	*M. truncatula* chloroplast
	Putative ID	*M. truncatula* chloroplast	*Rhizobium tropici*	*M. truncatula* chloroplast	*Ochrobactrum* sp.	No Hits	*M. truncatula* chloroplast
Row C	Contig 1	Class Gammaproteobacterium	No Hits	*M. truncatula chloroplast*	*Ralstonia* sp.	Unknown	*Rhizobium tropici*
	Contig 2	Unknown	Unknown	*M. truncatula chloroplast*	No Data	*Rhizobium* sp.	No Data
	Putative ID	Class Gammaproteobacterium	Unknown	*M. truncatula chloroplast*	*Ralstonia* sp.	*Rhizobium* sp.	*Rhizobium tropici*
Row D	Contig 1	*M. truncatula chloroplast*	No Hits	*Bacillus* sp.	No Hits	Unknown	Unknown
	Contig 2	*M. truncatula chloroplast*	No Hits	No Data	No Hits	No Hits	No Hits
	Putative ID	*M. truncatula chloroplast*	No Hits	*Bacillus* sp.	No Hits	Unknown	Unknown
Row E	Contig 1	No Hits	*Rhizobium tropici*	No Data	No Data	*Rhizobium tropici*	*Pseudomonas kilonensis*
	Contig 2	No Hits	No Hits	No Data	No Data	*Rhizobium* sp.	No Hits
	Putative ID	No Hits	*Rhizobium tropici*	No Data	No Data	*Rhizobium tropici*	*Pseudomonas kilonensis*
Row F	Contig 1	No Hits	No Hits	No Data	No Data	No Hits	Unknown
	Contig 2	No Hits	No Hits	No Data	No Data	No Hits	*Bradyrhizobium japonicum*
	Putative ID	No Hits	No Hits	No Data	No Data	No Hits	*Bradyrhizobium japonicum*
Row G	Contig 1	*M. truncatula* chloroplast	No Data	*Bradyrhizobium* sp.	*Dyella marensis*	No Data	No Data
	Contig 2	*M. truncatula* chloroplast	No Data	*Bradyrhizobium japonicum*	No Hits	No Data	No Data
	Putative ID	*M. truncatula* chloroplast	No Data	*Bradyrhizobium japonicum*	*Dyella marensis*	No Data	No Data
Row H	Contig 1	No Hits	*Rhizobium tropici*	No Data	No Hits	No Data	No Data
	Contig 2	No Hits	*Rhizobium tropici*	No Data	No Hits	No Data	No Data
	Putative ID	No Hits	*Rhizobium tropici*	No Data	No Hits	No Data	No Data

		Column					
		13	14	15	16	17	18
Row A	Contig 1	*M. truncatula* chloroplast	No Data	No Hits	*Rhizobium tropici*	No Data	No Hits
	Contig 2	*M. truncatula* chloroplast	No Data	No Hits	*Rhizobium tropici*	No Data	No Data
	Putative ID	*M. truncatula* chloroplast	No Data	No Hits	*Rhizobium tropici*	No Data	No Hits
Row B	Contig 1	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Streptomyces* sp.	No Hits	No Hits	No Data
	Contig 2	*M. truncatula* chloroplast	No Hits	No Hits	No Data	No Data	No Data
	Putative ID	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Streptomyces* sp.	No Hits	No Hits	No Data
Row C	Contig 1	No Data	*Rhizobium tropici*	No Hits	No Hits	No Hits	No Hits
	Contig 2	No Data	No Data	No Data	No Data	No Data	No Hits
	Putative ID	No Data	*Rhizobium tropici*	No Hits	No Hits	No Hits	No Hits
Row D	Contig 1	No Hits	Unknown	No Data	*M. truncatula* chloroplast	No Hits	*Ralstonia* sp.
	Contig 2	No Data	No Data	No Data	No Hits	No Hits	No Data
	Putative ID	No Hits	Unknown	No Data	*M. truncatula* chloroplast	No Hits	*Ralstonia* sp.
Row E	Contig 1	*M. truncatula* chloroplast	*Sphingomonas* sp.	No Data	No Hits	Phylum Actinobacteria	No Hits
	Contig 2	*Hyphomicrobium facile*	*Sphingomonas pruni*	No Data	No Hits	Family Micromonosporaceae	No Hits
	Putative ID	*M. truncatula* chloroplast	*Sphingomonas pruni*	No Data	No Hits	Phylum Actinobacteria	No Hits
Row F	Contig 1	No Hits	*Rhizobium tropici*	Class Gammaproteobacterium	No Hits	No Hits	No Hits
	Contig 2	No Data	No Hits	Unknown	No Hits	No Hits	No Hits
	Putative ID	No Hits	*Rhizobium tropici*	Class Gammaproteobacterium	No Hits	No Hits	No Hits
Row G	Contig 1	Unknown	Unknown	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Pantoea agglomerans*	No Hits
	Contig 2	No Hits	No Data	*M. truncatula* chloroplast	*M. truncatula* chloroplast	Unknown	No Data
	Putative ID	Unknown	Unknown	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Pantoea agglomerans*	No Hits
Row H	Contig 1	*Rhizobium* sp.	No Hits	No Data	*Pseudomonas corrugata*	*M. truncatula* chloroplast	*Rhizobium tropici*
	Contig 2	No Data	No Hits	No Data	Unknown	*M. truncatula* chloroplast	No Data
	Putative ID	*Rhizobium* sp.	No Hits	No Data	*Pseudomonas corrugata*	*M. truncatula* chloroplast	*Rhizobium tropici*

		Column					
		19	20	21	22	23	24
Row A	Contig 1	No Hits	*Bacillus* sp.	Unknown	Unknown	Unknown	Unknown
	Contig 2	No Hits	No Hits	No Hits	Unknown	No Hits	No Hits
	Putative ID	No Hits	*Bacillus* sp.	Unknown	Unknown	Unknown	Unknown
Row B	Contig 1	Unknown	Unknown	Unknown	No Hits	No Hits	Unknown
	Contig 2	No Hits	No Data	*Rhizobium tropici*	Unknown	No Hits	No Hits
	Putative ID	Unknown	Unknown	*Rhizobium tropici*	Unknown	No Hits	Unknown
Row C	Contig 1	*Escherichia coli*	Unknown	Unknown	No Hits	*Bacillus* sp.	*Bradyrhizobium japonicum*
	Contig 2	No Data	No Hits	No Hits	No Data	No Hits	No Hits
	Putative ID	*Escherichia coli*	Unknown	Unknown	No Hits	*Bacillus* sp.	*Bradyrhizobium japonicum*
Row D	Contig 1	No Hits	*M. truncatula* chloroplast	Class Alphaproteobacterium	Unknown	*Bacillus sp.*	*M. truncatula* chloroplast
	Contig 2	No Data	No Data	No Data	No Hits	*Bacillus sp.*	*M. truncatula* chloroplast
	Putative ID	No Hits	*M. truncatula* chloroplast	Class Alphaproteobacterium	Unknown	*Bacillus sp.*	*M. truncatula* chloroplast
Row E	Contig 1	No Hits	*M. truncatula* chloroplast	No Data	No Data	No Data	*M. truncatula* chloroplast
	Contig 2	No Hits	No Hits	No Data	No Data	No Data	*M. truncatula* chloroplast
	Putative ID	No Hits	*M. truncatula* chloroplast	No Data	No Data	No Data	*M. truncatula* chloroplast
Row F	Contig 1	No Hits	No Hits	No Data	No Data	Class Sphingobacteria	No Data
	Contig 2	*M. truncatula* chloroplast	No Hits	No Data	No Data	No Hits	No Data
	Putative ID	*M. truncatula* chloroplast	No Hits	No Data	No Data	Class Sphingobacteria	No Data
Row G	Contig 1	No Hits	Class Gammaproteobacterium	*Rhizobium tropici*	Class Alphaproteobacterium	*M. truncatula* chloroplast	*Bradyrhizobium* sp.
	Contig 2	No Hits	Unknown	*Rhizobium tropici*	No Hits	No Hits	Unknown
	Putative ID	No Hits	Class Gammaproteobacterium	*Rhizobium tropici*	Class Alphaproteobacterium	*M. truncatula* chloroplast	*Bradyrhizobium* sp.
Row H	Contig 1	Class Alphaproteobacterium	No Hits	*Rhizobium tropici*	*Rhizobium tropici*	*Burkholderia* sp.	*M. truncatula* chloroplast
	Contig 2	No Hits	No Hits	*Rhizobium* sp.	*Rhizobium tropici*	No Data	No Hits
	Putative ID	Class Alphaproteobacterium	No Hits	*Rhizobium tropici*	*Rhizobium tropici*	*Burkholderia* sp.	*M. truncatula* chloroplast

Table A3.3: BLAST® results used for putative identification of 16S rDNA insert sequences from the KNS endophyte clonal library.

STW

		Column					
		1	2	3	4	5	6
Row I	Contig 1	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	No Hits	No Data	No Data
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	No Data	No Data
Row J	Contig 1	No Hits	*Sinorhizobium meliloti*	*Rhizobium leguminosarum*	No Data	No Data	*Sinorhizobium* sp.
	Contig 2	No Data	*Sinorhizobium meliloti*	*Pseudomonas syringae*	No Data	No Data	No Data
	Putative ID	No Hits	*Sinorhizobium meliloti*	*Rhizobium leguminosarum*	No Data	No Data	*Sinorhizobium* sp.
Row K	Contig 1	No Hits	*M. truncatula* chloroplast	No Data	No Data	No Data	No Data
	Contig 2	*M. truncatula* chloroplast	No Data	No Data	No Data	No Data	No Data
	Putative ID	*M. truncatula* chloroplast	*M. truncatula* chloroplast	No Data	No Data	No Data	No Data
Row L	Contig 1	*Sinorhizobium meliloti*	No Hits	No Data	No Data	No Data	No Data
	Contig 2	*Sinorhizobium meliloti*	No Hits	No Data	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	No Hits	No Data	No Data	No Data	No Data
Row M	Contig 1	No Data	No Hits	No Data	No Hits	No Hits	*Sinorhizobium sp.*
	Contig 2	No Data	No Data	No Data	No Data	No Hits	No Data
	Putative ID	No Data	No Hits	No Data	No Hits	No Hits	*Sinorhizobium sp.*
Row N	Contig 1	No Data	*M. truncatula* chloroplast	No Hits	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium* sp.	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	No Data	*Sinorhizobium* sp.	No Hits	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row O	Contig 1	*Sinorhizobium meliloti*	*Pseudomonas* sp.	No Data	No Data	No Data	No Hits
	Contig 2	*Sinorhizobium meliloti*	*Pseudomonas* sp.	No Data	No Data	No Data	No Hits
	Putative ID	*Sinorhizobium meliloti*	*Pseudomonas* sp.	No Data	No Data	No Data	No Hits
Row P	Contig 1	No Data	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium meliloti*	No Data	No Data	Order Alpha Proteobacterium	No Data
	Putative ID	No Data	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*

		Column					
		7	8	9	10	11	12
Row I	Contig 1	*Sinorhizobium meliloti*	No Hits	No Data	Class Gammaproteobacterium	No Hits	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Data	No Data	No Hits	*Sinorhizobium meliloti*	No Data
	Putative ID	*Sinorhizobium meliloti*	No Hits	No Data	Class Gammaproteobacterium	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row J	Contig 1	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	No Hits	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
Row K	Contig 1	No Data	No Data	*Mesorhizobium amorphae*	*Sinorhizobium meliloti*	No Data	No Data
	Contig 2	No Data	No Data	Class Betaproteobacterium	*M. truncatula* chloroplast	No Data	No Data
	Putative ID	No Data	No Data	*Mesorhizobium amorphae*	*Sinorhizobium meliloti*	No Data	No Data
Row L	Contig 1	No Data	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data	No Data
	Contig 2	No Data	No Data	*Sinorhizobium meliloti*	No Data	No Data	No Data
	Putative ID	No Data	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data	No Data
Row M	Contig 1	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.	*M. truncatula* chloroplast
	Contig 2	*Niastella koreensis*	*Sinorhizobium meliloti*	No Data	No Hits	No Data	No Hits
	Putative ID	*Niastella koreensis*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.	*M. truncatula* chloroplast
Row N	Contig 1	No Hits	*Sinorhizobium meliloti*	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Hits	No Hits	No Hits	*M. truncatula* chloroplast	No Hits	*Sinorhizobium meliloti*
	Putative ID	No Hits	*Sinorhizobium meliloti*	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row O	Contig 1	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Hits	No Hits	*Sinorhizobium meliloti*	No Hits
	Contig 2	No Data	No Hits	No Hits	No Hits	*Sinorhizobium meliloti*	No Data
	Putative ID	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Hits	No Hits	*Sinorhizobium meliloti*	No Hits
Row P	Contig 1	No Data	No Data	No Hits	*M. truncatula* chloroplast	No Data	No Data
	Contig 2	No Data	No Data	No Hits	*M. truncatula* chloroplast	No Data	No Data
	Putative ID	No Data	No Data	No Hits	*M. truncatula* chloroplast	No Data	No Data

		Column					
		13	14	15	16	17	18
Row I	Contig 1	Class Betaproteobacterium	*Sinorhizobium meliloti*	No Hits	No Hits	No Data	No Data
	Contig 2	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data	No Data
	Putative ID	Class Betaproteobacterium	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	No Data	No Data
Row J	Contig 1	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	No Data	No Data
	Contig 2	No Data	*M. truncatula* chloroplast	No Data	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	No Data	No Data
Row K	Contig 1	*Streptomyces* sp.	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
	Contig 2	*Streptomyces* sp.	No Data	No Data	*Sinorhizobium* sp.	No Hits	No Data
	Putative ID	*Streptomyces* sp.	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
Row L	Contig 1	Unknown	Unknown	No Data	No Hits	No Hits	*Sinorhizobium meliloti*
	Contig 2	No Data	Unknown	No Data	No Data	No Data	No Hits
	Putative ID	Unknown	Unknown	No Data	No Hits	No Hits	*Sinorhizobium meliloti*
Row M	Contig 1	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Hits
	Contig 2	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium* sp.	No Hits	No Data	Class Alphaproteobacterium
	Putative ID	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	Class Alphaproteobacterium
Row N	Contig 1	*Sinorhizobium meliloti*	No Data	No Data	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
	Contig 2	No Hits	No Data	No Data	No Hits	*Sinorhizobium meliloti*	No Data
	Putative ID	*Sinorhizobium meliloti*	No Data	No Data	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
Row O	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Hits	No Data	No Hits	No Hits	No Hits
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
Row P	Contig 1	*Sinorhizobium meliloti*	Unknown	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
	Contig 2	*Sinorhizobium meliloti*	No Hits	No Data	No Hits	No Hits	No Hits
	Putative ID	*Sinorhizobium meliloti*	Unknown	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast

		Column					
		19	20	21	22	23	24
Row I	Contig 1	No Hits	No Hits	*Sinorhizobium meliloti*	Unknown	*M. truncatula* chloroplast	No Data
	Contig 2	No Hits	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	No Hits	No Data	No Data
	Putative ID	No Hits	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	Unknown	*M. truncatula* chloroplast	No Data
Row J	Contig 1	Class Betaproteobacterium	*M. truncatula* chloroplast	Class Betaproteobacterium	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Hits
	Contig 2	No Hits	*M. truncatula* chloroplast	No Hits	No Hits	*Sinorhizobium meliloti*	No Hits
	Putative ID	Class Betaproteobacterium	*M. truncatula* chloroplast	Class Betaproteobacterium	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Hits
Row K	Contig 1	No Hits	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*M. truncatula* chloroplast	No Data	No Data
	Contig 2	No Data	*Sinorhizobium* sp.	*M. truncatula* chloroplast	*M. truncatula* chloroplast	No Data	No Data
	Putative ID	No Hits	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*M. truncatula* chloroplast	No Data	No Data
Row L	Contig 1	*Sinorhizobium meliloti*	Unknown	No Hits	No Hits	No Data	No Data
	Contig 2	No Hits	No Data	Class Betaproteobacterium	No Hits	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	Unknown	Class Betaproteobacterium	No Hits	No Data	No Data
Row M	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
	Contig 2	*Sinorhizobium meliloti*	No Data	No Hits	*Sinorhizobium meliloti*	No Data	*Rhizobium etli*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Rhizobium etli*
Row N	Contig 1	*Sinorhizobium* sp.	*Sinorhizobium* sp.	*Niastella jeongjuensis*	*Sinorhizobium meliloti*	*M. truncatula chloroplast*	*M. truncatula chloroplast*
	Contig 2	No Data	*Sinorhizobium meliloti*	No Data	No Data	No Hits	No Data
	Putative ID	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	*Niastella jeongjuensis*	*Sinorhizobium meliloti*	*M. truncatula chloroplast*	*M. truncatula chloroplast*
Row O	Contig 1	No Data	No Data	No Hits	*Sinorhizobium meliloti*	No Data	No Data
	Contig 2	No Data	No Data	No Data	No Hits	No Data	No Data
	Putative ID	No Data	No Data	No Hits	*Sinorhizobium meliloti*	No Data	No Data
Row P	Contig 1	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data
	Contig 2	No Data	No Data	*Sinorhizobium meliloti*	No Data	No Data	No Data
	Putative ID	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data

Table A3.4: BLAST® results used for putative identification of 16S rDNA insert sequences from the STW endophyte clonal library.

PAW

		Column					
		1	2	3	4	5	6
Row A	Contig 1	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	No Hits	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*
Row B	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row C	Contig 1	*Sinorhizobium meliloti*	Class Alphaproteobacterium	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Data	No Data	No Data	No Data	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	Class Alphaproteobacterium	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
Row D	Contig 1	No Data	No Data	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	No Data	No Data	No Data
	Putative ID	No Data	No Data	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row E	Contig 1	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Hits
	Putative ID	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row F	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
Row G	Contig 1	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.
	Contig 2	No Data	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
	Putative ID	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.
Row H	Contig 1	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.	*Sinorhizobium* sp.	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.	*Sinorhizobium meliloti*

		Column					
		7	8	9	10	11	12
Row A	Contig 1	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Matsuebacter chitosanotabidus*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Pseudomonas saccharophila*	*Sinorhizobium meliloti*
	Putative ID	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Matsuebacter chitosanotabidus*	*Sinorhizobium meliloti*
Row B	Contig 1	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data
	Putative ID	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row C	Contig 1	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Burkholderia* sp.	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data
	Contig 2	*Sinorhizobium meliloti*	No Hits	No Hits	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data
	Putative ID	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Burkholderia* sp.	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data
Row D	Contig 1	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Data	No Hits
	Contig 2	No Data	No Data	No Data	No Hits	No Data	No Data
	Putative ID	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Data	No Hits
Row E	Contig 1	No Hits	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
	Contig 2	No Data	No Data	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	No Hits
	Putative ID	No Hits	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
Row F	Contig 1	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	No Data
	Contig 2	No Data	No Data	*Sinorhizobium meliloti*	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	No Data
Row G	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data
Row H	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Unknown	No Data	No Data
	Contig 2	No Hits	*Sinorhizobium meliloti*	No Data	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Unknown	No Data	No Data

		Column					
		13	14	15	16	17	18
Row A	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
Row B	Contig 1	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	No Data	*Sinorhizobium meliloti*
	Putative ID	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
Row C	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
Row D	Contig 1	No Data	No Data	No Data	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	No Hits	No Data	*Sinorhizobium meliloti*
	Putative ID	No Data	No Data	No Data	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row E	Contig 1	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Hits	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data	No Data	No Data
	Putative ID	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row F	Contig 1	*M. truncatula* chloroplast	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*M. truncatula* chloroplast	No Data	No Data	No Data	No Data	No Data
	Putative ID	*M. truncatula* chloroplast	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row G	Contig 1	Phylum Bacteroidetes	Unknown	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	Class Alphaproteobacterium
	Contig 2	No Data	*Burkholderia* sp.	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data
	Putative ID	Phylum Bacteroidetes	*Burkholderia* sp.	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	Class Alphaproteobacterium
Row H	Contig 1	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*

		Column					
		19	20	21	22	23	24
Row A	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*M. truncatula* chloroplast	No Hits	*M. truncatula* chloroplast
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Hits	No Data	*M. truncatula* chloroplast
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*M. truncatula* chloroplast	No Hits	*M. truncatula* chloroplast
Row B	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Hits	*Sinorhizobium meliloti*	No Hits
	Contig 2	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
Row C	Contig 1	*M. truncatula* chloroplast	*M. truncatula* chloroplast	No Data	No Data	*Sinorhizobium* sp.	*M. truncatula* chloroplast
	Contig 2	No Data	*M. truncatula* chloroplast	No Data	No Data	No Data	No Data
	Putative ID	*M. truncatula* chloroplast	*M. truncatula* chloroplast	No Data	No Data	*Sinorhizobium* sp.	*M. truncatula* chloroplast
Row D	Contig 1	No Hits	No Hits	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium* sp.	No Hits	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium* sp.	No Hits	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row E	Contig 1	Class Alphaproteobacterium	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data
	Contig 2	No Hits	No Data	No Data	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	Class Alphaproteobacterium	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data
Row F	Contig 1	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data
	Contig 2	*Sinorhizobium meliloti*	No Hits	No Hits	No Data	*Sinorhizobium meliloti*	No Data
	Putative ID	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data
Row G	Contig 1	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	Class Alphaproteobacterium	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium meliloti*	No Data	No Data	No Data	No Data
	Putative ID	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	Class Alphaproteobacterium	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row H	Contig 1	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	No Data
	Putative ID	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*

Table A3.5: BLAST® results used for putative identification of 16S rDNA insert sequences from the PAW endophyte clonal library.

GDW

		Column					
		1	2	3	4	5	6
Row I	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row J	Contig 1	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.
	Contig 2	No Data	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium* sp.	No Hits	No Data
	Putative ID	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.
Row K	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	Unknown	*Sinorhizobium meliloti*	No Data
	Contig 2	No Data	*Sinorhizobium meliloti*	No Hits	Unknown	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	Unknown	*Sinorhizobium meliloti*	No Data
Row L	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Unknown	No Data	No Hits	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Hits	*Sinorhizobium meliloti*
Row M	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Hits	No Data	Unknown	*Sinorhizobium meliloti*	No Hits
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row N	Contig 1	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Burkholderia phytofirmans*	*Sinorhizobium* sp.	*Lactobacillus mobilis*
	Contig 2	*Pseudoxanthomonas mexicana*	No Data	No Data	*Burkholderia phytofirmans*	No Data	No Data
	Putative ID	*Pseudoxanthomonas mexicana*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Burkholderia phytofirmans*	*Sinorhizobium* sp.	*Lactobacillus mobilis*
Row O	Contig 1	No Data	No Data	No Hits	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	No Data	No Data	No Hits	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row P	Contig 1	*Sinorhizobium meliloti*	No Data	No Data	No Data	No Data	No Data
	Contig 2	*Sinorhizobium meliloti*	No Data	No Data	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	No Data	No Data	No Data	No Data	No Data

		Column					
		7	8	9	10	11	12
Row I	Contig 1	*Sinorhizobium meliloti*	No Data	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Data	No Data	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	No Data	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row J	Contig 1	No Hits	Unknown	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	Unknown	Unknown	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	Unknown	Unknown	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row K	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Sinorhizobium* sp.	*Sinorhizobium* sp.	No Hits
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
Row L	Contig 1	*Dokdonella* sp.	Phylum Bacteroidetes	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium* sp.
	Putative ID	*Dokdonella* sp.	Phylum Bacteroidetes	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row M	Contig 1	No Data	No Data	*Sinorhizobium meliloti*	No Data	Unknown	Unknown
	Contig 2	No Data	No Data	*Sinorhizobium* sp.	No Data	*Sinorhizobium meliloti*	No Data
	Putative ID	No Data	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	Unknown
Row N	Contig 1	No Data	No Data	*Sinorhizobium meliloti*	Class Alphaproteobacterium	Phylum Bacteroidetes	Unknown
	Contig 2	No Data	No Data	No Data	No Hits	No Data	No Data
	Putative ID	No Data	No Data	*Sinorhizobium meliloti*	Class Alphaproteobacterium	Phylum Bacteroidetes	Unknown
Row O	Contig 1	*Sinorhizobium meliloti*	*Niastella jeongjuensis*	*M. truncatula* chloroplast	*M. truncatula* chloroplast	No Hits	No Hits
	Contig 2	*Sinorhizobium meliloti*	No Hits	*M. truncatula* chloroplast	*M. truncatula* chloroplast	Unknown	No Hits
	Putative ID	*Sinorhizobium meliloti*	*Niastella jeongjuensis*	*M. truncatula* chloroplast	*M. truncatula* chloroplast	Unknown	No Hits
Row P	Contig 1	No Data	*Sinorhizobium meliloti*	No Hits	No Data	No Data	*Glaucimonas multicolorus*
	Contig 2	No Data	*Sinorhizobium meliloti*	No Hits	No Data	No Data	No Data
	Putative ID	No Data	*Sinorhizobium meliloti*	No Hits	No Data	No Data	*Glaucimonas multicolorus*

		Column					
		13	14	15	16	17	18
Row I	Contig 1	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Class Betaproteobacterium	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Class Betaproteobacterium	*Sinorhizobium meliloti*
Row J	Contig 1	No Data	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Contig 2	No Data	No Hits	Unknown	*Sinorhizobium meliloti*	No Data	*Sinorhizobium* sp.
	Putative ID	No Data	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
Row K	Contig 1	*Pseudomonas fluorescens*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	No Hits
	Contig 2	No Data	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	*Pseudomonas fluorescens*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row L	Contig 1	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
	Contig 2	*M. truncatula* chloroplast	No Hits	No Data	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
	Putative ID	*M. truncatula* chloroplast	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
Row M	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium fredii*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium fredii*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row N	Contig 1	*Sinorhizobium meliloti*	No Data	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Data	No Data	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	No Data	No Hits	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row O	Contig 1	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
Row P	Contig 1	No Data	Unknown	No Data	Unknown	*Pseudomonas saccharophila*	No Data
	Contig 2	No Data	Unknown	No Data	No Hits	No Data	No Data
	Putative ID	No Data	Unknown	No Data	Unknown	*Pseudomonas saccharophila*	No Data

		Column					
		19	20	21	22	23	24
Row I	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Contig 2	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*
Row J	Contig 1	No Hits	*Sinorhizobium meliloti*	No Data	No Data	*Sinorhizobium meliloti*	No Data
	Contig 2	No Hits	No Data	No Hits	No Data	No Data	No Data
	Putative ID	No Hits	*Sinorhizobium meliloti*	No Hits	No Data	*Sinorhizobium meliloti*	No Data
Row K	Contig 1	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row L	Contig 1	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	No Data	Unknown	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	No Data	No Hits	No Data	Unknown	No Data
	Putative ID	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	No Data	Unknown	*Sinorhizobium meliloti*
Row M	Contig 1	*Sinorhizobium* sp.	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
	Contig 2	No Data	*M. truncatula* chloroplast	No Data	*Sinorhizobium* sp.	No Hits	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium* sp.	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*
Row N	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	No Hits	Unknown	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Hits	No Hits	No Data	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	No Hits	Unknown	*Sinorhizobium meliloti*
Row O	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	Unknown	*Sinorhizobium meliloti*	Class Alphaproteobacterium
	Contig 2	No Data	*Sinorhizobium meliloti*	No Data	No Hits	No Hits	Unknown
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	Unknown	*Sinorhizobium meliloti*	Class Alphaproteobacterium
Row P	Contig 1	No Data	No Data	Unknown	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	Unknown	No Data	*Sinorhizobium meliloti*	No Data
	Putative ID	No Data	No Data	Unknown	No Data	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*

Table A3.6: BLAST® results used for putative identification of 16S rDNA insert sequences from the GDW endophyte clonal library.

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		Column					
		1	2	3	4	5	6
Row A	Contig 1	*Sinorhizobium meliloti*	*Frateuria* sp.	*Sinorhizobium meliloti*	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	No Hits
	Contig 2	*Sinorhizobium meliloti*	*Frateuria* sp.	*Sinorhizobium meliloti*	No Hits	No Data	*Herbaspirillum seropedicae*
	Putative ID	*Sinorhizobium meliloti*	*Frateuria* sp.	*Sinorhizobium meliloti*	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	*Herbaspirillum seropedicae*
Row B	Contig 1	*Herbaspirillum seropedicae*	*Herbaspirillum seropedicae*	*M. truncatula* chloroplast	Class Betaproteobacterium	Phylum Bacteroidetes	No Data
	Contig 2	No Hits	No Data	*M. truncatula* chloroplast	No Data	Unknown	No Data
	Putative ID	*Herbaspirillum seropedicae*	*Herbaspirillum seropedicae*	*M. truncatula* chloroplast	Class Betaproteobacterium	Phylum Bacteroidetes	No Data
Row C	Contig 1	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
	Contig 2	No Data	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
Row D	Contig 1	No Hits	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	Class Betaproteobacterium	*Acidovorax* sp.
	Contig 2	No Hits	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	No Hits	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	Class Betaproteobacterium	*Acidovorax* sp.
Row E	Contig 1	*Pantoea ananatis*	Class Alphaproteobacterium	No Hits	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data
	Contig 2	*Pantoea agglomerans*	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	*Pantoea* sp.	Class Alphaproteobacterium	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data
Row F	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Family Oxalobacteraceae	*Sinorhizobium* sp.	No Hits
	Contig 2	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	No Hits
Row G	Contig 1	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	No Hits	*Lactobacillus mobilis*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	No Data	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	No Hits	*Lactobacillus mobilis*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row H	Contig 1	*Sinorhizobium meliloti*	*Herbaspirillum seropedicae*	Class Betaproteobacterium	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	Class Betaproteobacterium	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*Herbaspirillum seropedicae*	Class Betaproteobacterium	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*

		Column					
		7	8	9	10	11	12
Row A	Contig 1	*Mesorhizobium* sp.	Family Flexibacteraceae	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	No Hits
	Contig 2	*Mesorhizobium plurifarium*	*Niastella jeongjuensis*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	*Mesorhizobium plurifarium*	*Niastella jeongjuensis*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	No Hits
Row B	Contig 1	No Data	No Hits	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	No Data	No Data	Unknown	*Sinorhizobium meliloti*	No Data	No Data
	Putative ID	No Data	No Hits	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row C	Contig 1	No Data	Class Alphaproteobacterium	Class Betaproteobacterium	*M. truncatula* chloroplast	No Hits	No Hits
	Contig 2	No Data	No Hits	No Data	No Data	No Data	No Hits
	Putative ID	No Data	Class Alphaproteobacterium	Class Betaproteobacterium	*M. truncatula* chloroplast	No Hits	No Hits
Row D	Contig 1	*Sinorhizobium meliloti*	No Data	No Hits	No Hits	Unknown	Phylum Planctomycetes
	Contig 2	*Sinorhizobium meliloti*	No Data	No Hits	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	No Data	No Hits	No Hits	Unknown	Phylum Planctomycetes
Row E	Contig 1	No Hits	*Thermomonas fusca*	*M. truncatula* chloroplast	Class Betaproteobacterium	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	Unknown	*Thermomonas* sp.	*M. truncatula* chloroplast	No Hits	No Data	*Niastella jeongjuensis*
	Putative ID	Unknown	*Thermomonas fusca*	*M. truncatula* chloroplast	Class Betaproteobacterium	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row F	Contig 1	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	Unknown	Unknown	*Sinorhizobium meliloti*	No Hits
	Contig 2	*Sinorhizobium meliloti*	No Data	Phylum Bacteroidetes	No Data	No Data	No Data
	Putative ID	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	Phylum Bacteroidetes	Unknown	*Sinorhizobium meliloti*	No Hits
Row G	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Hits	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	No Data	*Niastella koreensis*	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*Niastella koreensis*	*Sinorhizobium meliloti*
Row H	Contig 1	*Sinorhizobium meliloti*	No Hits	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Asticcacaulis taihuensis*
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data	*M. truncatula* chloroplast	*Asticcacaulis* sp.
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Asticcacaulis taihuensis*

		Column					
		13	14	15	16	17	18
Row A	Contig 1	No Hits	No Hits	No Hits	No Hits	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
	Contig 2	No Data	Family Xanthomonadaceae	No Data	No Data	No Data	*M. truncatula* chloroplast
	Putative ID	No Hits	Family Xanthomonadaceae	No Hits	No Hits	*Sinorhizobium meliloti*	*M. truncatula* chloroplast
Row B	Contig 1	Unknown	Family Oxalobacteraceae	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Herbaspirillum seropedicae*	*Herbaspirillum seropedicae*	No Data	No Data	No Data	No Data
	Putative ID	*Herbaspirillum seropedicae*	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row C	Contig 1	*Acidovorax* sp.	No Hits	Unknown	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*
	Contig 2	*Acidovorax* sp.	*Herbaspirillum seropedicae*	No Hits	No Data	No Data	*Sinorhizobium meliloti*
	Putative ID	*Acidovorax* sp.	*Herbaspirillum seropedicae*	Unknown	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*
Row D	Contig 1	Unknown	*M. truncatula* chloroplast	Unknown	Unknown	*Pantoea agglomerans*	*Sinorhizobium meliloti*
	Contig 2	*Burkholderia phytofirmans*	*M. truncatula* chloroplast	*Bacillus megaterium*	No Data	No Data	No Data
	Putative ID	*Burkholderia phytofirmans*	*M. truncatula* chloroplast	*Bacillus megaterium*	Unknown	*Pantoea agglomerans*	*Sinorhizobium meliloti*
Row E	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	Family Oxalobacteraceae
	Contig 2	No Hits	No Data	*Sinorhizobium meliloti*	Unknown	No Data	*Herbaspirillum seropedicae*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits	*Herbaspirillum seropedicae*
Row F	Contig 1	No Hits	*M. truncatula chloroplast*	*Pseudomonas* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
	Contig 2	No Data	No Data	*Pseudomonas fluorescens*	No Data	No Data	*Sinorhizobium meliloti*
	Putative ID	No Hits	*M. truncatula chloroplast*	*Pseudomonas fluorescens*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row G	Contig 1	No Hits	No Hits	Class Alphaproteobacterium	*Herbaspirillum seropedicae*	No Data	*Sinorhizobium meliloti*
	Contig 2	*Herbaspirillum seropedicae*	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	Family Oxalobacteraceae	No Data	*Sinorhizobium meliloti*
	Putative ID	*Herbaspirillum seropedicae*	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	*Herbaspirillum seropedicae*	No Data	*Sinorhizobium meliloti*
Row H	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Rhizobium* sp.	Family Oxalobacteraceae	*M. truncatula* chloroplast	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Rhizobium leguminosarum*	*Herbaspirillum seropedicae*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Rhizobium leguminosarum*	*Herbaspirillum seropedicae*	*M. truncatula* chloroplast	*Sinorhizobium meliloti*

		Column					
		19	20	21	22	23	24
Row A	Contig 1	*Sinorhizobium meliloti*	*Niastella koreensis*	Family Oxalobacteraceae	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Unknown
	Contig 2	*Sinorhizobium meliloti*	*Flavobacterium* sp.	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Dyadobacter fermentans*
	Putative ID	*Sinorhizobium meliloti*	*Niastella koreensis*	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Dyadobacter fermentans*
Row B	Contig 1	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	No Data	*Sinorhizobium meliloti*	No Data
	Putative ID	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*M. truncatula* chloroplast	*Sinorhizobium* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row C	Contig 1	No Data	*Sinorhizobium meliloti*	*Novosphingobium pentaromativorans*	Family Oxalobacteraceae	Class Betaproteobacterium	*Bacillus cereus*
	Contig 2	No Data	*Sinorhizobium meliloti*	*Sphingomonas* sp.	*Herbaspirillum seropedicae*	No Hits	No Data
	Putative ID	No Data	*Sinorhizobium meliloti*	*Novosphingobium pentaromativorans*	*Herbaspirillum seropedicae*	Class Betaproteobacterium	*Bacillus cereus*
Row D	Contig 1	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Hits	No Hits	*M. truncatula* chloroplast
	Contig 2	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Hits	*M. truncatula* chloroplast	*M. truncatula* chloroplast
	Putative ID	*Sinorhizobium meliloti*	No Data	*Sinorhizobium meliloti*	No Hits	*M. truncatula* chloroplast	*M. truncatula* chloroplast
Row E	Contig 1	No Hits	Unknown	*Pantoea* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Contig 2	*Herbaspirillum seropedicae*	*Hyphomicrobium facile*	*Enterobacter hormaechei*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
	Putative ID	*Herbaspirillum seropedicae*	*Hyphomicrobium facile*	*Pantoea* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*
Row F	Contig 1	*Acidovorax* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data
	Contig 2	*Acidovorax* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	Unknown	*Sinorhizobium meliloti*	No Data
	Putative ID	*Acidovorax* sp.	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Data
Row G	Contig 1	No Hits	Unknown	*Niastella koreensis*	No Hits	*Sinorhizobium meliloti*	*Asticcacaulis* sp.
	Contig 2	*Sinorhizobium meliloti*	No Hits	Unknown	*Herbaspirillum seropedicae*	No Data	Family Caulobacteraceae
	Putative ID	*Sinorhizobium meliloti*	Unknown	*Niastella koreensis*	*Herbaspirillum seropedicae*	*Sinorhizobium meliloti*	*Asticcacaulis* sp.
Row H	Contig 1	*Sinorhizobium meliloti*	Unknown	Unknown	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
	Contig 2	*Sinorhizobium meliloti*	Unknown	Class Gammaproteobacterium	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits
	Putative ID	*Sinorhizobium meliloti*	Unknown	Class Gammaproteobacterium	*Sinorhizobium meliloti*	*Sinorhizobium meliloti*	No Hits

Table A3.7: BLAST® results used for putative identification of 16S rDNA insert sequences from the MM366 endophyte clonal library.

UNK

		Column					
		1	2	3	4	5	6
Row I	Contig 1	Unknown	*Bacillus cereus*	*Bacillus cereus*	*Bacillus sphaericus*	No Data	No Data
	Contig 2	*Bacillus megaterium*	Unknown	Unknown	No Data	No Data	No Data
	Putative ID	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus sphaericus*	No Data	No Data
Row J	Contig 1	*Bacillus* sp.	*Bacillus* sp.	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus sphaericus*	*Bacillus sphaericus*
	Contig 2	*Bacillus megaterium*	*Bacillus subtilis*	*Bacillus* sp.	*Bacillus cereus*	*Bacillus fusiformis*	No Data
	Putative ID	*Bacillus megaterium*	*Bacillus subtilis*	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus* sp.	*Bacillus sphaericus*
Row K	Contig 1	*Lysinibacillus sphaericus*	No Data	Unknown	*Bacillus cereus*	*Bacillus megaterium*	*Bacillus cereus*
	Contig 2	No Data	No Data	*Bacillus cereus*	Unknown	*Bacillus* sp.	Unknown
	Putative ID	*Lysinibacillus sphaericus*	No Data	*Bacillus cereus*	*Bacillus cereus*	*Bacillus megaterium*	*Bacillus cereus*
Row L	Contig 1	Unknown	*Bacillus cereus*	*Bacillus* sp.	*Bacillus megaterium*	*Bacillus megaterium*	*Bacillus sphaericus*
	Contig 2	No Data	No Data	*Bacillus megaterium*	No Data	No Data	No Data
	Putative ID	Unknown	*Bacillus cereus*	*Bacillus megaterium*	*Bacillus megaterium*	*Bacillus megaterium*	*Bacillus sphaericus*
Row M	Contig 1	*Bacillus* sp.	*Bacillus cereus*	Unknown	*Bacillus sphaericus*	Unknown	*Bacillus cereus*
	Contig 2	No Data	No Data	No Data	*Bacillus sphaericus*	No Data	No Data
	Putative ID	*Bacillus* sp.	*Bacillus cereus*	Unknown	*Bacillus sphaericus*	Unknown	*Bacillus cereus*
Row N	Contig 1	*Bacillus* sp.	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus* sp.	*Bacillus* sp.	*Bacillus cereus*
	Contig 2	No Data	No Data	*Bacillus fusiformis*	*Bacillus megaterium*	No Data	No Data
	Putative ID	*Bacillus* sp.	*Bacillus sphaericus*	*Bacillus* sp.	*Bacillus megaterium*	*Bacillus* sp.	*Bacillus cereus*
Row O	Contig 1	*Bacillus megaterium*	No Hits	*Bacillus cereus*	Unknown	No Hits	*Bacillus sphaericus*
	Contig 2	No Data	No Data	No Data	No Data	*Bacillus megaterium*	No Data
	Putative ID	*Bacillus megaterium*	No Hits	*Bacillus cereus*	Unknown	*Bacillus megaterium*	*Bacillus sphaericus*
Row P	Contig 1	No Data	Unknown	Unknown	No Data	*Bacillus megaterium*	*Bacillus* sp.
	Contig 2	No Data	No Data	No Data	No Data	No Data	No Data
	Putative ID	No Data	Unknown	Unknown	No Data	*Bacillus megaterium*	*Bacillus* sp.

		Column					
		7	8	9	10	11	12
Row I	Contig 1	*Bacillus* sp.	No Data	*Bacillus cereus*	*Bacillus pseudomycoides*	*Bacillus sphaericus*	Unknown
	Contig 2	No Data	No Data	No Data	*Bacillus megaterium*	No Data	No Data
	Putative ID	*Bacillus* sp.	No Data	*Bacillus cereus*	*Bacillus* sp.	*Bacillus sphaericus*	Unknown
Row J	Contig 1	*Bacillus sphaericus*	*Bacillus megaterium*	*Sinorhizobium meliloti*	No Hits	*Bacillus megaterium*	Unknown
	Contig 2	*Bacillus sphaericus*	*Bacillus* sp.	*Sinorhizobium meliloti*	No Data	*Bacillus sphaericus*	No Data
	Putative ID	*Bacillus sphaericus*	*Bacillus megaterium*	*Sinorhizobium meliloti*	No Hits	*Bacillus* sp.	Unknown
Row K	Contig 1	*Bacillus flexus*	No Data	*Bacillus sphaericus*	*Bacillus cereus*	*Bacillus sphaericus*	*Bacillus sphaericus*
	Contig 2	*Bacillus sphaericus*	No Data	No Data	No Data	*Bacillus megaterium*	*Bacillus megaterium*
	Putative ID	*Bacillus* sp.	No Data	*Bacillus sphaericus*	*Bacillus cereus*	*Bacillus* sp.	*Bacillus* sp.
Row L	Contig 1	*Bacillus cereus*	Unknown	*Bacillus* sp.	*Bacillus cereus*	Unknown	No Hits
	Contig 2	*Bacillus cereus*	No Data	*Bacillus* sp.	*Bacillus cereus*	No Data	No Data
	Putative ID	*Bacillus cereus*	Unknown	*Bacillus* sp.	*Bacillus cereus*	Unknown	No Hits
Row M	Contig 1	*Bacillus cereus*	No Hits	*Bacillus sphaericus*	*Bacillus megaterium*	No Hits	Unknown
	Contig 2	Unknown	No Data	*Bacillus sphaericus*	Unknown	No Data	No Data
	Putative ID	*Bacillus cereus*	No Hits	*Bacillus sphaericus*	*Bacillus megaterium*	No Hits	Unknown
Row N	Contig 1	*Bacillus cereus*	*Bacillus* sp.	*Bacillus* sp.	*Bacillus subtilis*	*Bacillus cereus*	*Bacillus* sp.
	Contig 2	*Bacillus* sp.	No Data	No Hits	*Bemisia tabaci*	*Bacillus cereus*	No Data
	Putative ID	*Bacillus cereus*	*Bacillus* sp.	*Bacillus* sp.	*Bacillus subtilis*	*Bacillus cereus*	*Bacillus* sp.
Row O	Contig 1	*Bacillus* sp.	Unknown	No Data	*Bacillus megaterium*	*Bacillus sphaericus*	*Bacillus subtilis*
	Contig 2	*Bacillus megaterium*	*Bacillus megaterium*	No Data	No Data	*Bacillus sphaericus*	No Data
	Putative ID	*Bacillus megaterium*	*Bacillus megaterium*	No Data	*Bacillus megaterium*	*Bacillus sphaericus*	*Bacillus subtilis*
Row P	Contig 1	Unknown	No Hits	*Bacillus sphaericus*	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus cereus*
	Contig 2	No Hits	No Data	No Data	No Data	No Data	No Data
	Putative ID	Unknown	No Hits	*Bacillus sphaericus*	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus cereus*

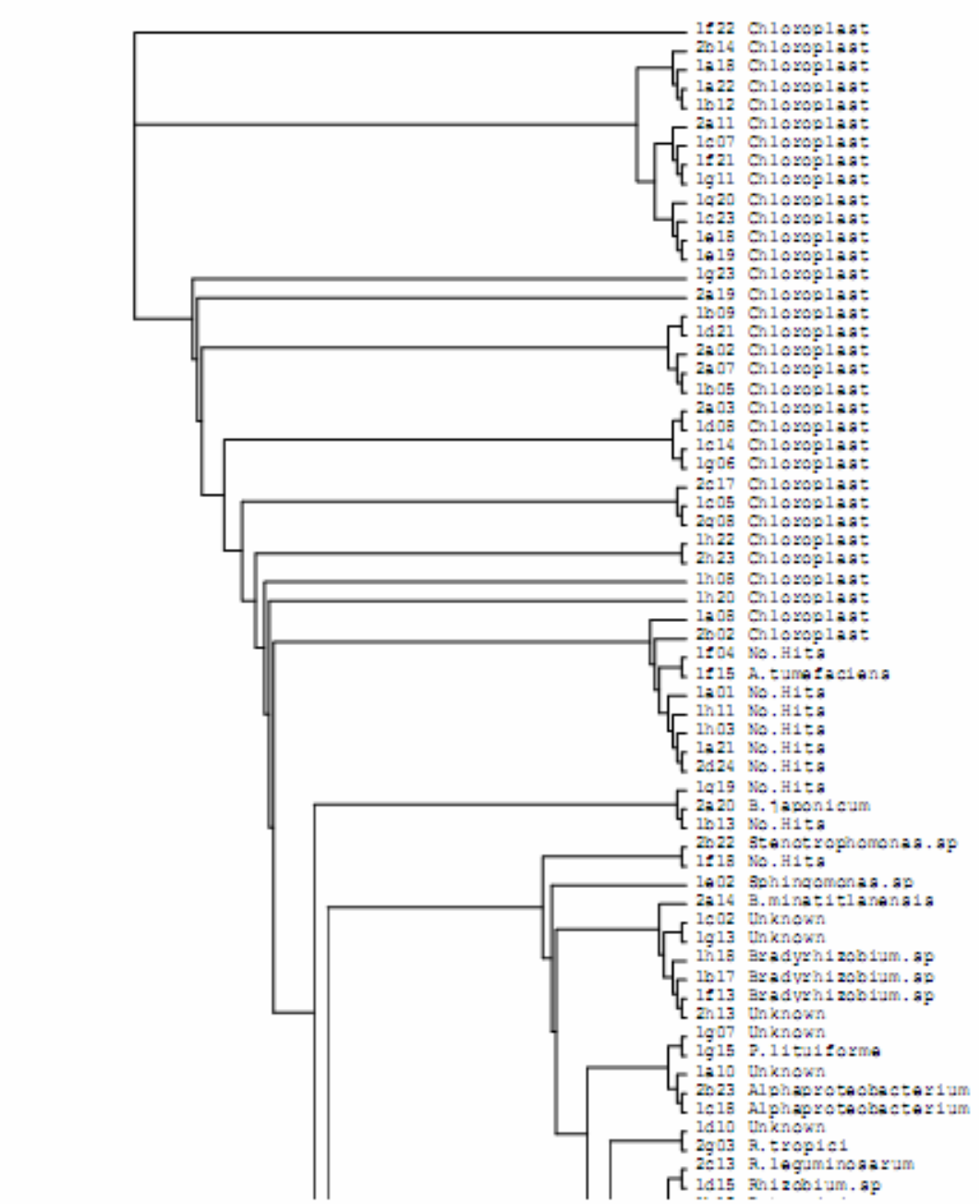
		Column					
		13	14	15	16	17	18
Row I	Contig 1	No Hits	*Bacillus sphaericus*	Unknown	*Bacillus* sp.	*Bacillus* sp.	*Bacillus cereus*
	Contig 2	*Bacillus* sp.	No Data	No Data	*Bacillus megaterium*	*Bacillus megaterium*	Unknown
	Putative ID	*Bacillus* sp.	*Bacillus sphaericus*	Unknown	*Bacillus megaterium*	*Bacillus megaterium*	*Bacillus cereus*
Row J	Contig 1	*Bacillus cereus*	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus megaterium*	*Bacillus cereus*	No Data
	Contig 2	Unknown	No Data	*Bacillus sphaericus*	*Bacillus* sp.	No Data	No Data
	Putative ID	*Bacillus cereus*	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus megaterium*	*Bacillus cereus*	No Data
Row K	Contig 1	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus megaterium*	*Bacillus megaterium*	Unknown	*Bacillus cereus*
	Contig 2	Unknown	No Hits	No Data	No Data	No Data	No Data
	Putative ID	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus megaterium*	*Bacillus megaterium*	Unknown	*Bacillus cereus*
Row L	Contig 1	*Bacillus* sp.	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus megaterium*	*Bacillus fusiformis*	No Data
	Contig 2	No Data	No Data	No Data	No Data	*Bacillus cereus*	No Data
	Putative ID	*Bacillus* sp.	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus megaterium*	*Bacillus* sp.	No Data
Row M	Contig 1	*Bacillus megaterium*	*Bacillus* sp.	*Bacillus sphaericus*	*Bacillus subtilis*	*Bacillus megaterium*	*Bacillus sphaericus*
	Contig 2	*Bacillus* sp.	No Hits	*Bacillus sphaericus*	*Bacillus megaterium*	Unknown	No Hits
	Putative ID	*Bacillus megaterium*	*Bacillus* sp.	*Bacillus sphaericus*	*Bacillus* sp.	*Bacillus megaterium*	*Bacillus sphaericus*
Row N	Contig 1	*Bacillus* sp.	*Bacillus cereus*	*Bacillus cereus*	*Bacillus subtilis*	*Bacillus cereus*	*Bacillus* sp.
	Contig 2	*Bacillus cereus*	*Bacillus* sp.	Unknown	No Data	Unknown	*Bacillus megaterium*
	Putative ID	*Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus subtilis*	*Bacillus cereus*	*Bacillus megaterium*
Row O	Contig 1	Unknown	*Bacillus cereus*	*Bacillus cereus*	*Bacillus* sp.	*Bacillus* sp.	*Bacillus sphaericus*
	Contig 2	*Bacillus cereus*	Unknown	*Bacillus cereus*	*Bacillus cereus*	*Bacillus* sp.	No Data
	Putative ID	*Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus* sp.	*Bacillus sphaericus*
Row P	Contig 1	No Hits	*Bacillus* sp.	*Bacillus cereus*	No Data	*Bacillus cereus*	No Hits
	Contig 2	No Hits	*Bacillus* sp.	*Bacillus cereus*	No Data	*Bacillus cereus*	Unknown
	Putative ID	No Hits	*Bacillus* sp.	*Bacillus cereus*	No Data	*Bacillus cereus*	Unknown

		Column					
		19	20	21	22	23	24
Row I	Contig 1	*Bacillus* sp.	*Bacillus cereus*	*Bacillus* sp.	Unknown	*Bacillus* sp.	*Bacillus* sp.
	Contig 2	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus* sp.	*Bacillus subtilis*
	Putative ID	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus* sp.	*Bacillus subtilis*
Row J	Contig 1	*Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*	No Hits	No Hits	*Bacillus* sp.
	Contig 2	*Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus* sp.	*Bacillus megaterium*	No Hits
	Putative ID	*Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus* sp.	*Bacillus megaterium*	*Bacillus* sp.
Row K	Contig 1	*Bacillus* sp.	*Bacillus sphaericus*	No Data	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus cereus*
	Contig 2	*Bacillus megaterium*	*Bacillus* sp.	No Data	*Bacillus megaterium*	*Bacillus sphaericus*	*Bacillus megaterium*
	Putative ID	*Bacillus megaterium*	*Bacillus sphaericus*	No Data	*Bacillus* sp.	*Bacillus sphaericus*	*Bacillus* sp.
Row L	Contig 1	No Data	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus fusiformis*	*Paenibacillus polymyxa*
	Contig 2	No Data	No Data	*Bacillus cereus*	*Bacillus cereus*	*Bacillus sphaericus*	*Paenibacillus polymyxa*
	Putative ID	No Data	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus* sp.	*Paenibacillus polymyxa*
Row M	Contig 1	Unknown	*Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*	Unknown	No Hits
	Contig 2	*Bacillus cereus*	No Data	*Bacillus cereus*	Unknown	No Data	No Hits
	Putative ID	*Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus cereus*	Unknown	No Hits
Row N	Contig 1	*Bacillus sphaericus*	*Bacillus fusiformis*	No Hits	No Data	*Bacillus megaterium*	Family Flexibacteraceae
	Contig 2	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus sphaericus*	No Data	*Bacillus sphaericus*	*Niastella jeongjuensis*
	Putative ID	*Bacillus sphaericus*	*Bacillus* sp.	*Bacillus sphaericus*	No Data	*Bacillus* sp.	*Niastella jeongjuensis*
Row O	Contig 1	*Bacillus* sp.	*Bacillus sphaericus*	*Bacillus cereus*	*Bacillus megaterium*	*Bacillus* sp.	*Bacillus sphaericus*
	Contig 2	*Bacillus cereus*	*Bacillus* sp.	*Bacillus subtilis*	Unknown	No Hits	*Bacillus megaterium*
	Putative ID	*Bacillus cereus*	*Bacillus sphaericus*	*Bacillus* sp.	*Bacillus megaterium*	*Bacillus* sp.	*Bacillus* sp.
Row P	Contig 1	No Hits	*Bacillus cereus*	Unknown	*Bacillus cereus*	*Bacillus cereus*	*Bacillus thuringiensis*
	Contig 2	No Hits	*Bacillus sphaericus*	No Hits	*Bacillus megaterium*	*Bacillus cereus*	*Bacillus fusiformis*
	Putative ID	No Hits	*Bacillus* sp.	Unknown	*Bacillus* sp.	*Bacillus cereus*	*Bacillus* sp.

Table A3.8: BLAST® results used for putative identification of 16S rDNA insert sequences from the UNK clonal library.

Appendix 4. The following figures are ClustalW2-generated cladograms of the forward and reverse 16S rDNA sequences for each sample series. All sequences were edited prior to alignment in order to remove extraneous 5' flanking pGEM®-T Easy vector sequence.

WLB Forward



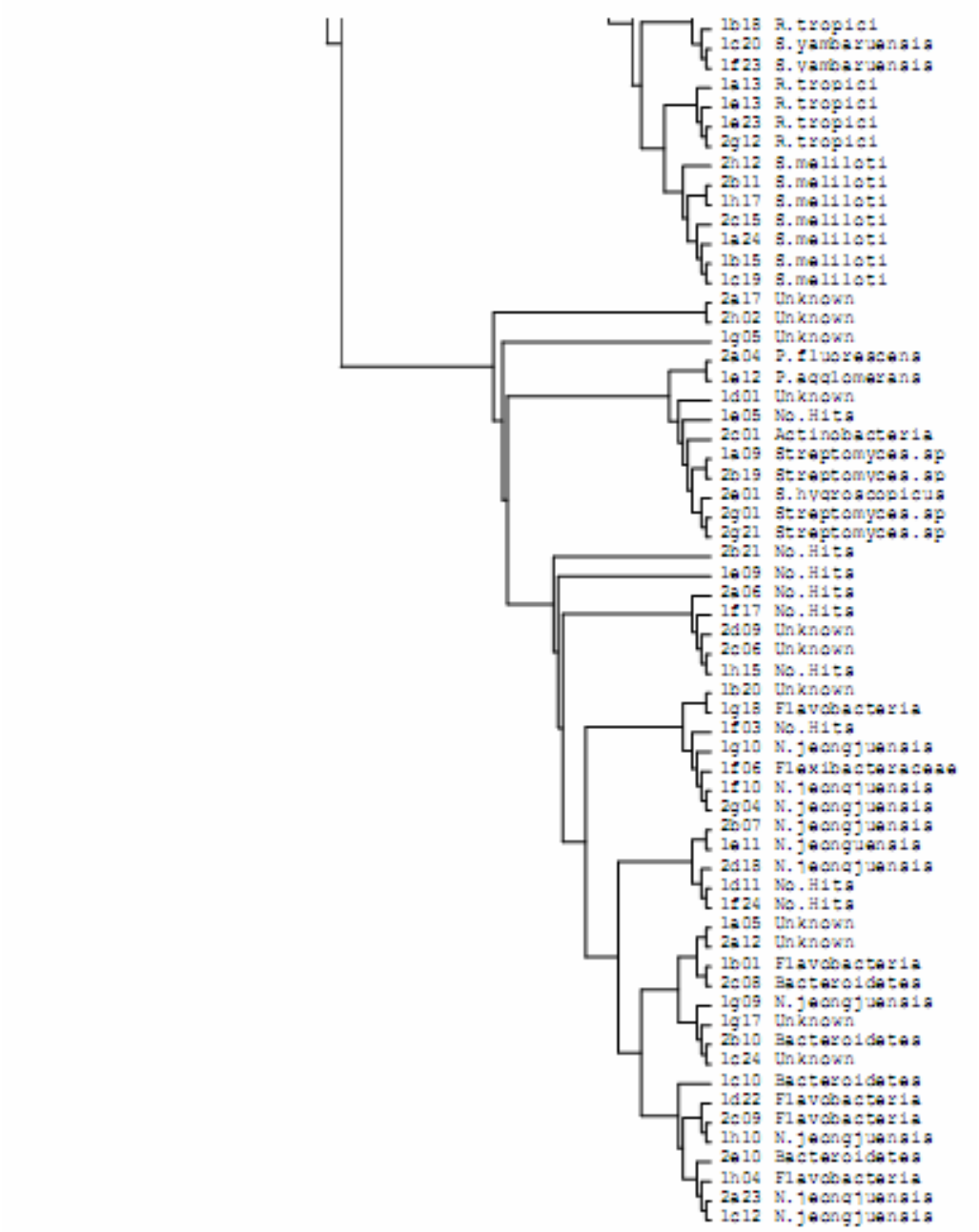
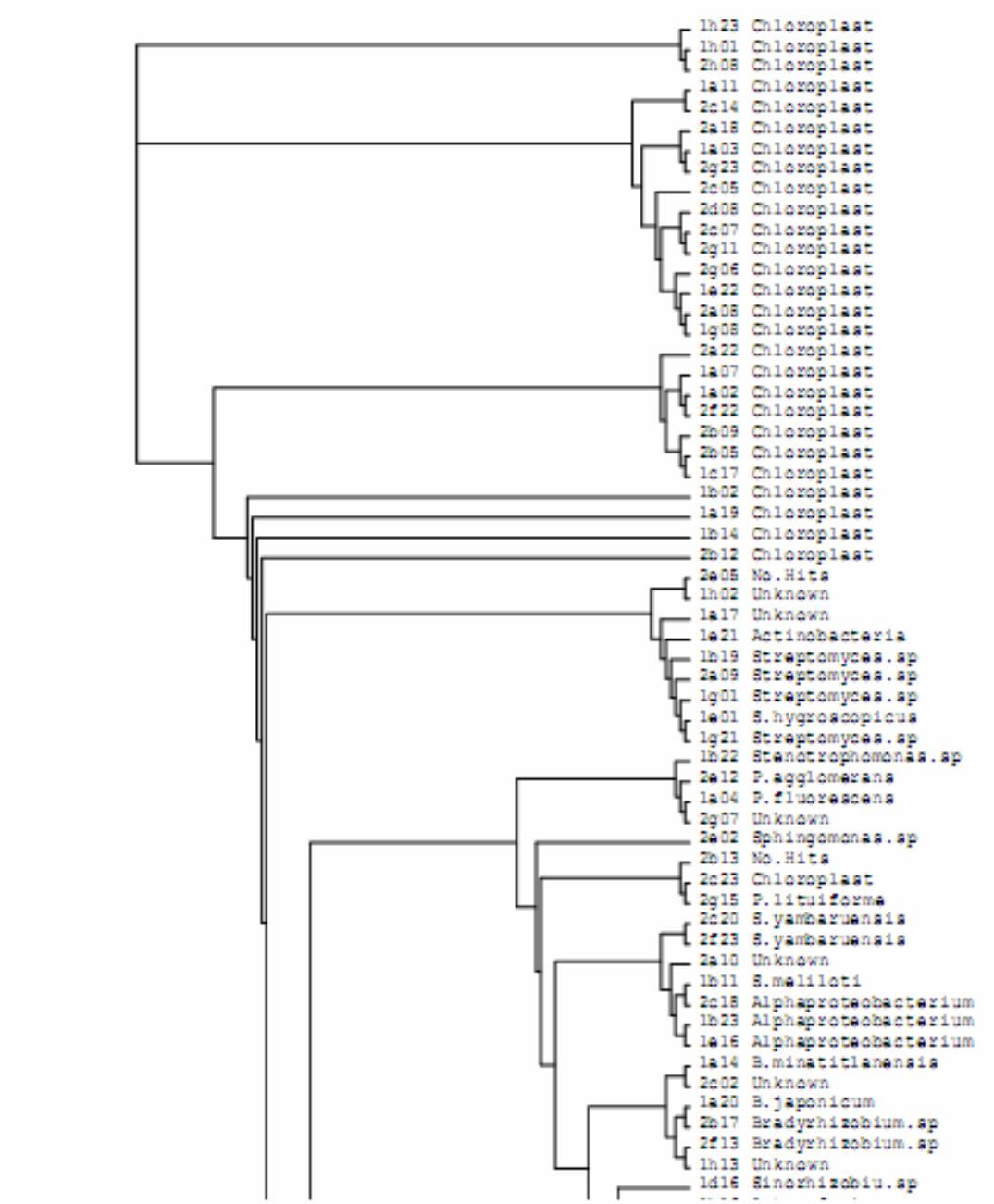


Figure A4.1: Cladogram of forward 16S rDNA insert sequences from the WLB sample series.

WLB Reverse


1h23 Chloroplast
1h01 Chloroplast
2h08 Chloroplast
1a11 Chloroplast
2c14 Chloroplast
2a18 Chloroplast
1a03 Chloroplast
2g23 Chloroplast
2c05 Chloroplast
2d08 Chloroplast
2c07 Chloroplast
2g11 Chloroplast
2g06 Chloroplast
1e22 Chloroplast
2a08 Chloroplast
1g08 Chloroplast
2a22 Chloroplast
1a07 Chloroplast
1a02 Chloroplast
2f22 Chloroplast
2b09 Chloroplast
2b05 Chloroplast
1c17 Chloroplast
1b02 Chloroplast
1a19 Chloroplast
1b14 Chloroplast
2b12 Chloroplast
2e05 No.Hits
1h02 Unknown
1a17 Unknown
1e21 Actinobacteria
1b19 Streptomyces.sp
2a09 Streptomyces.sp
1g01 Streptomyces.sp
1e01 S.hygroscopicus
1g21 Streptomyces.sp
1b22 Stenotrophomonas.sp
2e12 P.agglomerans
1a04 P.fluorescens
2g07 Unknown
2e02 Sphingomonas.sp
2b13 No.Hits
2c23 Chloroplast
2g15 P.lituiforme
2c20 S.yambaruensis
2f23 S.yambaruensis
2a10 Unknown
1b11 S.melliloti
2c18 Alphaproteobacterium
1b23 Alphaproteobacterium
1e16 Alphaproteobacterium
1a14 B.minatitlanensis
2c02 Unknown
1a20 B.japonicum
2b17 Bradyrhizobium.sp
2f13 Bradyrhizobium.sp
1h13 Unknown
1d16 Sinorhizobiu.sp

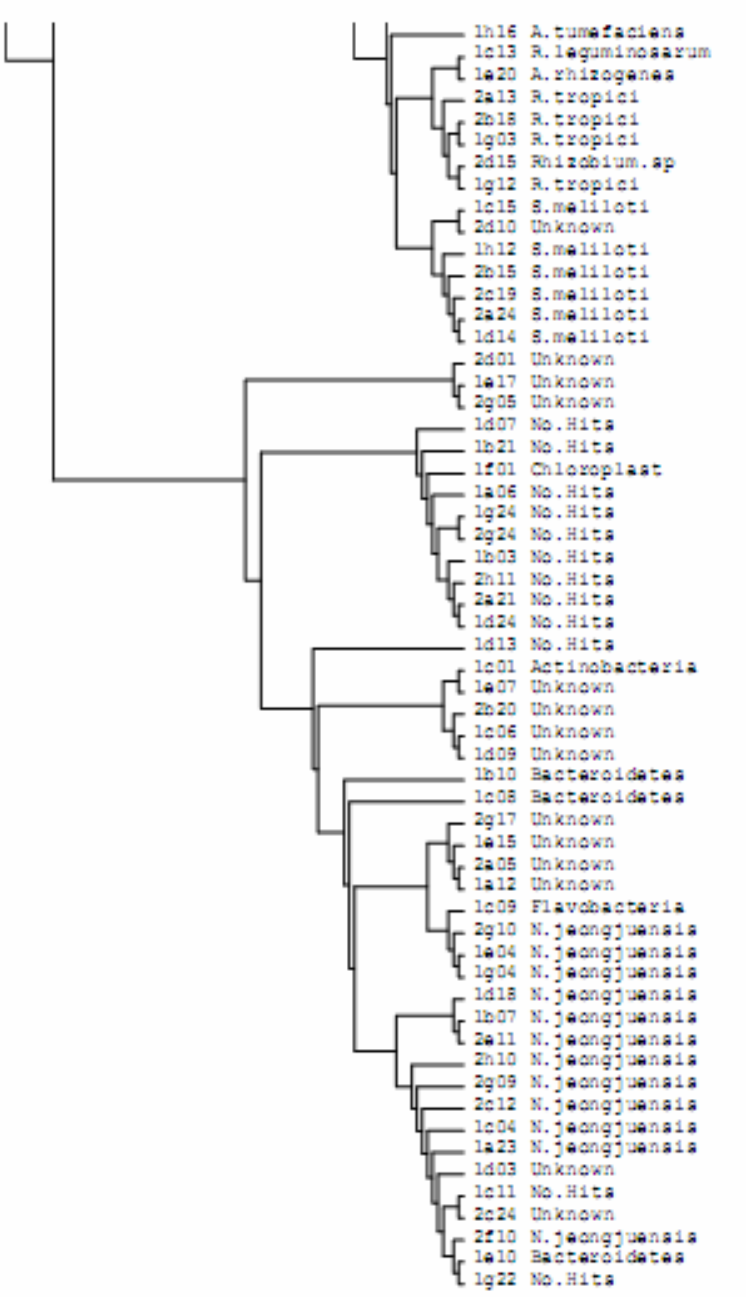
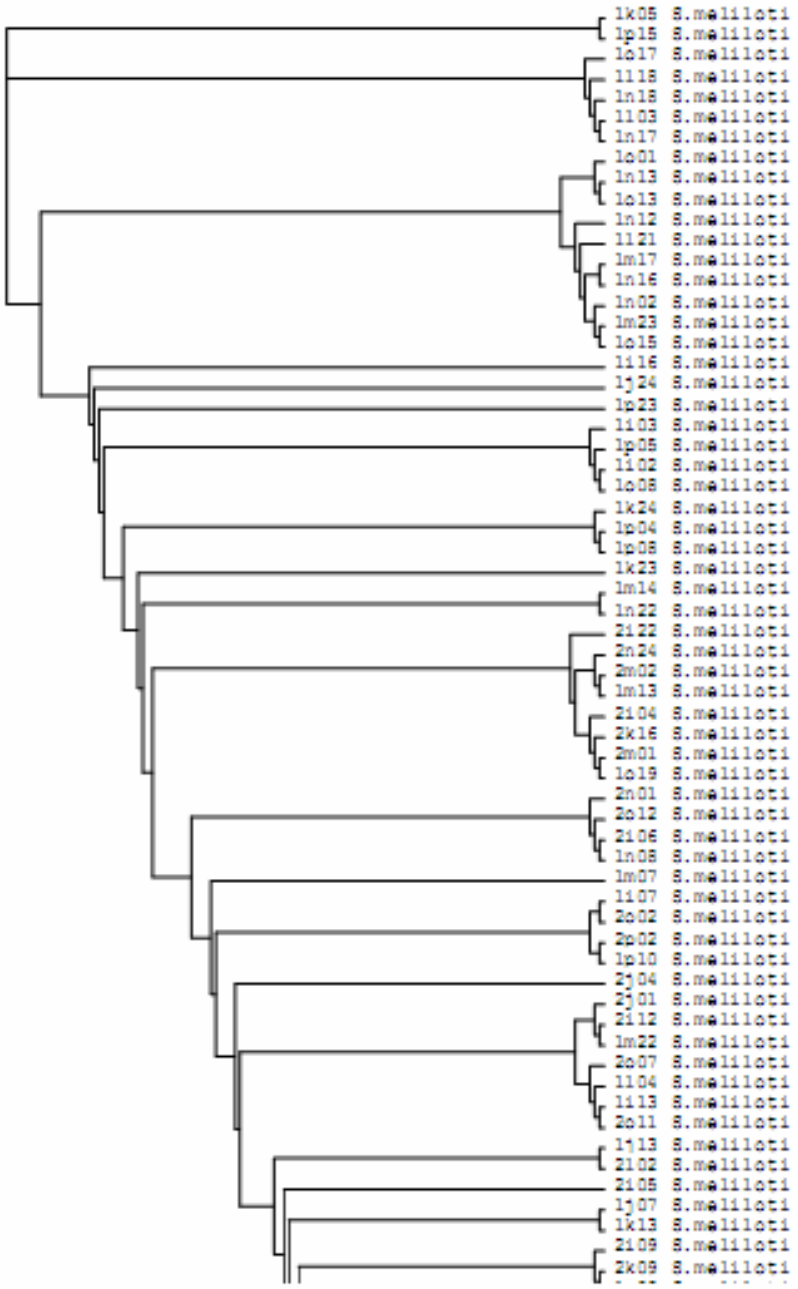


Figure A4.2: Cladogram of reverse 16S rDNA insert sequences from the WLB sample series.

WDW Forward


1k05 S.melilloti
1p15 S.melilloti
1o17 S.melilloti
1l18 S.melilloti
1n18 S.melilloti
1l03 S.melilloti
1n17 S.melilloti
1o01 S.melilloti
1n13 S.melilloti
1o13 S.melilloti
1n12 S.melilloti
1l21 S.melilloti
1m17 S.melilloti
1n16 S.melilloti
1n02 S.melilloti
1m23 S.melilloti
1o15 S.melilloti
1l16 S.melilloti
1j24 S.melilloti
1p23 S.melilloti
1l03 S.melilloti
1p05 S.melilloti
1l02 S.melilloti
1o08 S.melilloti
1k24 S.melilloti
1p04 S.melilloti
1p08 S.melilloti
1k23 S.melilloti
1m14 S.melilloti
1n22 S.melilloti
2l22 S.melilloti
2n24 S.melilloti
2m02 S.melilloti
1m13 S.melilloti
2l04 S.melilloti
2k16 S.melilloti
2m01 S.melilloti
1o19 S.melilloti
2n01 S.melilloti
2o12 S.melilloti
2l06 S.melilloti
1n08 S.melilloti
1m07 S.melilloti
1l07 S.melilloti
2o02 S.melilloti
2p02 S.melilloti
1p10 S.melilloti
2j04 S.melilloti
2j01 S.melilloti
2l12 S.melilloti
1m22 S.melilloti
2o07 S.melilloti
1l04 S.melilloti
1l13 S.melilloti
2o11 S.melilloti
1l13 S.melilloti
2l02 S.melilloti
2l05 S.melilloti
1j07 S.melilloti
1k13 S.melilloti
2l09 S.melilloti
2k09 S.melilloti

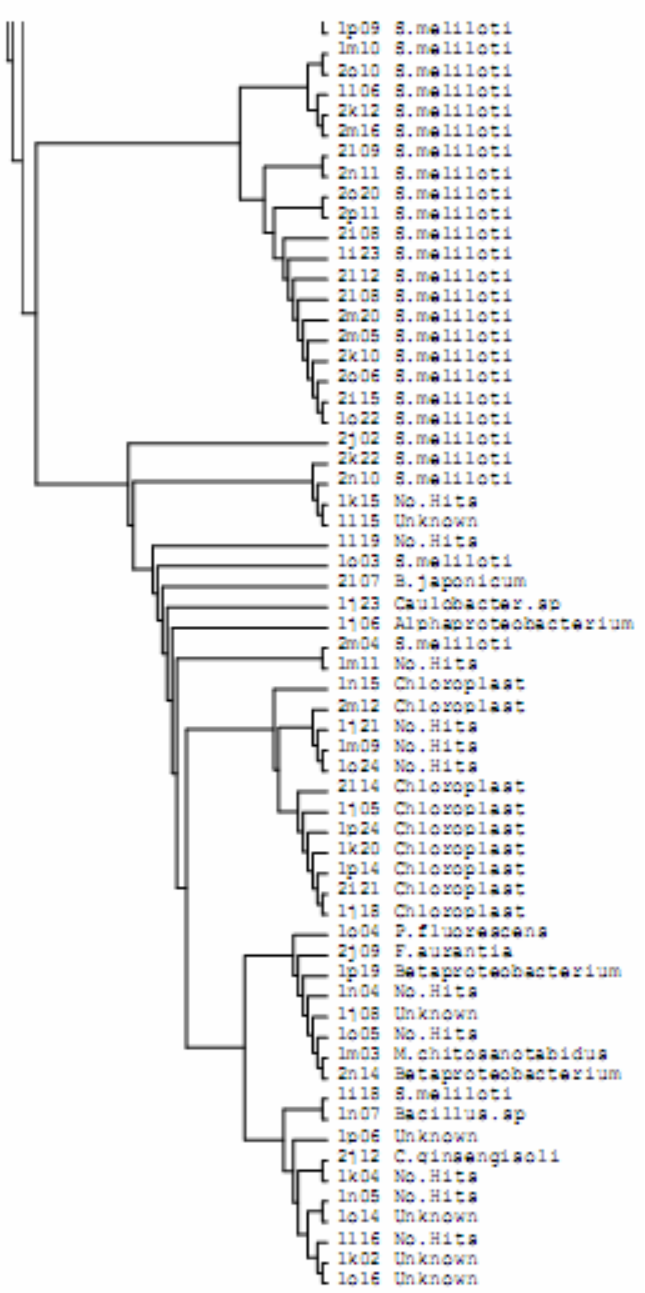
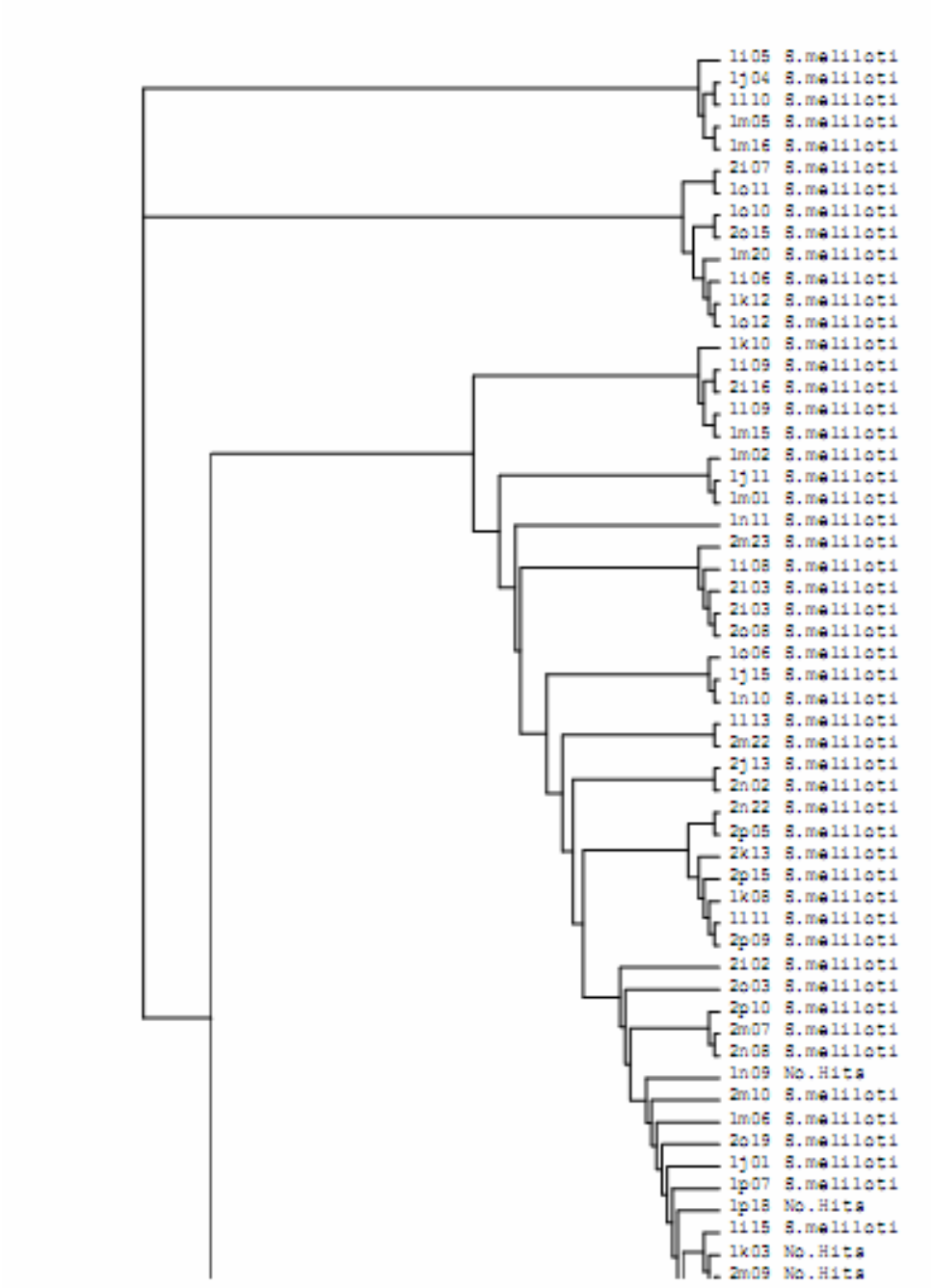


Figure A4.3: Cladogram of forward 16S rDNA insert sequences from the WDW sample series.

WDW Reverse


1i05 S.melliloti
1j04 S.melliloti
1l10 S.melliloti
1m05 S.melliloti
1m16 S.melliloti
2i07 S.melliloti
1o11 S.melliloti
1o10 S.melliloti
2o15 S.melliloti
1m20 S.melliloti
1i06 S.melliloti
1k12 S.melliloti
1o12 S.melliloti
1k10 S.melliloti
1i09 S.melliloti
2i16 S.melliloti
1l09 S.melliloti
1m15 S.melliloti
1m02 S.melliloti
1j11 S.melliloti
1m01 S.melliloti
1n11 S.melliloti
2m23 S.melliloti
1i08 S.melliloti
2l03 S.melliloti
2i03 S.melliloti
2o08 S.melliloti
1o06 S.melliloti
1j15 S.melliloti
1n10 S.melliloti
1l13 S.melliloti
2m22 S.melliloti
2j13 S.melliloti
2n02 S.melliloti
2n22 S.melliloti
2p05 S.melliloti
2k13 S.melliloti
2p15 S.melliloti
1k08 S.melliloti
1l11 S.melliloti
2p09 S.melliloti
2i02 S.melliloti
2o03 S.melliloti
2p10 S.melliloti
2m07 S.melliloti
2n08 S.melliloti
1n09 No.Hits
2m10 S.melliloti
1m06 S.melliloti
2o19 S.melliloti
1j01 S.melliloti
1p07 S.melliloti
1p18 No.Hits
1i15 S.melliloti
1k03 No.Hits
2m09 No.Hits

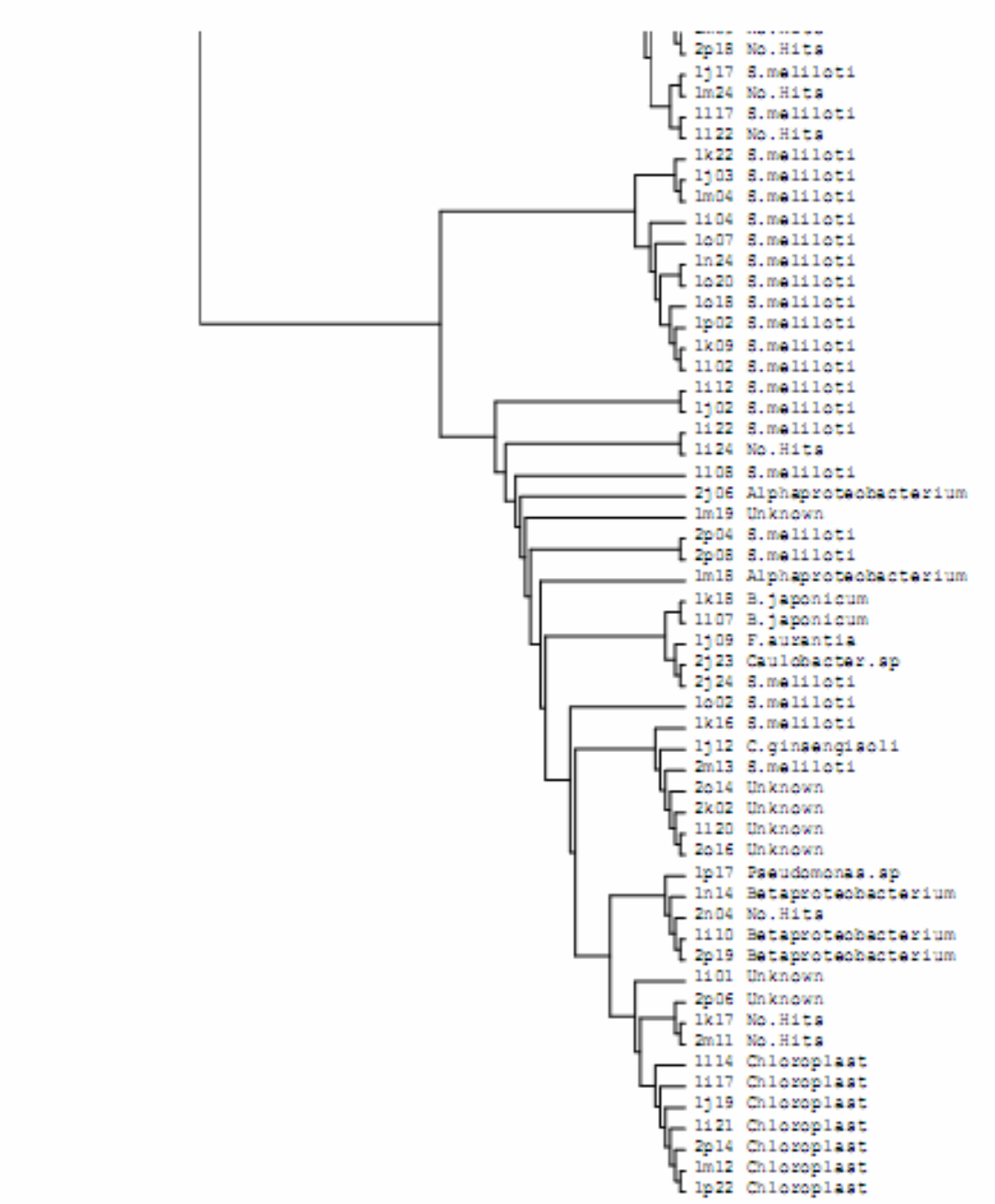
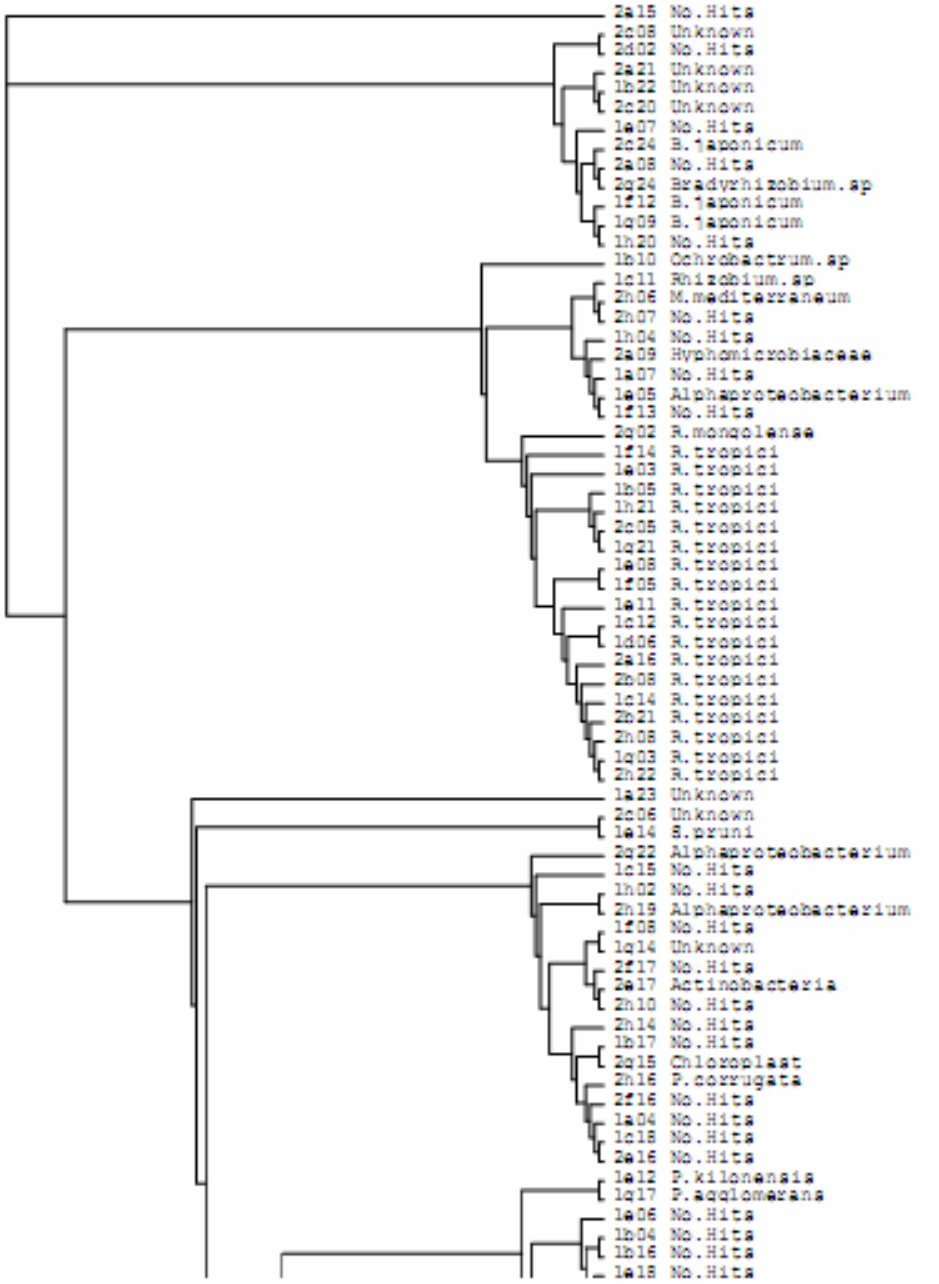


Figure A4.4: Cladogram of reverse 16S rDNA insert sequences from the WDW sample series.

KNS Forward


2a15 No.Hits
2c08 Unknown
2d02 No.Hits
2a21 Unknown
1b22 Unknown
2c20 Unknown
1a07 No.Hits
2c24 B.japonicum
2a08 No.Hits
2g24 Bradyrhizobium.sp
1f12 B.japonicum
1g09 B.japonicum
1h20 No.Hits
1b10 Ochrobactrum.sp
1c11 Rhizobium.sp
2h06 M.mediterraneum
2h07 No.Hits
1h04 No.Hits
2a09 Hyphomicrobiaceae
1a07 No.Hits
1a05 Alphaproteobacterium
1f13 No.Hits
2g02 R.mongolense
1f14 R.tropici
1a03 R.tropici
1b05 R.tropici
1h21 R.tropici
2c05 R.tropici
1g21 R.tropici
1a08 R.tropici
1f05 R.tropici
1a11 R.tropici
1c12 R.tropici
1d06 R.tropici
2a16 R.tropici
2h08 R.tropici
1c14 R.tropici
2b21 R.tropici
2h08 R.tropici
1g03 R.tropici
2h22 R.tropici
1a23 Unknown
2c06 Unknown
1a14 S.pruni
2g22 Alphaproteobacterium
1c15 No.Hits
1h02 No.Hits
2h19 Alphaproteobacterium
1f08 No.Hits
1g14 Unknown
2f17 No.Hits
2a17 Actinobacteria
2h10 No.Hits
2h14 No.Hits
1b17 No.Hits
2g15 Chloroplast
2h16 P.corrugata
2f16 No.Hits
1a04 No.Hits
1c18 No.Hits
2a16 No.Hits
1a12 P.kilonensis
1g17 P.agglomerans
1a06 No.Hits
1b04 No.Hits
1b16 No.Hits
1a18 No.Hits

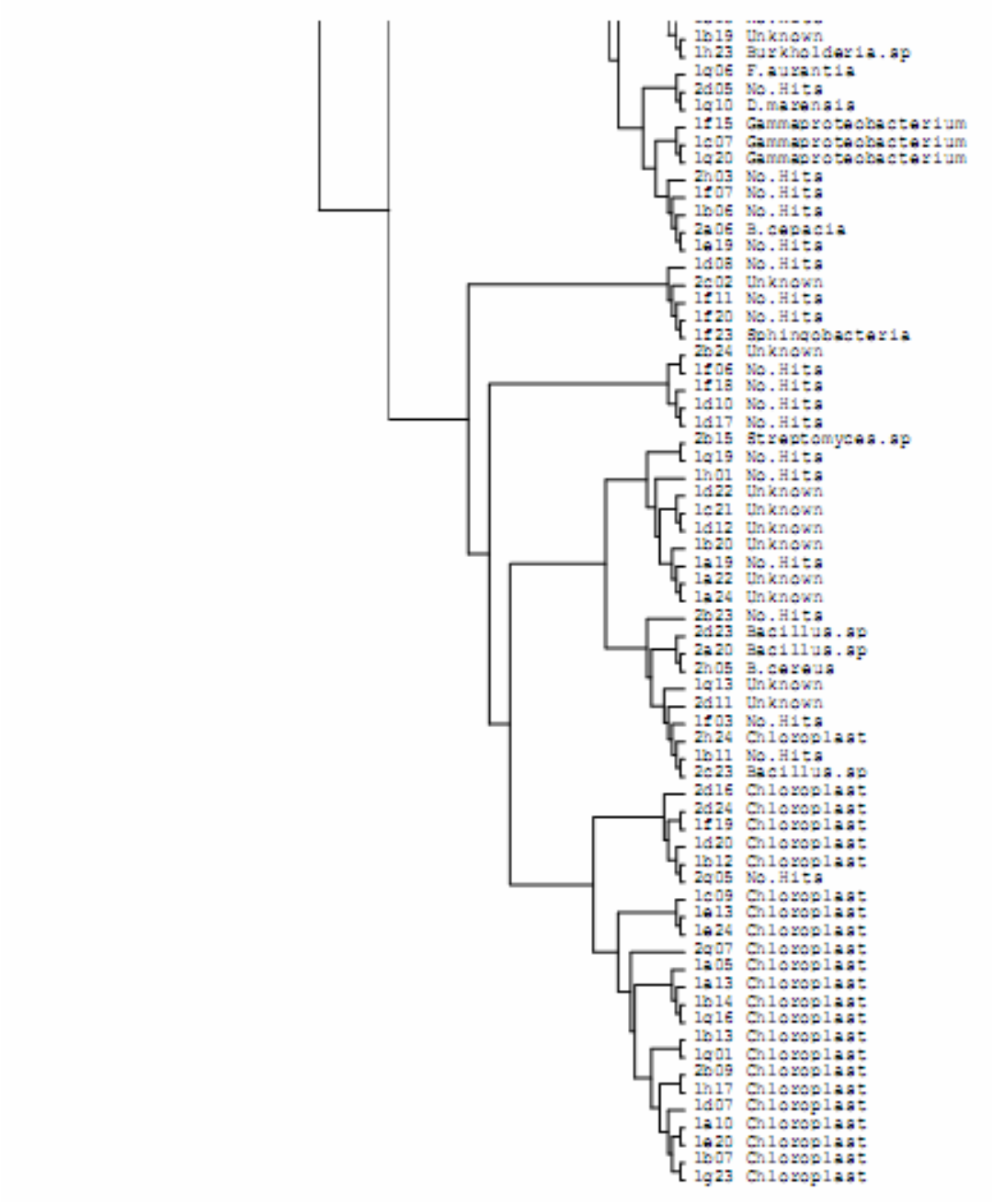
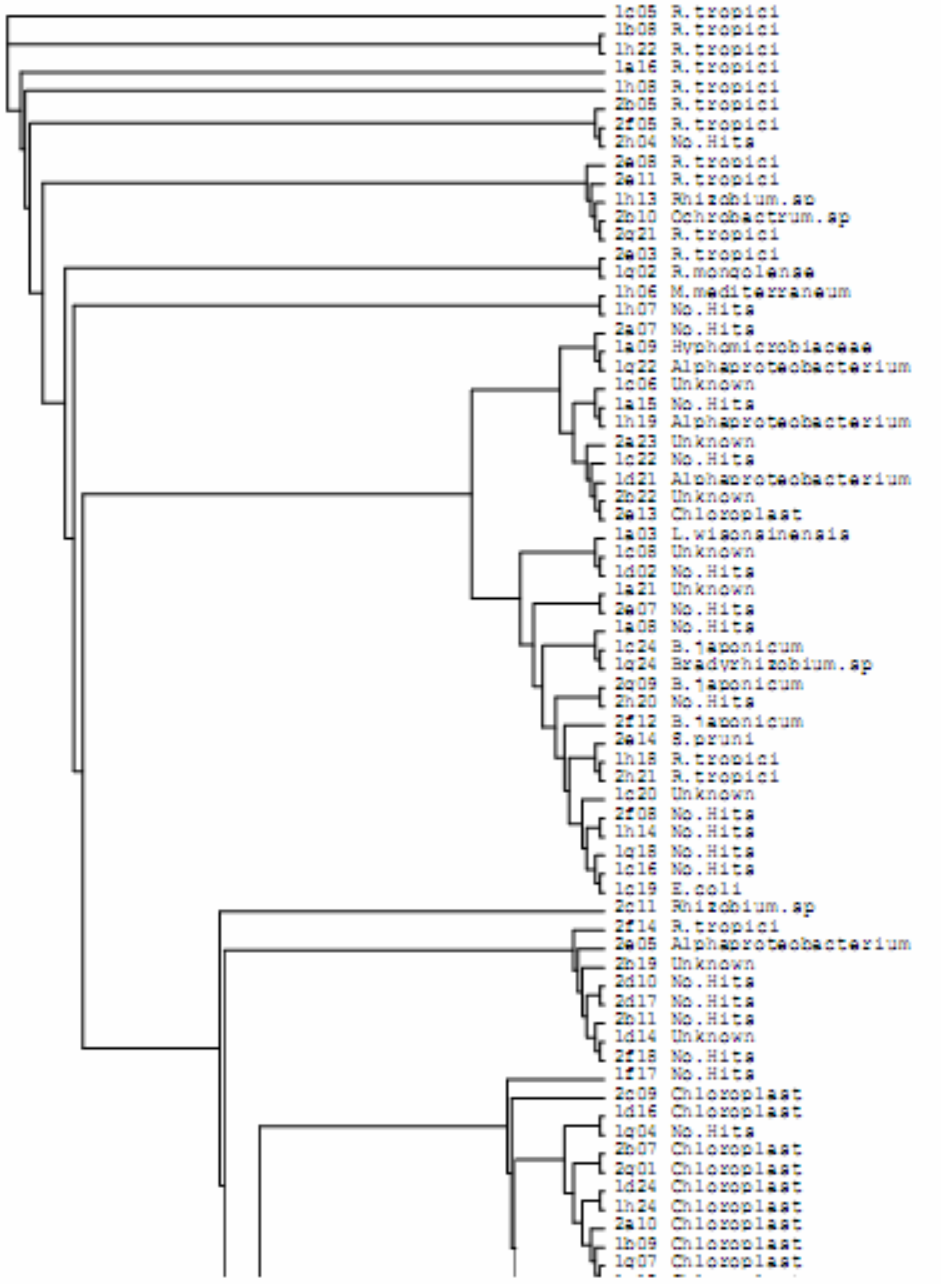


Figure A4.5: Cladogram of forward 16S rDNA insert sequences from the KNS sample series.

KNS Reverse


1c05 R.tropici
1b08 R.tropici
1h22 R.tropici
1a16 R.tropici
1h08 R.tropici
2b05 R.tropici
2f05 R.tropici
2h04 No.Hits
2a08 R.tropici
2a11 R.tropici
1h13 Rhizobium.sp
2b10 Ochrobactrum.sp
2g21 R.tropici
2a03 R.tropici
1g02 R.mongolense
1h06 M.mediterraneum
1h07 No.Hits
2a07 No.Hits
1a09 Hyphomicrobiaceae
1g22 Alphaproteobacterium
1c06 Unknown
1a15 No.Hits
1h19 Alphaproteobacterium
2a23 Unknown
1c22 No.Hits
1d21 Alphaproteobacterium
2b22 Unknown
2a13 Chloroplast
1a03 L.wisconsinensis
1c05 Unknown
1d02 No.Hits
1a21 Unknown
2a07 No.Hits
1a08 No.Hits
1c24 B.japonicum
1g24 Bradyrhizobium.sp
2g09 B.japonicum
2h20 No.Hits
2f12 B.japonicum
2a14 S.pruni
1h18 R.tropici
2h21 R.tropici
1c20 Unknown
2f08 No.Hits
1h14 No.Hits
1g18 No.Hits
1c16 No.Hits
1c19 E.coli
2c11 Rhizobium.sp
2f14 R.tropici
2a05 Alphaproteobacterium
2b19 Unknown
2d10 No.Hits
2d17 No.Hits
2b11 No.Hits
1d14 Unknown
2f18 No.Hits
1f17 No.Hits
2c09 Chloroplast
1d16 Chloroplast
1g04 No.Hits
2b07 Chloroplast
2g01 Chloroplast
1d24 Chloroplast
1h24 Chloroplast
2a10 Chloroplast
1b09 Chloroplast
1g07 Chloroplast

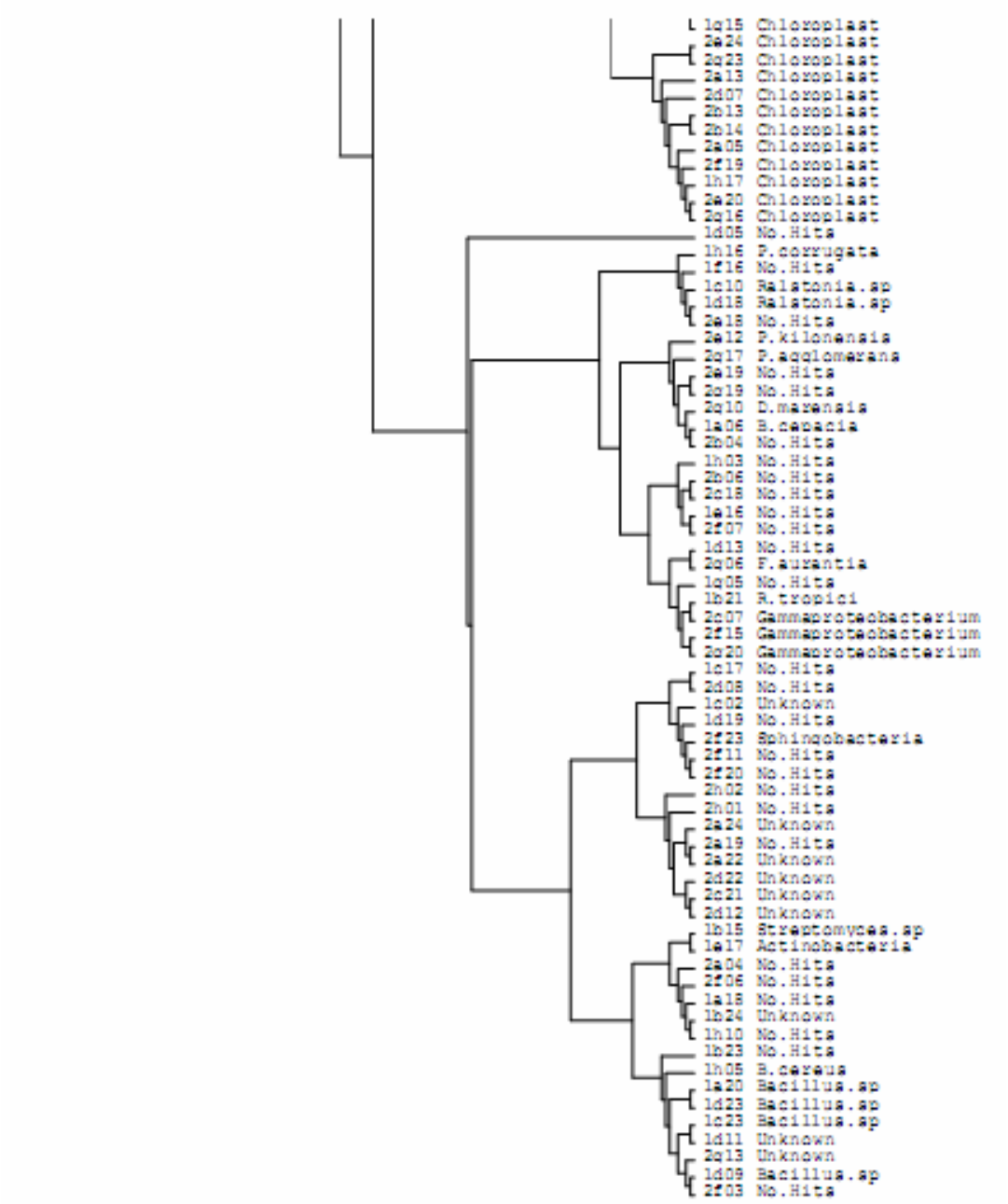
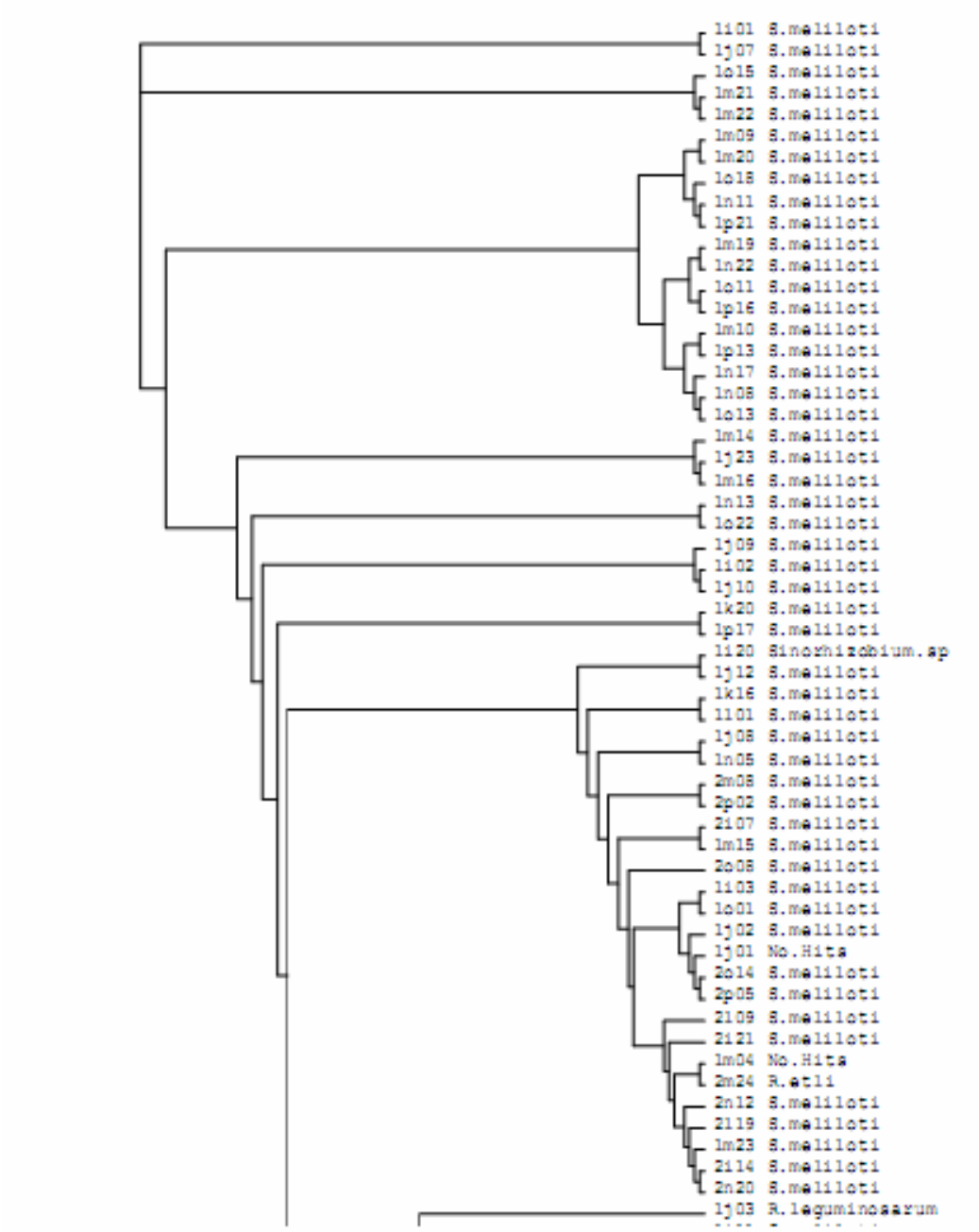


Figure A4.6: Cladogram of reverse 16S rDNA insert sequences from the KNS sample series.

STW Forward


1i01 S.meliloti
1j07 S.meliloti
1o15 S.meliloti
1m21 S.meliloti
1m22 S.meliloti
1m09 S.meliloti
1m20 S.meliloti
1o18 S.meliloti
1n11 S.meliloti
1p21 S.meliloti
1m19 S.meliloti
1n22 S.meliloti
1o11 S.meliloti
1p16 S.meliloti
1m10 S.meliloti
1p13 S.meliloti
1n17 S.meliloti
1n08 S.meliloti
1o13 S.meliloti
1m14 S.meliloti
1j23 S.meliloti
1m16 S.meliloti
1n13 S.meliloti
1o22 S.meliloti
1j09 S.meliloti
1i02 S.meliloti
1j10 S.meliloti
1k20 S.meliloti
1p17 S.meliloti
1i20 Sinorhizobium.sp
1j12 S.meliloti
1k16 S.meliloti
1l01 S.meliloti
1j08 S.meliloti
1n05 S.meliloti
2m08 S.meliloti
2p02 S.meliloti
2i07 S.meliloti
1m15 S.meliloti
2o08 S.meliloti
1i03 S.meliloti
1o01 S.meliloti
1j02 S.meliloti
1j01 No.Hits
2o14 S.meliloti
2p05 S.meliloti
2l09 S.meliloti
2i21 S.meliloti
1m04 No.Hits
2m24 R.etli
2n12 S.meliloti
2l19 S.meliloti
1m23 S.meliloti
2i14 S.meliloti
2n20 S.meliloti
1j03 R.leguminosarum

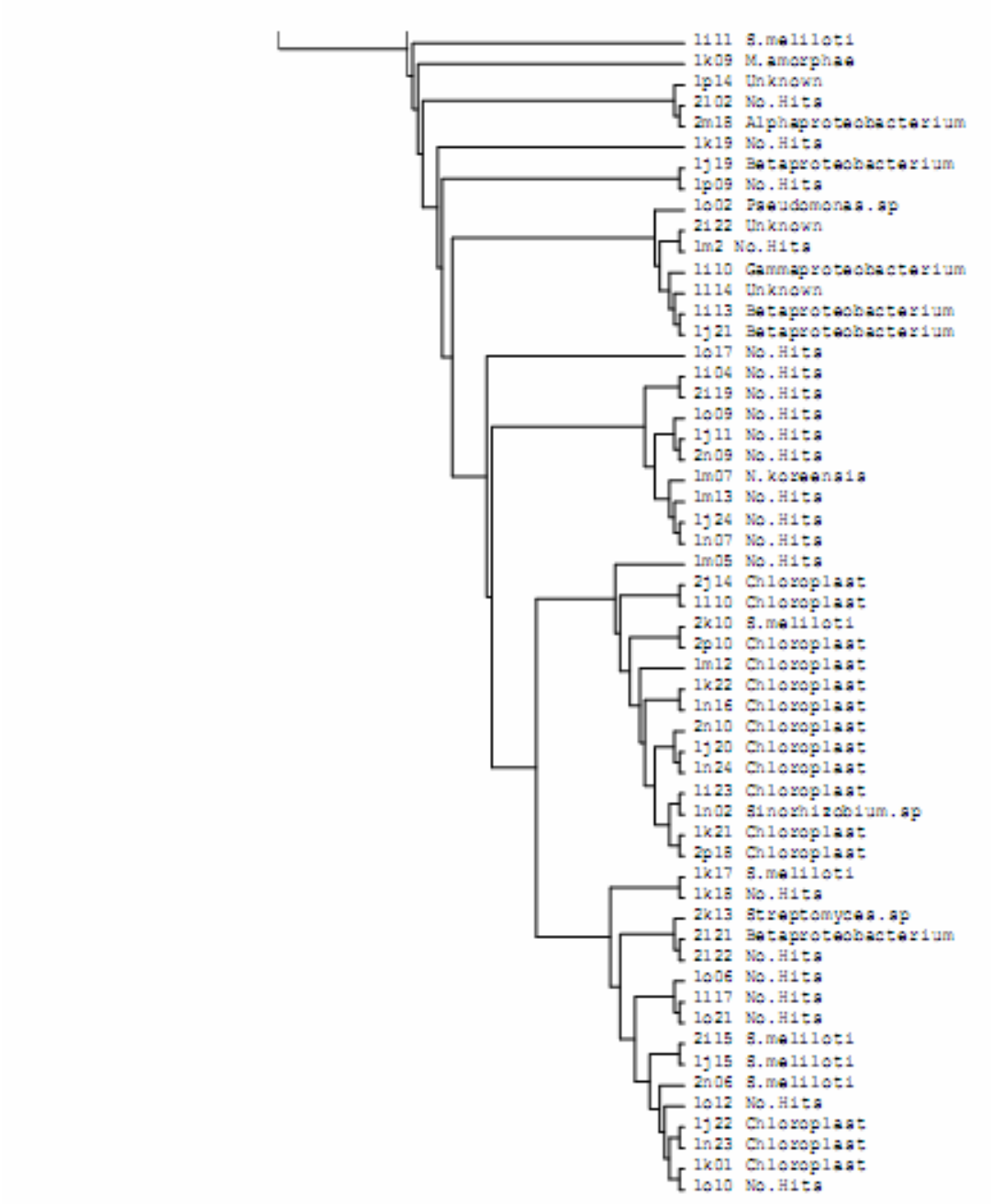
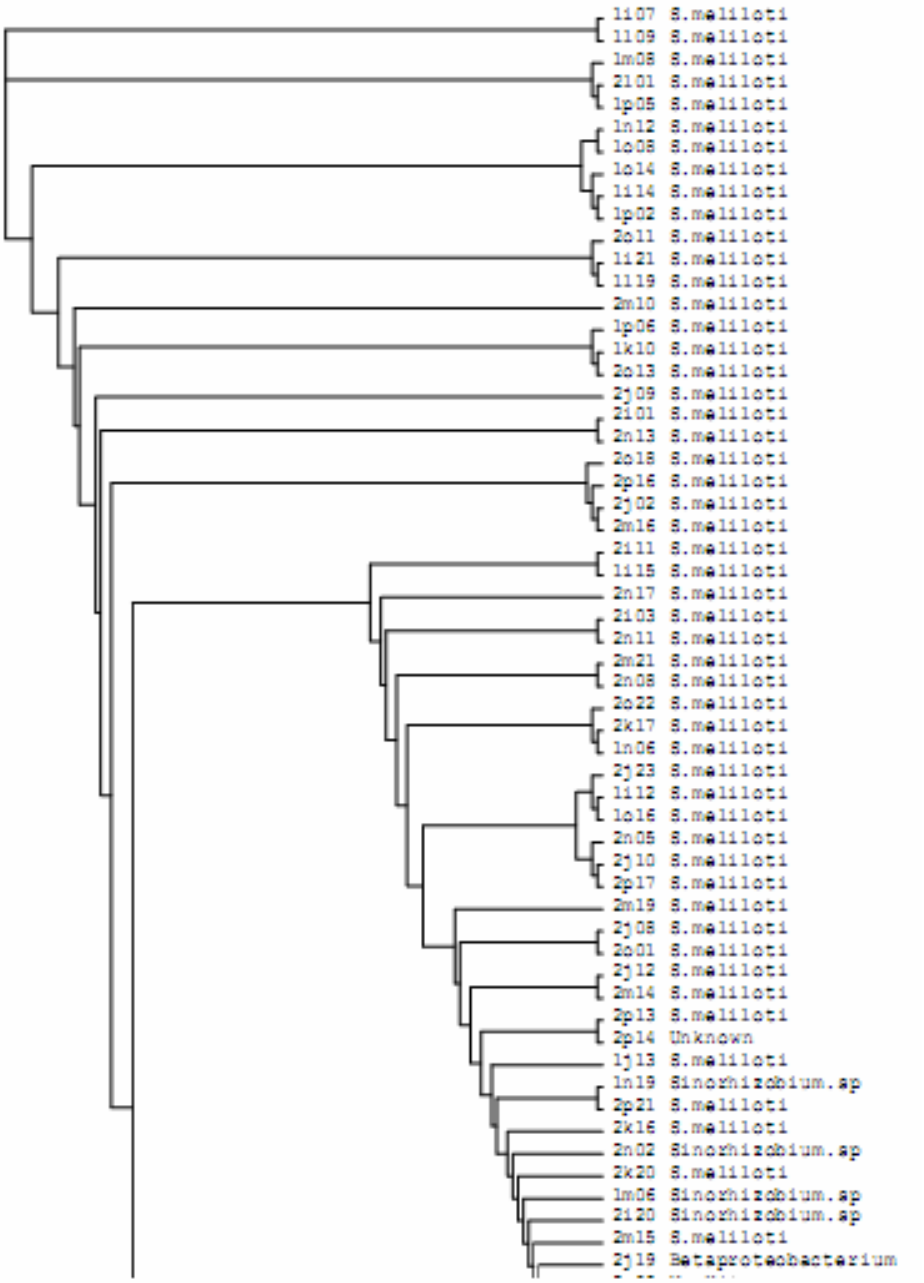


Figure A4.7: Cladogram of forward 16S rDNA insert sequences from the STW sample series.

STW Reverse


1i07 S.meliloti
1i09 S.meliloti
1m08 S.meliloti
2i01 S.meliloti
1p05 S.meliloti
1n12 S.meliloti
1o08 S.meliloti
1o14 S.meliloti
1i14 S.meliloti
1p02 S.meliloti
2o11 S.meliloti
1i21 S.meliloti
1i19 S.meliloti
2m10 S.meliloti
1p06 S.meliloti
1k10 S.meliloti
2o13 S.meliloti
2j09 S.meliloti
2i01 S.meliloti
2n13 S.meliloti
2o18 S.meliloti
2p16 S.meliloti
2j02 S.meliloti
2m16 S.meliloti
2i11 S.meliloti
1i15 S.meliloti
2n17 S.meliloti
2i03 S.meliloti
2n11 S.meliloti
2m21 S.meliloti
2n08 S.meliloti
2o22 S.meliloti
2k17 S.meliloti
1n06 S.meliloti
2j23 S.meliloti
1i12 S.meliloti
1o16 S.meliloti
2n05 S.meliloti
2j10 S.meliloti
2p17 S.meliloti
2m19 S.meliloti
2j08 S.meliloti
2o01 S.meliloti
2j12 S.meliloti
2m14 S.meliloti
2p13 S.meliloti
2p14 Unknown
1j13 S.meliloti
1n19 Sinorhizobium.sp
2p21 S.meliloti
2k16 S.meliloti
2n02 Sinorhizobium.sp
2k20 S.meliloti
1m06 Sinorhizobium.sp
2i20 Sinorhizobium.sp
2m15 S.meliloti
2j19 Betaproteobacterium

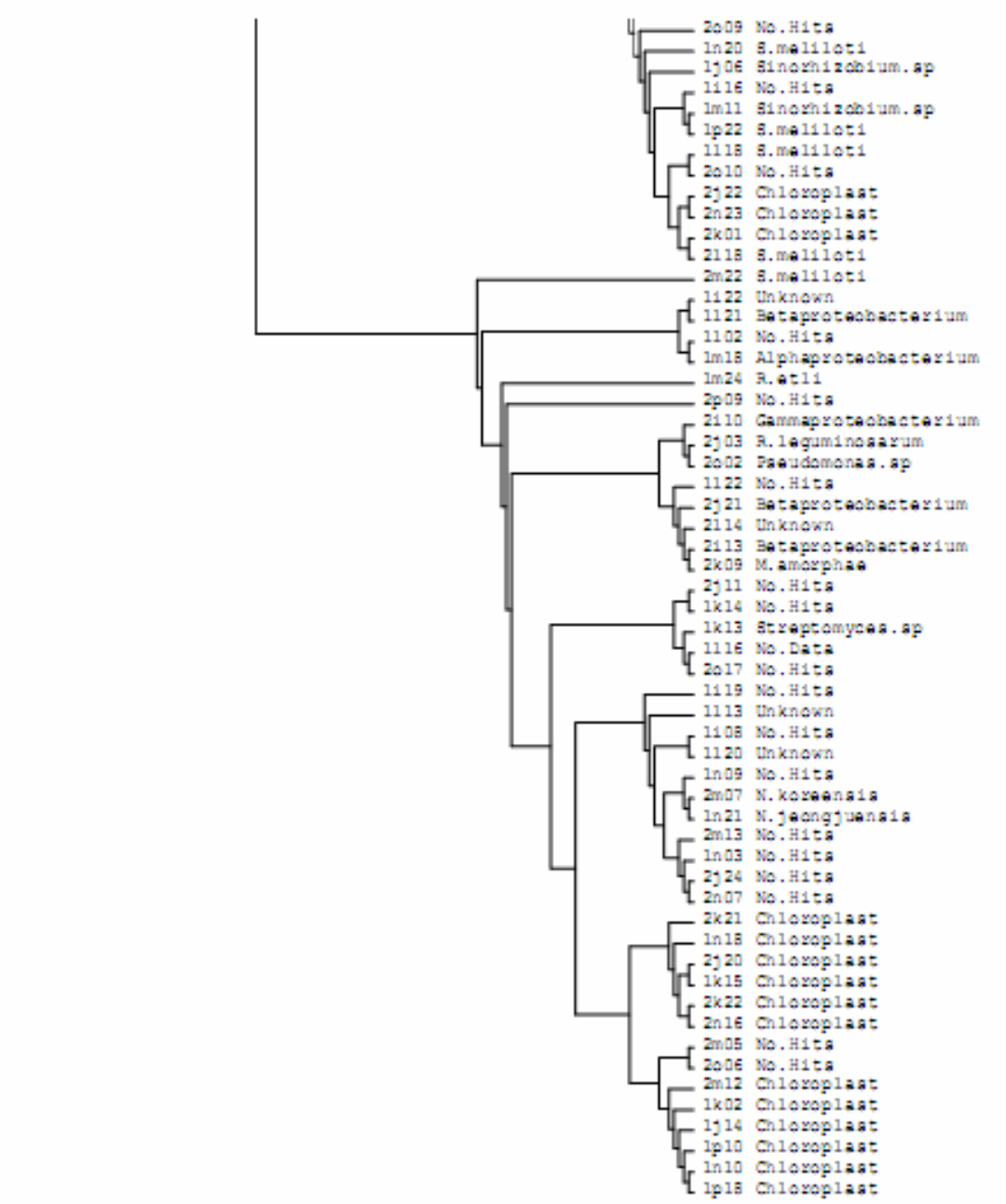
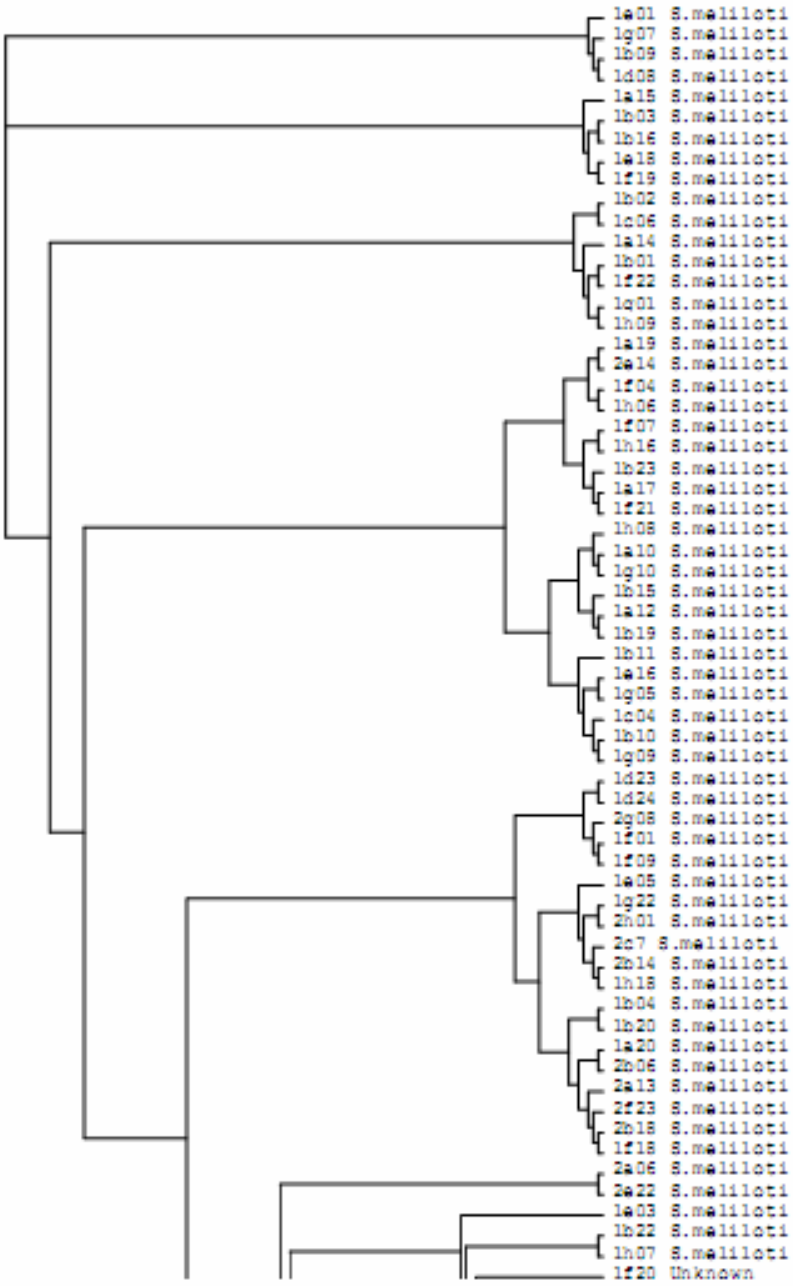


Figure A4.8: Cladogram of reverse 16S rDNA insert sequences from the STW sample series.

PAW Forward


1a01 S.mallilati
1g07 S.mallilati
1b09 S.mallilati
1d08 S.mallilati
1a15 S.mallilati
1b03 S.mallilati
1b16 S.mallilati
1a18 S.mallilati
1f19 S.mallilati
1b02 S.mallilati
1c06 S.mallilati
1a14 S.mallilati
1b01 S.mallilati
1f22 S.mallilati
1g01 S.mallilati
1h09 S.mallilati
1a19 S.mallilati
2a14 S.mallilati
1f04 S.mallilati
1h06 S.mallilati
1f07 S.mallilati
1h16 S.mallilati
1b23 S.mallilati
1a17 S.mallilati
1f21 S.mallilati
1h08 S.mallilati
1a10 S.mallilati
1g10 S.mallilati
1b15 S.mallilati
1a12 S.mallilati
1b19 S.mallilati
1b11 S.mallilati
1a16 S.mallilati
1g05 S.mallilati
1c04 S.mallilati
1h10 S.mallilati
1g09 S.mallilati
1d23 S.mallilati
1d24 S.mallilati
2g08 S.mallilati
1f01 S.mallilati
1f09 S.mallilati
1a05 S.mallilati
1g22 S.mallilati
2h01 S.mallilati
2c7 S.mallilati
2b14 S.mallilati
1h18 S.mallilati
1b04 S.mallilati
1b20 S.mallilati
1a20 S.mallilati
2b06 S.mallilati
2a13 S.mallilati
2f23 S.mallilati
2h18 S.mallilati
1f18 S.mallilati
2a06 S.mallilati
2a22 S.mallilati
1a03 S.mallilati
1b22 S.mallilati
1h07 S.mallilati
1f20 Unknown

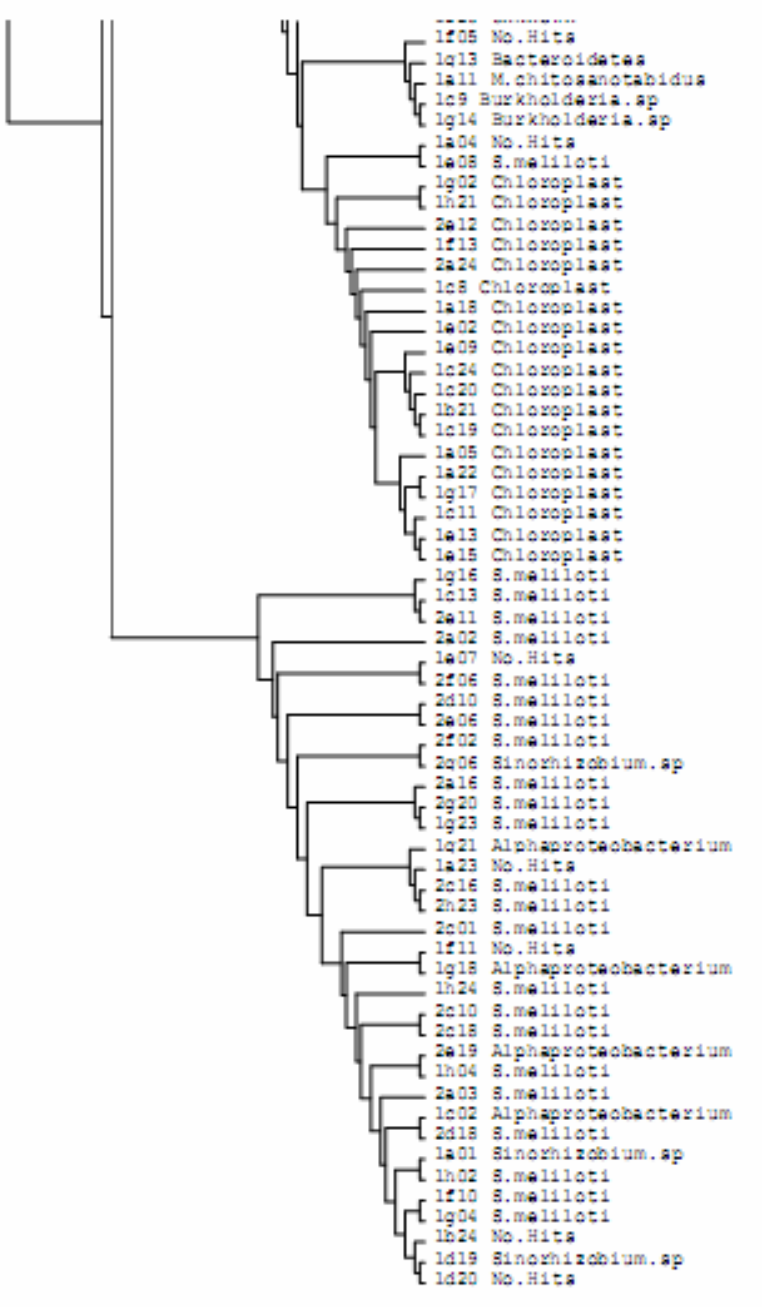
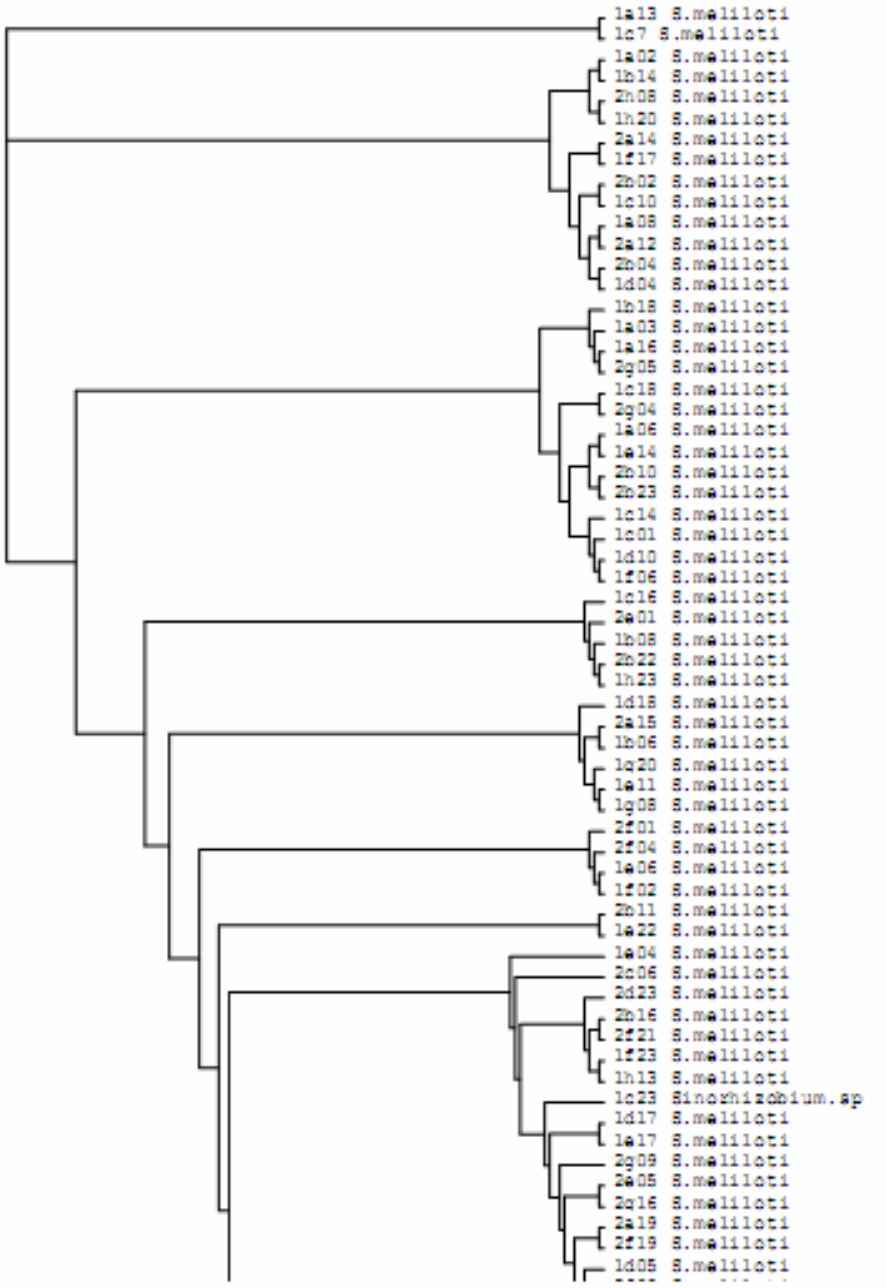


Figure A4.9: Cladogram of forward 16S rDNA insert sequences from the PAW sample series.

PAW Reverse


1a13 S.meliloti
1c7 S.meliloti
1a02 S.meliloti
1b14 S.meliloti
2h08 S.meliloti
1h20 S.meliloti
2a14 S.meliloti
1f17 S.meliloti
2b02 S.meliloti
1c10 S.meliloti
1a08 S.meliloti
2a12 S.meliloti
2b04 S.meliloti
1d04 S.meliloti
1b18 S.meliloti
1a03 S.meliloti
1a16 S.meliloti
2g05 S.meliloti
1c18 S.meliloti
2g04 S.meliloti
1a06 S.meliloti
1e14 S.meliloti
2b10 S.meliloti
2b23 S.meliloti
1c14 S.meliloti
1c01 S.meliloti
1d10 S.meliloti
1f06 S.meliloti
1c16 S.meliloti
2e01 S.meliloti
1b08 S.meliloti
2b22 S.meliloti
1h23 S.meliloti
1d18 S.meliloti
2a15 S.meliloti
1b06 S.meliloti
1g20 S.meliloti
1e11 S.meliloti
1g08 S.meliloti
2f01 S.meliloti
2f04 S.meliloti
1e06 S.meliloti
1f02 S.meliloti
2b11 S.meliloti
1e22 S.meliloti
1e04 S.meliloti
2c06 S.meliloti
2d23 S.meliloti
2b16 S.meliloti
2f21 S.meliloti
1f23 S.meliloti
1h13 S.meliloti
1c23 Sinorhizobium.sp
1d17 S.meliloti
1e17 S.meliloti
2g09 S.meliloti
2e05 S.meliloti
2g16 S.meliloti
2a19 S.meliloti
2f19 S.meliloti
1d05 S.meliloti

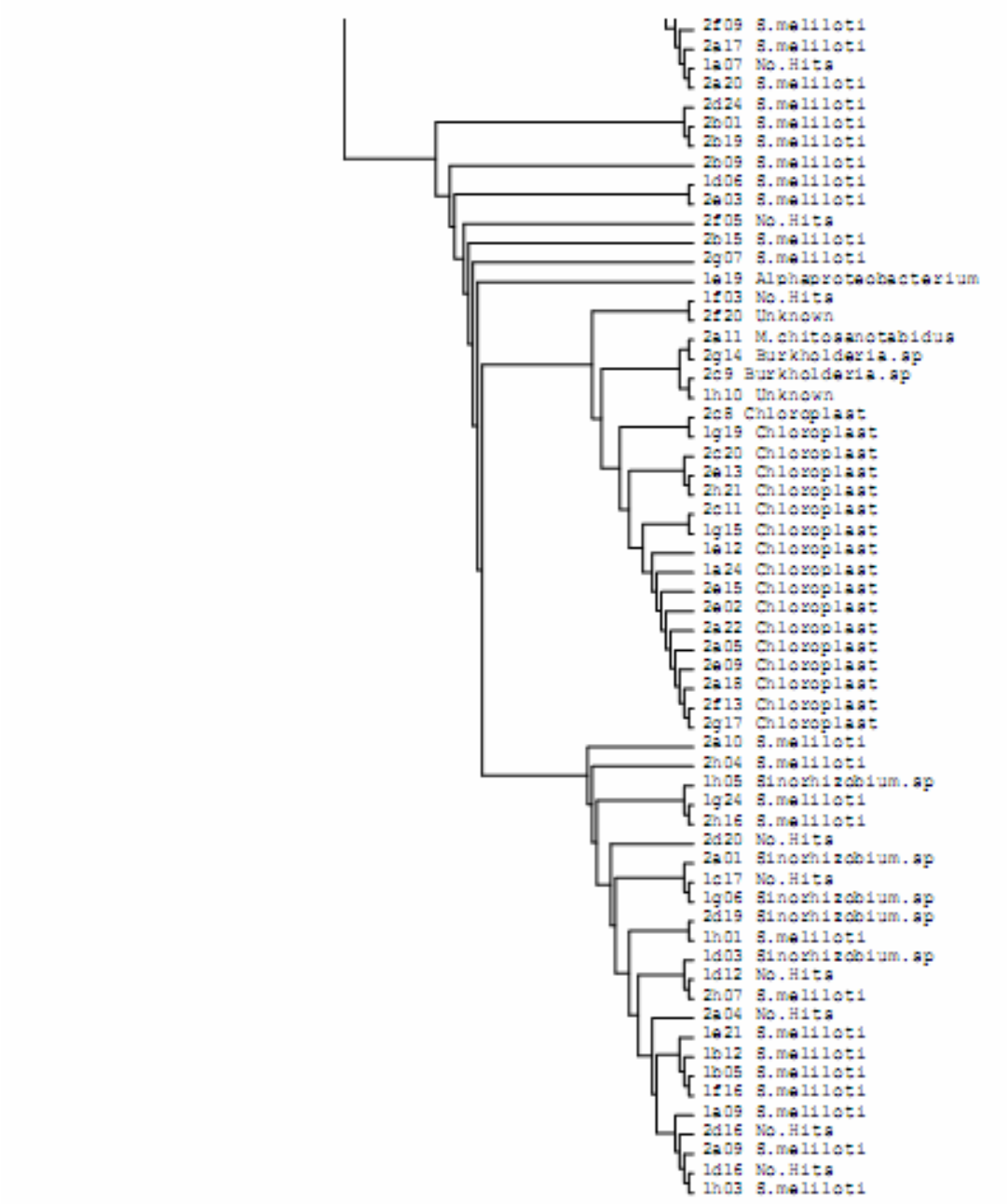
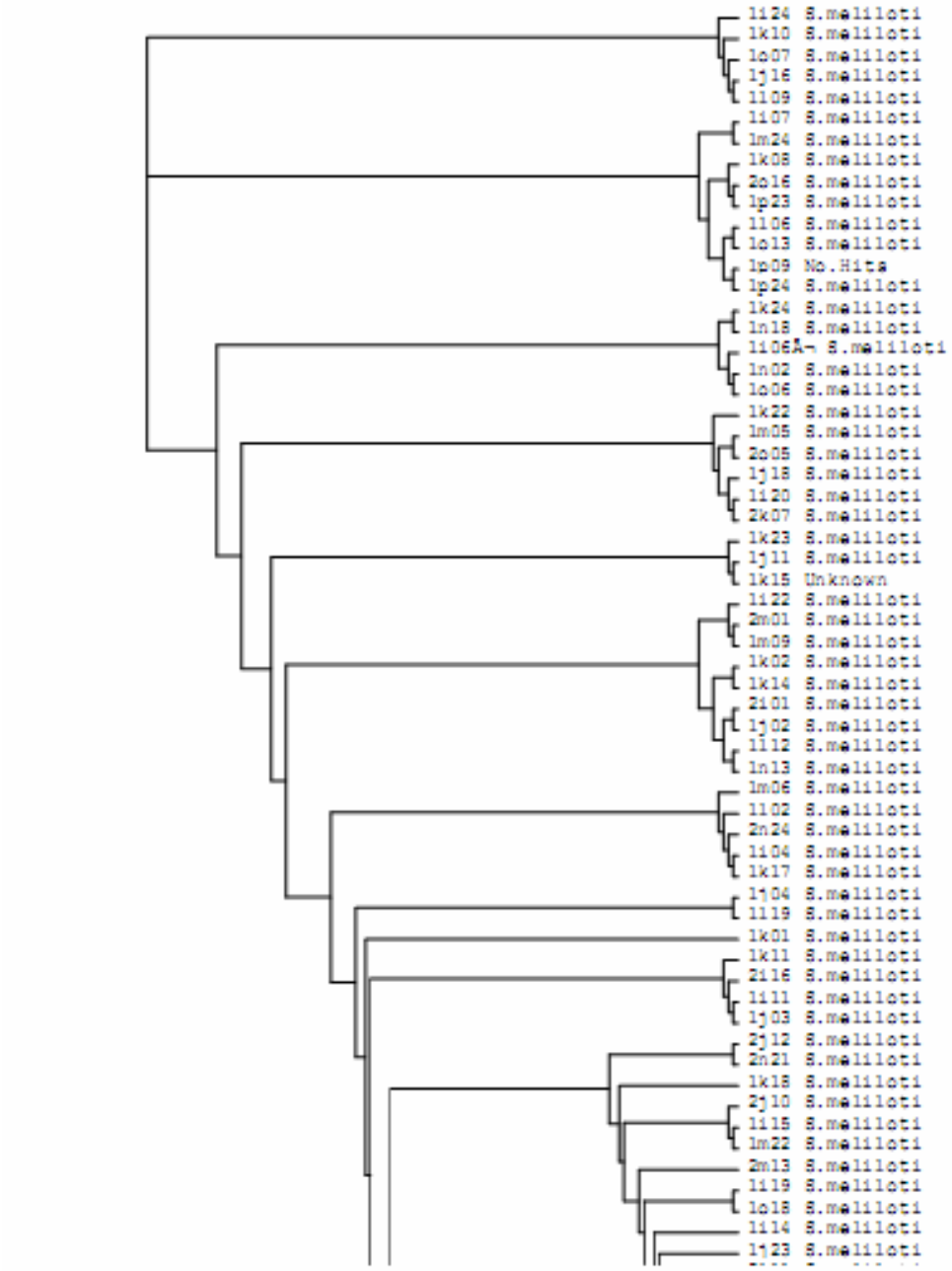


Figure A4.10: Cladogram of reverse 16S rDNA insert sequences from the PAW sample series.

GDW Forward


1i24 S.meliloti
1k10 S.meliloti
1o07 S.meliloti
1j16 S.meliloti
1l09 S.meliloti
1i07 S.meliloti
1m24 S.meliloti
1k08 S.meliloti
2o16 S.meliloti
1p23 S.meliloti
1l06 S.meliloti
1o13 S.meliloti
1p09 No.Hits
1p24 S.meliloti
1k24 S.meliloti
1n18 S.meliloti
1i06Â¬ S.meliloti
1n02 S.meliloti
1o06 S.meliloti
1k22 S.meliloti
1m05 S.meliloti
2o05 S.meliloti
1j18 S.meliloti
1i20 S.meliloti
2k07 S.meliloti
1k23 S.meliloti
1j11 S.meliloti
1k15 Unknown
1i22 S.meliloti
2m01 S.meliloti
1m09 S.meliloti
1k02 S.meliloti
1k14 S.meliloti
2i01 S.meliloti
1j02 S.meliloti
1l12 S.meliloti
1n13 S.meliloti
1m06 S.meliloti
1l02 S.meliloti
2n24 S.meliloti
1i04 S.meliloti
1k17 S.meliloti
1j04 S.meliloti
1l19 S.meliloti
1k01 S.meliloti
1k11 S.meliloti
2i16 S.meliloti
1i11 S.meliloti
1j03 S.meliloti
2j12 S.meliloti
2n21 S.meliloti
1k18 S.meliloti
2j10 S.meliloti
1i15 S.meliloti
1m22 S.meliloti
2m13 S.meliloti
1i19 S.meliloti
1o18 S.meliloti
1i14 S.meliloti
1j23 S.meliloti

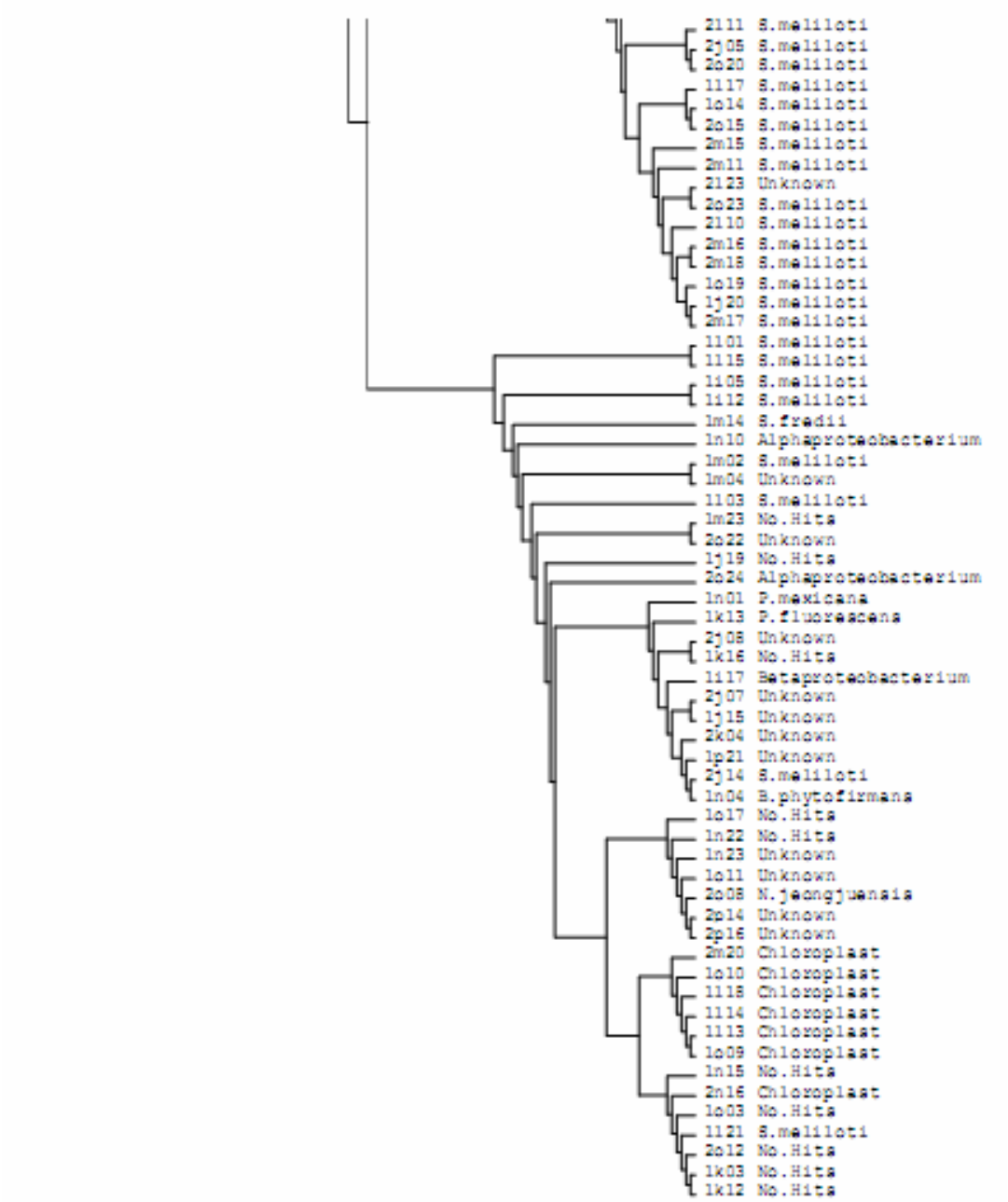
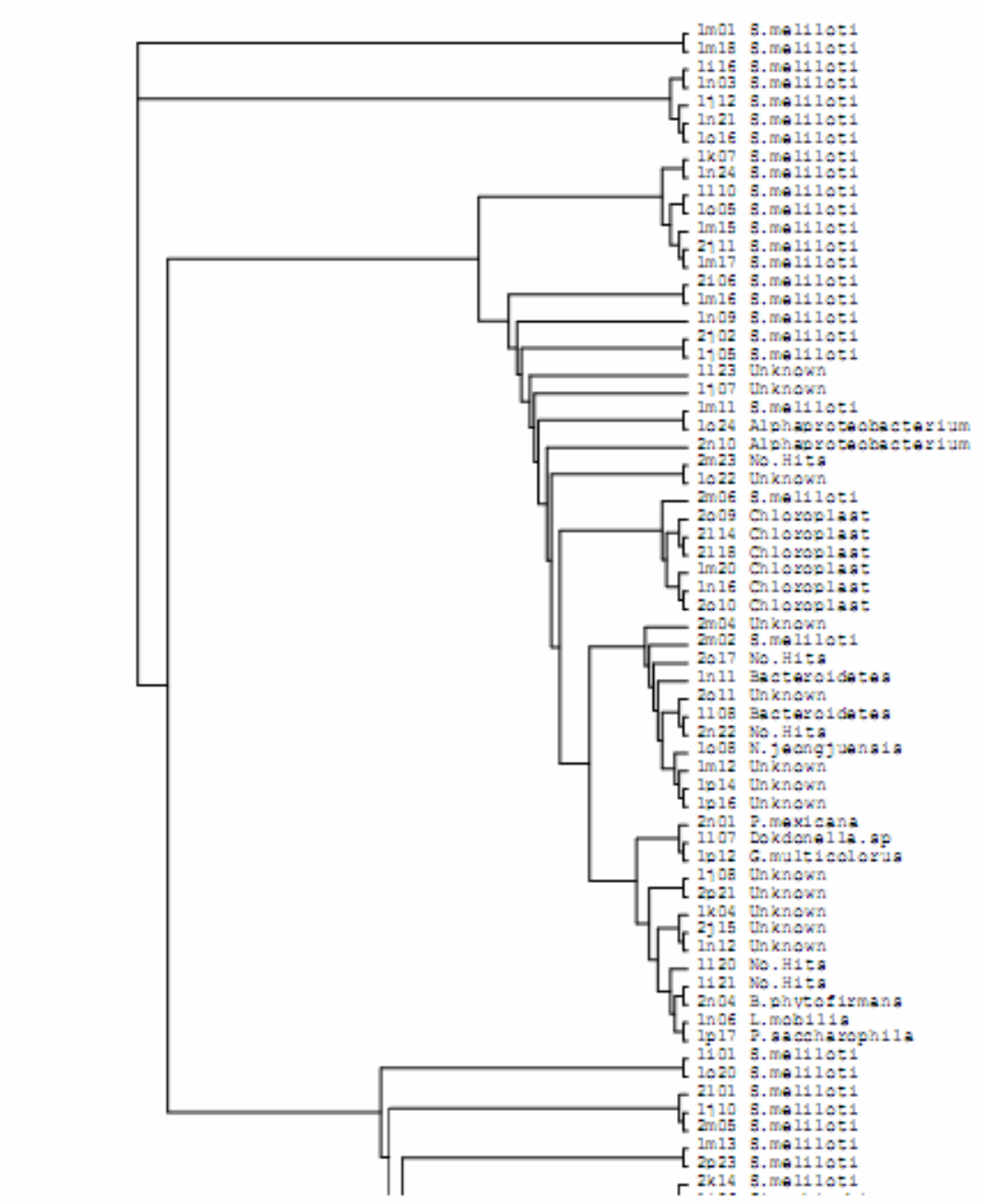


Figure A4.11: Cladogram of forward 16S rDNA insert sequences from the GDW sample series.

GDW Reverse


1m01 S.melliloti
1m18 S.melliloti
1i16 S.melliloti
1n03 S.melliloti
1j12 S.melliloti
1n21 S.melliloti
1o16 S.melliloti
1k07 S.melliloti
1n24 S.melliloti
1i10 S.melliloti
1o05 S.melliloti
1m15 S.melliloti
2j11 S.melliloti
1m17 S.melliloti
2i06 S.melliloti
1m16 S.melliloti
1n09 S.melliloti
2j02 S.melliloti
1j05 S.melliloti
1i23 Unknown
1j07 Unknown
1m11 S.melliloti
1o24 Alphaproteobacterium
2n10 Alphaproteobacterium
2m23 No.Hits
1o22 Unknown
2m06 S.melliloti
2o09 Chloroplast
2i14 Chloroplast
2i18 Chloroplast
1m20 Chloroplast
1n16 Chloroplast
2o10 Chloroplast
2m04 Unknown
2m02 S.melliloti
2o17 No.Hits
1n11 Bacteroidetes
2o11 Unknown
1i08 Bacteroidetes
2n22 No.Hits
1o08 N.jeongjuensis
1m12 Unknown
1p14 Unknown
1p16 Unknown
2n01 P.mexicana
1i07 Dokdonella.sp
1p12 C.multicolorus
1j08 Unknown
2p21 Unknown
1k04 Unknown
2j15 Unknown
1n12 Unknown
1i20 No.Hits
1i21 No.Hits
2n04 B.phytofirmans
1n06 L.mobilis
1p17 P.saccharophila
1i01 S.melliloti
1o20 S.melliloti
2i01 S.melliloti
1j10 S.melliloti
2m05 S.melliloti
1m13 S.melliloti
2p23 S.melliloti
2k14 S.melliloti

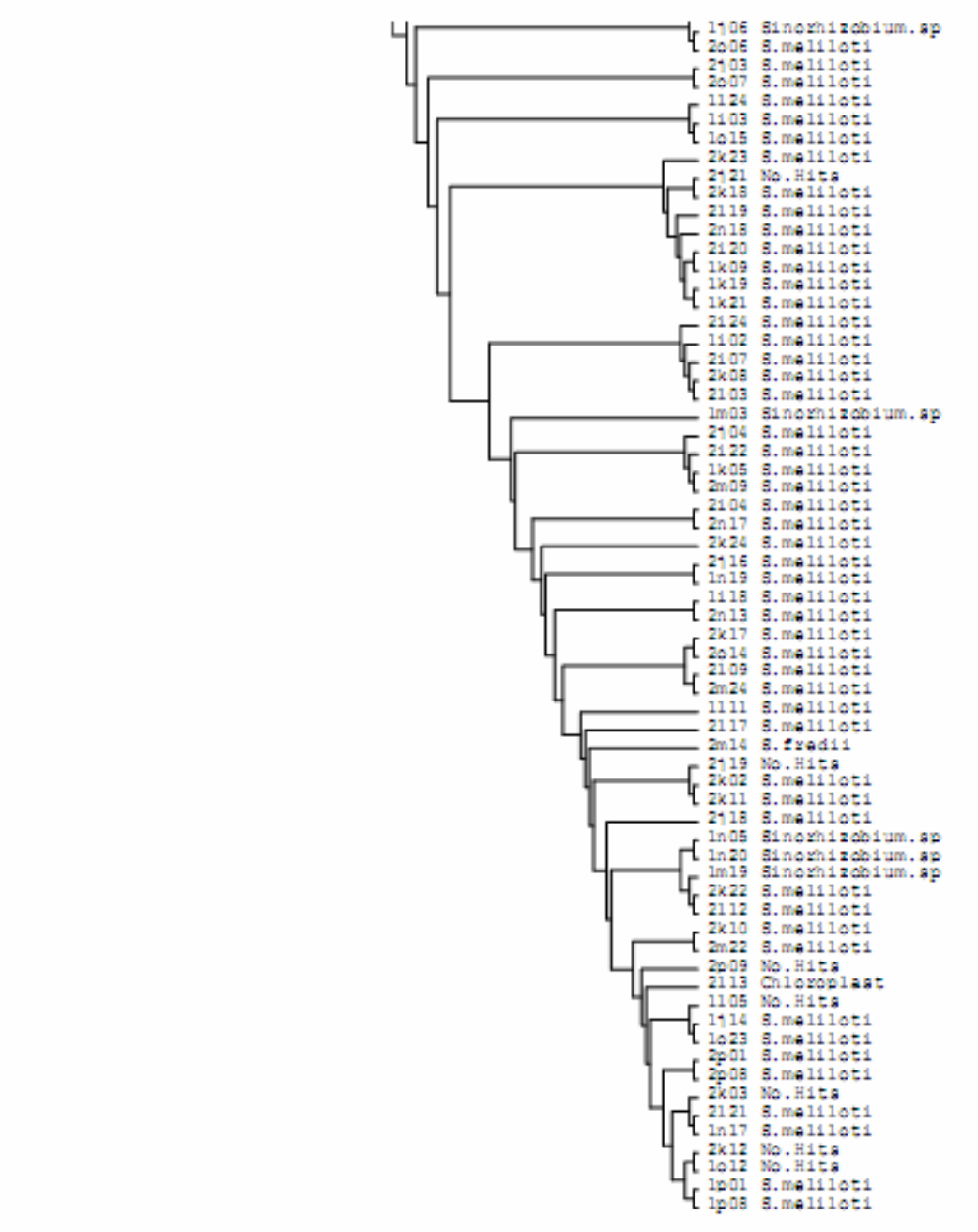


Figure A4.12: Cladogram of reverse 16S rDNA insert sequences from the GDW sample series.

MM366 Forward

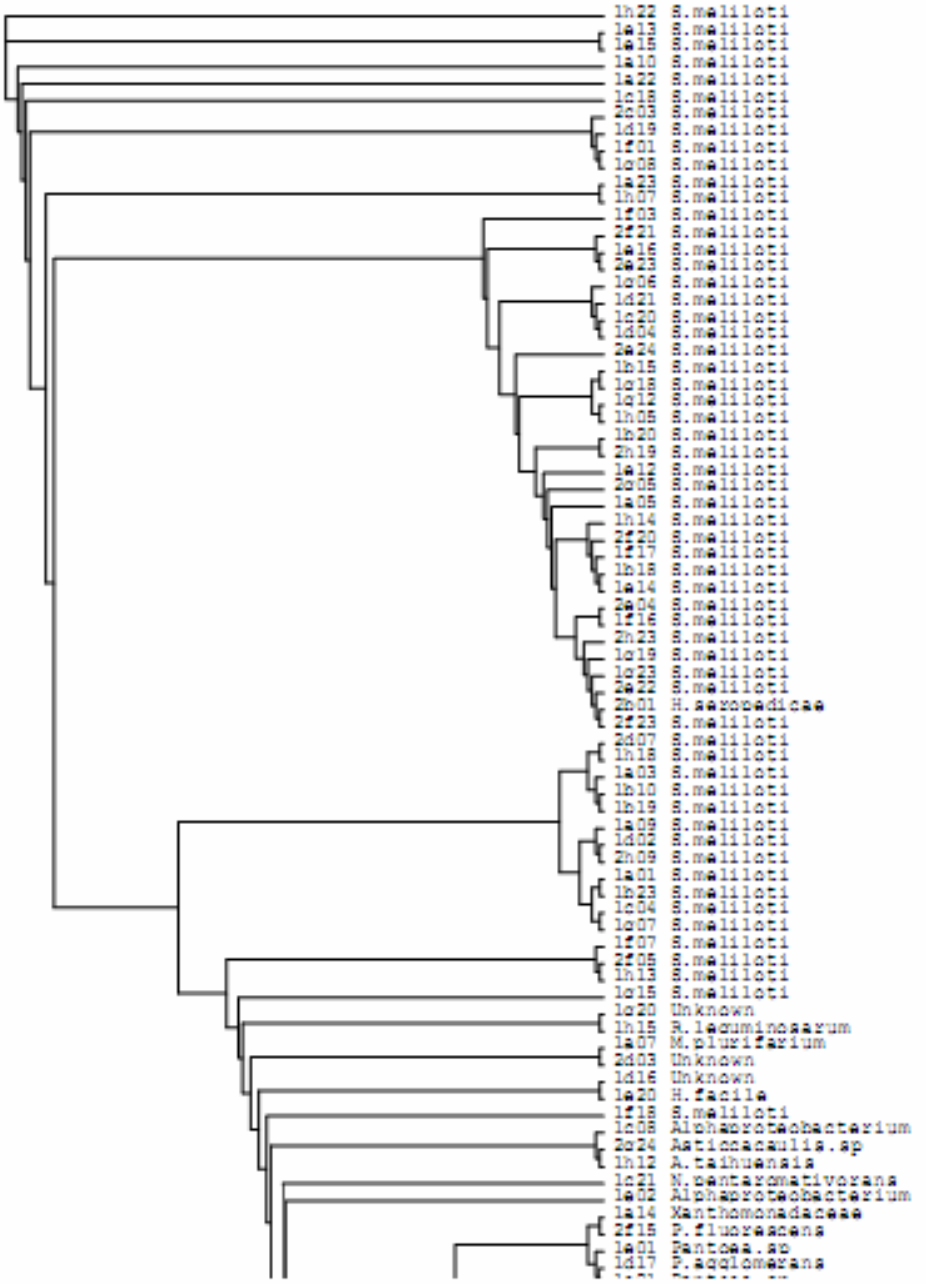
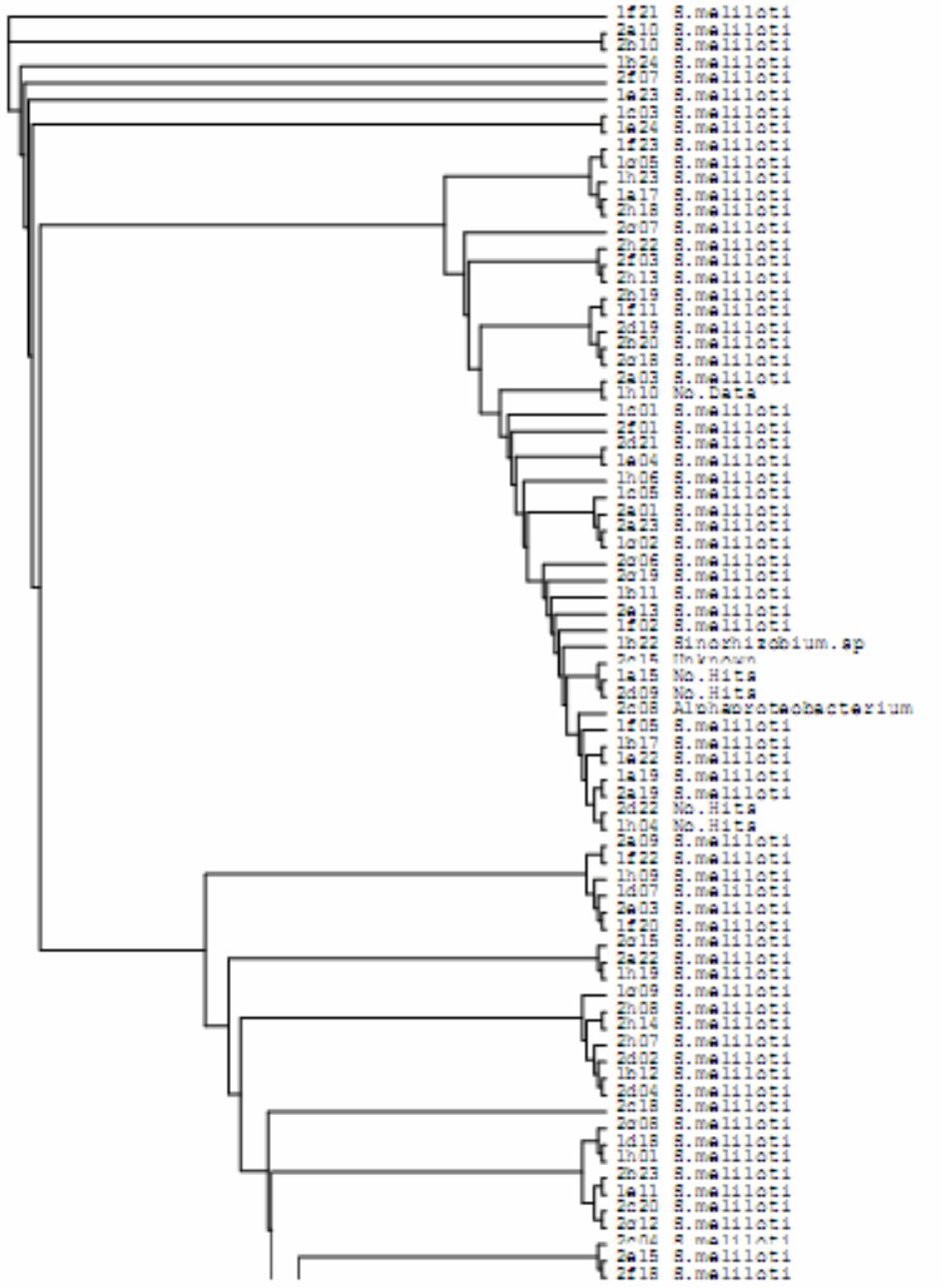


Figure A4.13: Cladogram of forward 16S rDNA insert sequences from the MM366 sample series.

MM366 Reverse



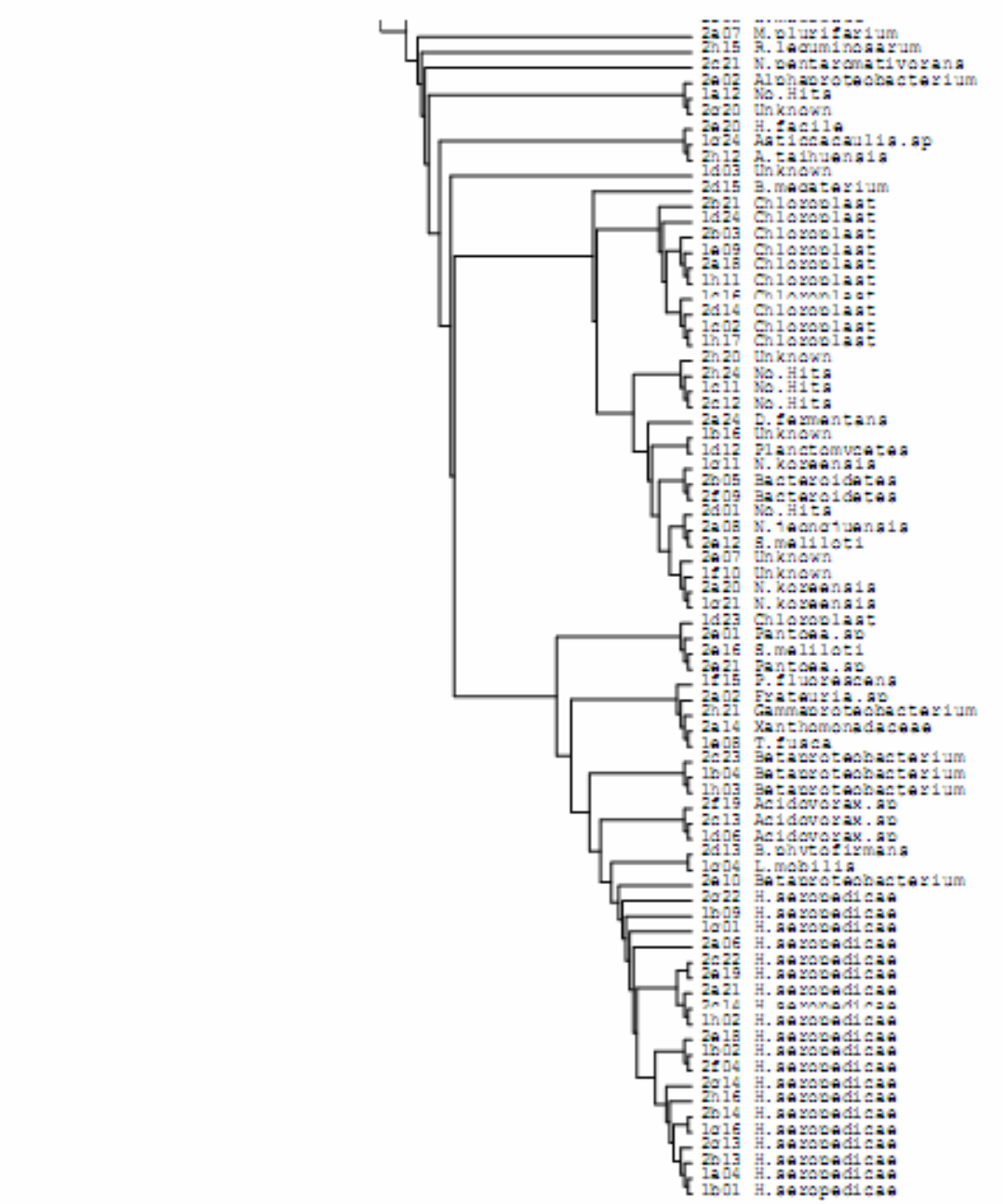
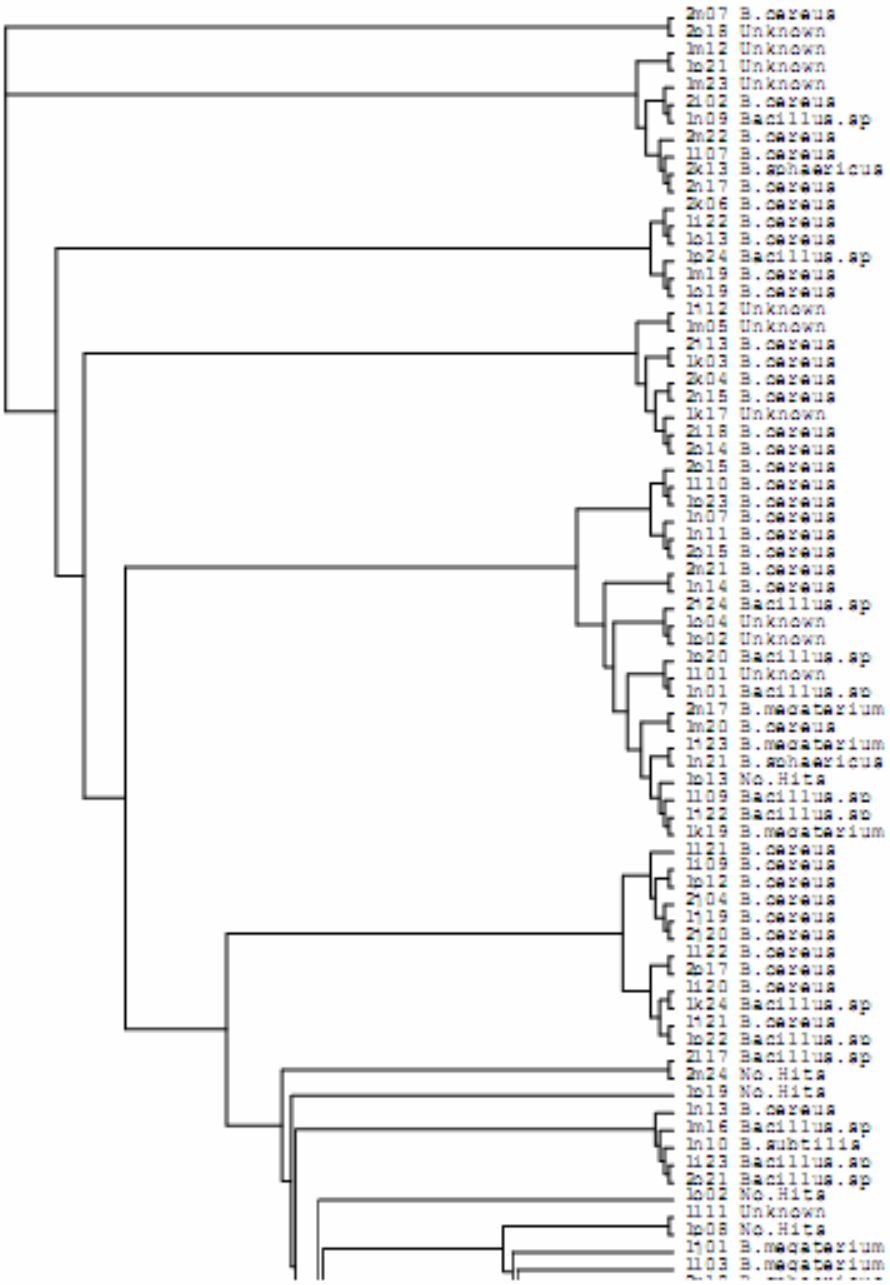


Figure A4.14: Cladogram of reverse 16S rDNA insert sequences from the MM366 sample series.

UNK Forward


2m07 B.cereus
2b18 Unknown
1m12 Unknown
1b21 Unknown
1m23 Unknown
2l02 B.cereus
1n09 Bacillus.sp
2m22 B.cereus
1l07 B.cereus
2k13 B.sphaericus
2n17 B.cereus
2k06 B.cereus
1l22 B.cereus
1b13 B.cereus
1b24 Bacillus.sp
1m19 B.cereus
1b19 B.cereus
1l12 Unknown
1m05 Unknown
2l13 B.cereus
1k03 B.cereus
2k04 B.cereus
2n15 B.cereus
1k17 Unknown
2l18 B.cereus
2b14 B.cereus
2b15 B.cereus
1l10 B.cereus
1b23 B.cereus
1n07 B.cereus
1n11 B.cereus
2b15 B.cereus
2m21 B.cereus
1n14 B.cereus
2l24 Bacillus.sp
1a04 Unknown
1b02 Unknown
1b20 Bacillus.sp
1l01 Unknown
1n01 Bacillus.sp
2m17 B.megaterium
1m20 B.cereus
1l23 B.megaterium
1n21 B.sphaericus
1b13 No.Hits
1l09 Bacillus.sp
1l22 Bacillus.sp
1k19 B.megaterium
1l21 B.cereus
1l09 B.cereus
1b12 B.cereus
2l04 B.cereus
1l19 B.cereus
2l20 B.cereus
1l22 B.cereus
2b17 B.cereus
1l20 B.cereus
1k24 Bacillus.sp
1l21 B.cereus
1b22 Bacillus.sp
2l17 Bacillus.sp
2m24 No.Hits
1b19 No.Hits
1n13 B.cereus
1m16 Bacillus.sp
1n10 B.subtilis
1l23 Bacillus.sp
2b21 Bacillus.sp
1b02 No.Hits
1l11 Unknown
1b08 No.Hits
1l01 B.megaterium
1l03 B.megaterium

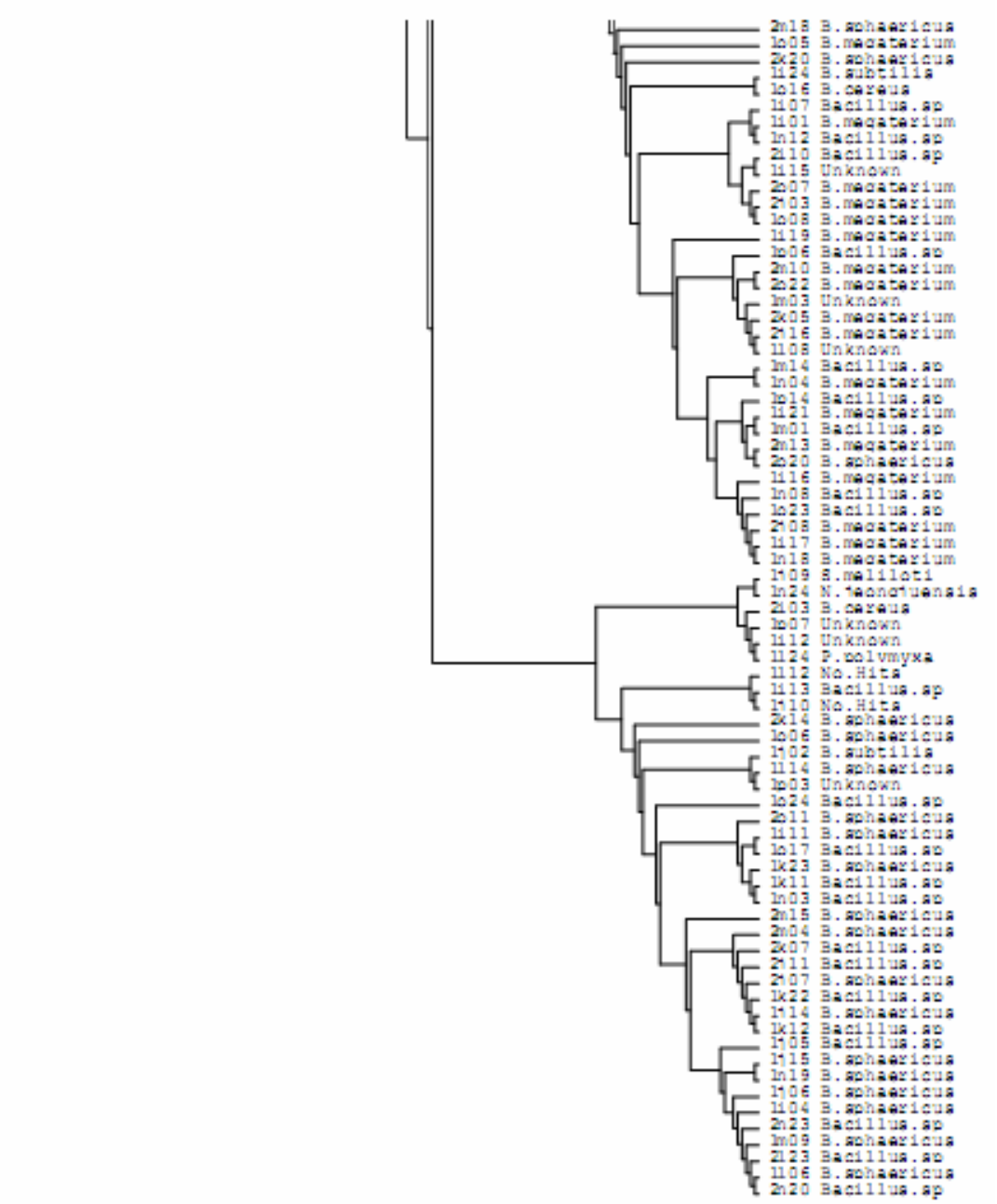
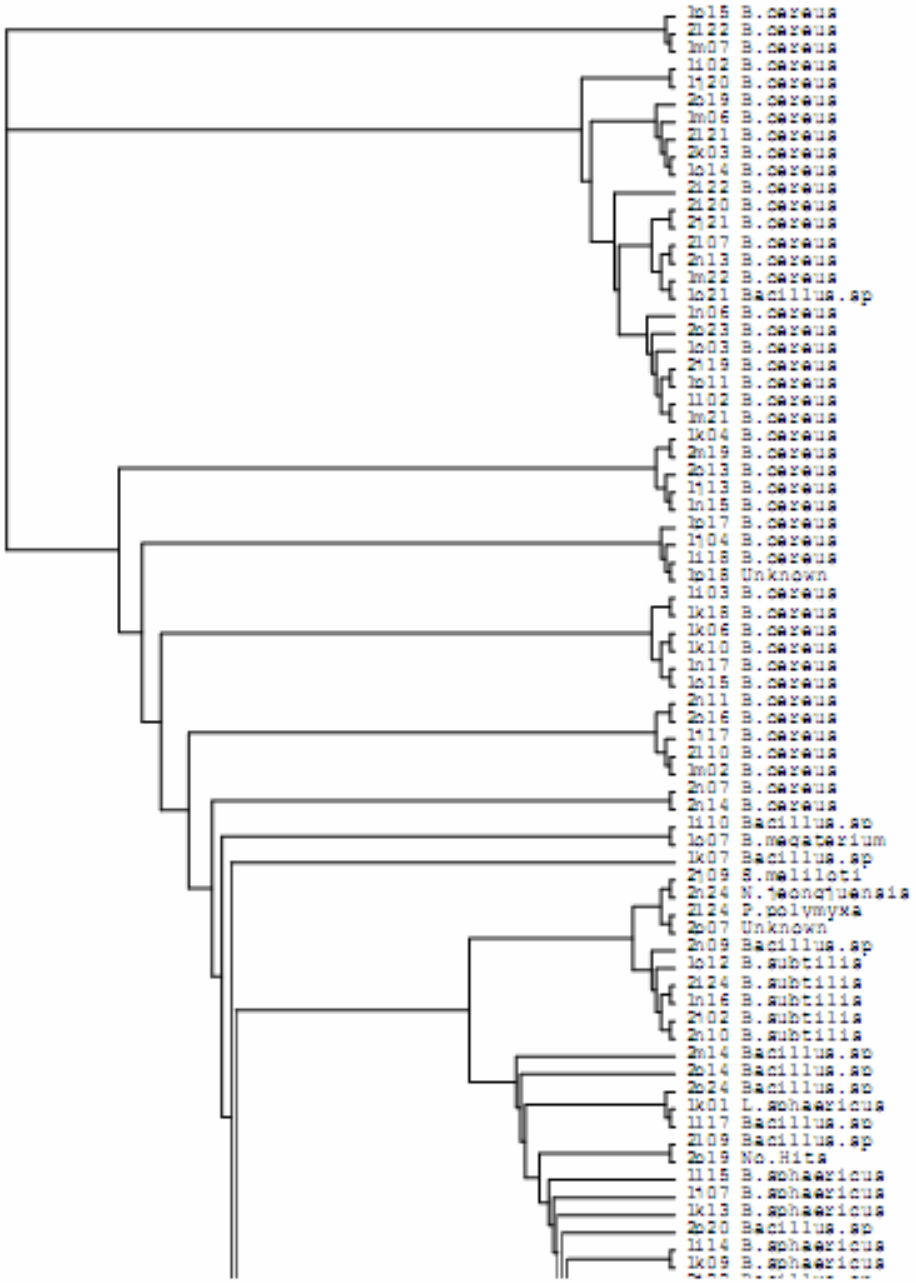


Figure A4.15: Clustal W-generated cladogram of forward 16S rDNA insert sequences from the UNK sample series.

UNK Reverse


1o15 B.cereus
2l22 B.cereus
1m07 B.cereus
1l02 B.cereus
1j20 B.cereus
2o19 B.cereus
1m06 B.cereus
2l21 B.cereus
2k03 B.cereus
1o14 B.cereus
2l22 B.cereus
2l20 B.cereus
2j21 B.cereus
2l07 B.cereus
2n13 B.cereus
1m22 B.cereus
1o21 Bacillus.sp
1n06 B.cereus
2o23 B.cereus
1o03 B.cereus
2j19 B.cereus
1o11 B.cereus
1l02 B.cereus
1m21 B.cereus
1k04 B.cereus
2m19 B.cereus
2o13 B.cereus
1j13 B.cereus
1n15 B.cereus
1o17 B.cereus
1j04 B.cereus
1l18 B.cereus
1o18 Unknown
1l03 B.cereus
1k18 B.cereus
1k06 B.cereus
1k10 B.cereus
1n17 B.cereus
1o15 B.cereus
2n11 B.cereus
2o16 B.cereus
1j17 B.cereus
2l10 B.cereus
1m02 B.cereus
2n07 B.cereus
2n14 B.cereus
1l10 Bacillus.sp
1o07 B.megaterium
1k07 Bacillus.sp
2j09 S.meliloti
2n24 N.jeonqjuensis
2l24 P.polymyxa
2o07 Unknown
2n09 Bacillus.sp
1o12 B.subtilis
2l24 B.subtilis
1n16 B.subtilis
2j02 B.subtilis
2n10 B.subtilis
2m14 Bacillus.sp
2o14 Bacillus.sp
2o24 Bacillus.sp
1k01 L.sphaericus
1l17 Bacillus.sp
2l09 Bacillus.sp
2o19 Wo.Hita
1l15 B.sphaericus
1j07 B.sphaericus
1k13 B.sphaericus
2o20 Bacillus.sp
1l14 B.sphaericus
1k09 B.sphaericus

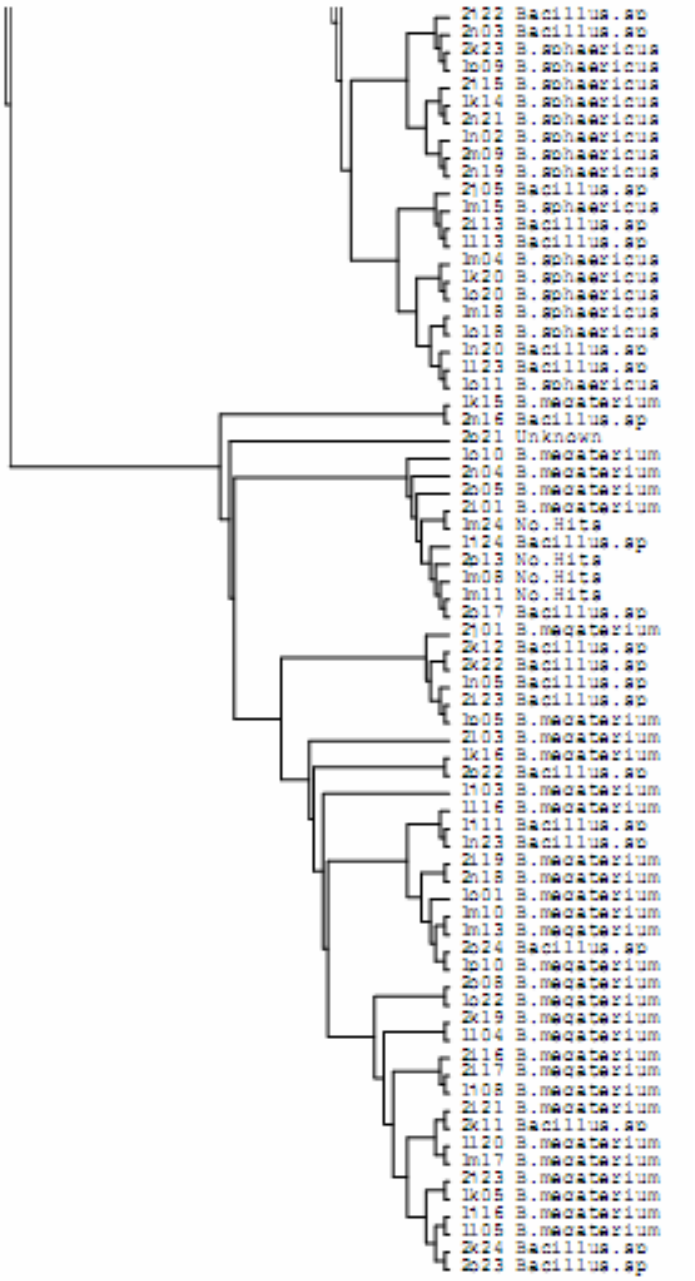


Figure A4.16: Clustal W-generated cladogram of reverse 16S rDNA insert sequences from the UNK sample series.

Appendix 5. The following figures are selected assembly and schematic diagrams of the TGGE apparatus constructed for this research. Diagrams were created using Autodesk QuickCAD 8 (Autodesk Inc., 2001).

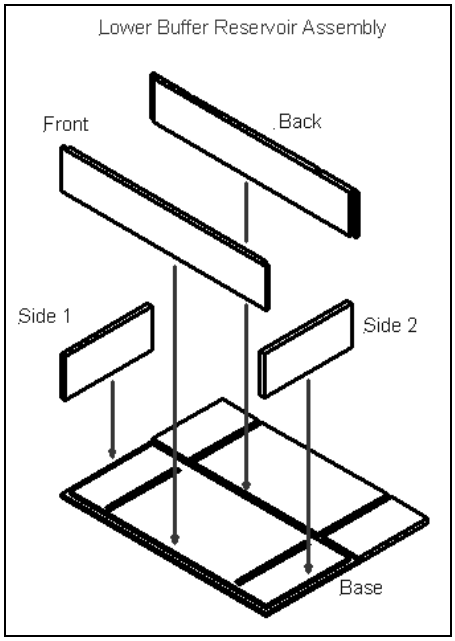


Figure A5.1: Assembly of the lower buffer reservoir of the TGGE apparatus

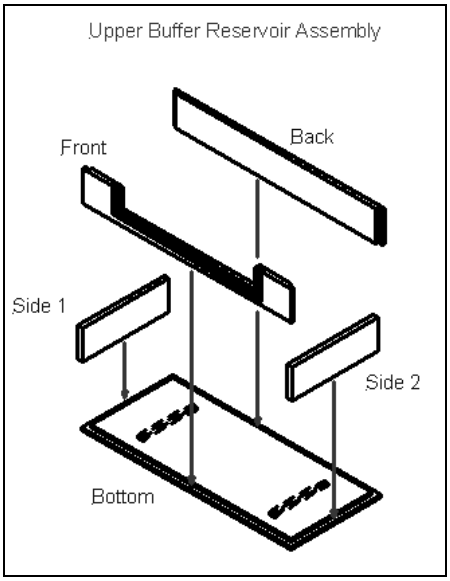


Figure A5.2: Assembly of the upper buffer reservoir of the TGGE apparatus. Note the notched front designed to mate with the notched glass plate.

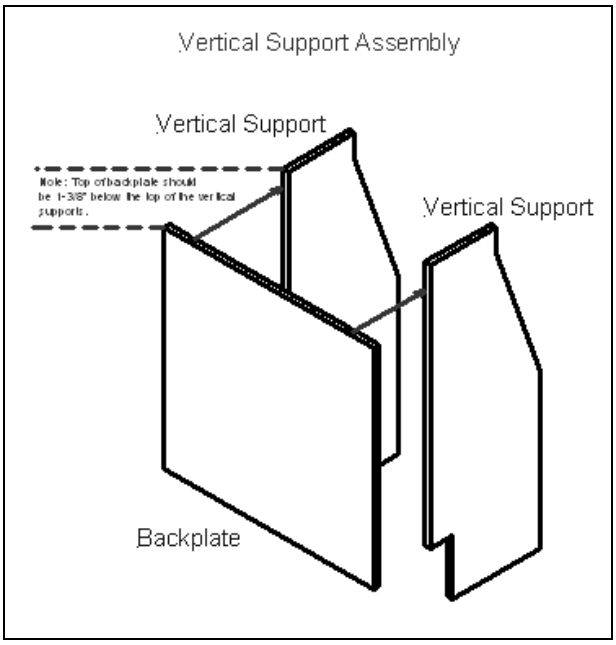


Figure A5.3: Assembly of the vertical support for the upper buffer reservoir and backplate for the gel cassette of the TGGE apparatus.

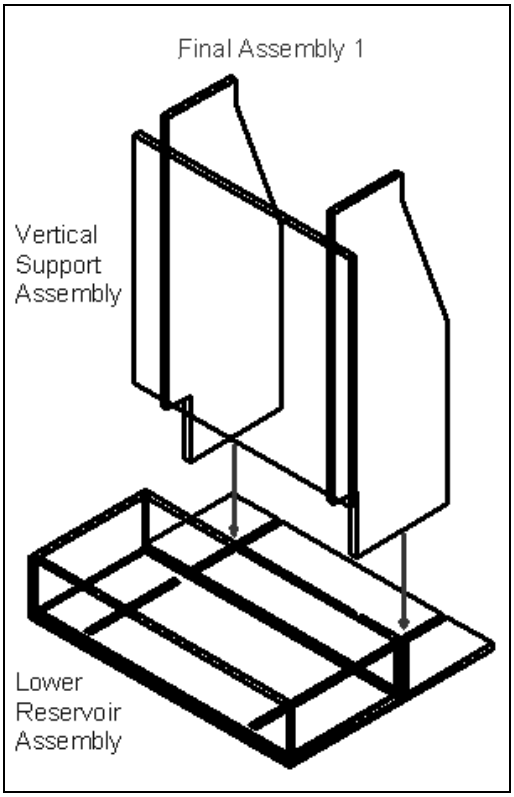


Figure A5.4: Attachment of the vertical support assembly to the lower reservoir assembly of the TGGE apparatus.

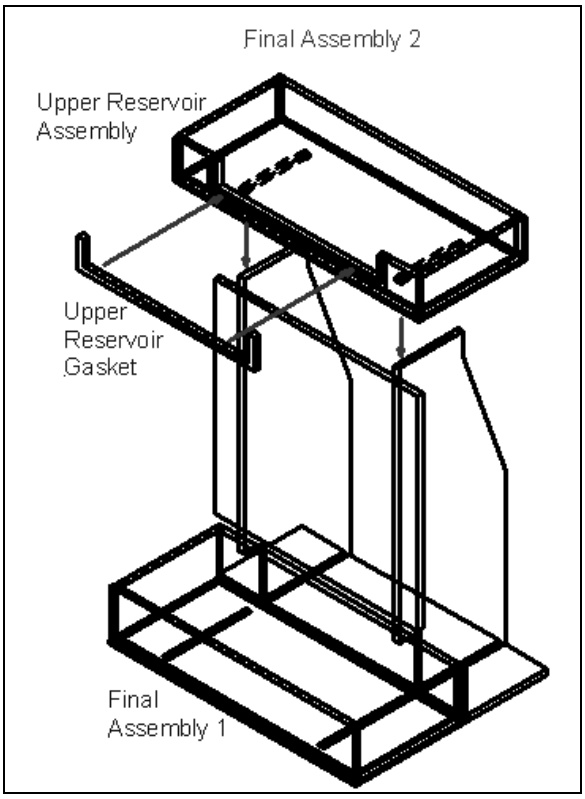


Figure A5.5: Attachment of the upper reservoir assembly to the vertical support assembly of the TGGE apparatus. Also shown is the gasket used to seal the upper reservoir to the notched glass plate.

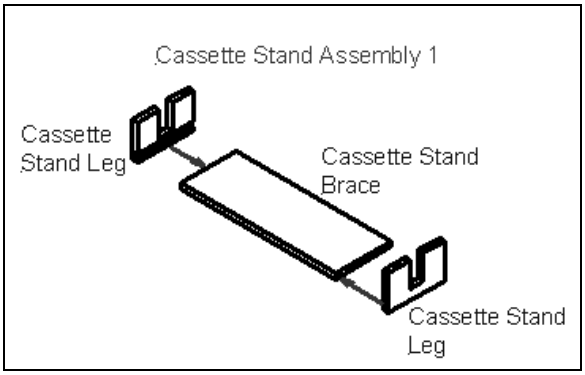


Figure A5.6: Assembly of the base of the cassette stand used to position the gel cassette at the proper height for mating of the notched glass plate with the upper reservoir assembly.

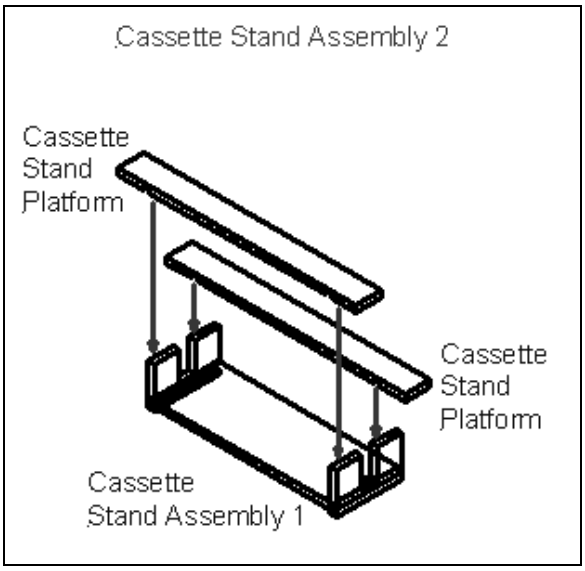


Figure A5.7: Final assembly of the cassette stand used to position the gel cassette at the proper height for mating of the notched glass plate with the upper reservoir assembly.

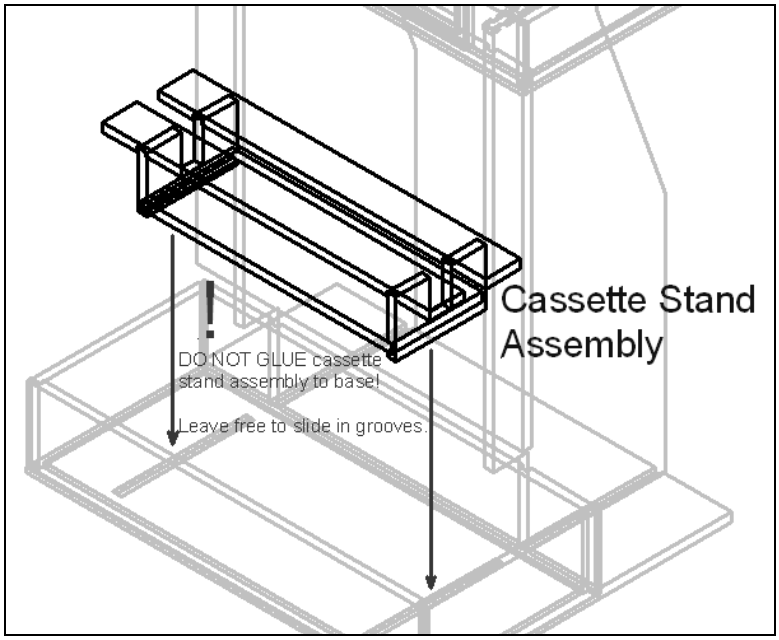


Figure A5.8: Placement of the completed cassette stand inside the lower buffer reservoir. The assembly was left unglued to allow for lateral movement as the notched glass plate was sealed against the upper buffer reservoir using woodworking clamps.

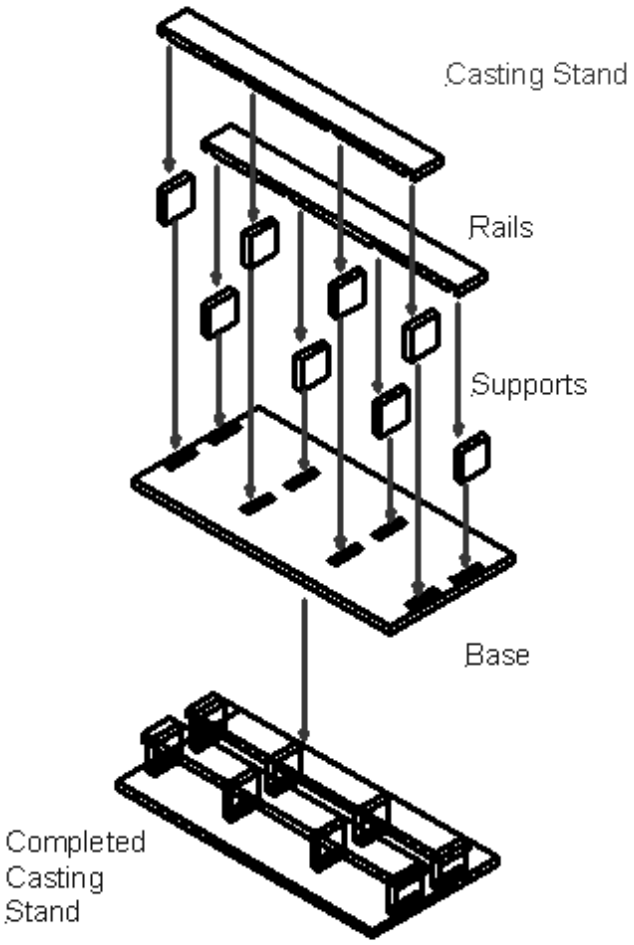


Figure A5.9: Assembly of the casting stand used to align the glass plates and thermal plates to their proper vertical positions prior to tightening of the three steel strap pairs that held the cassette firmly "sandwiched".

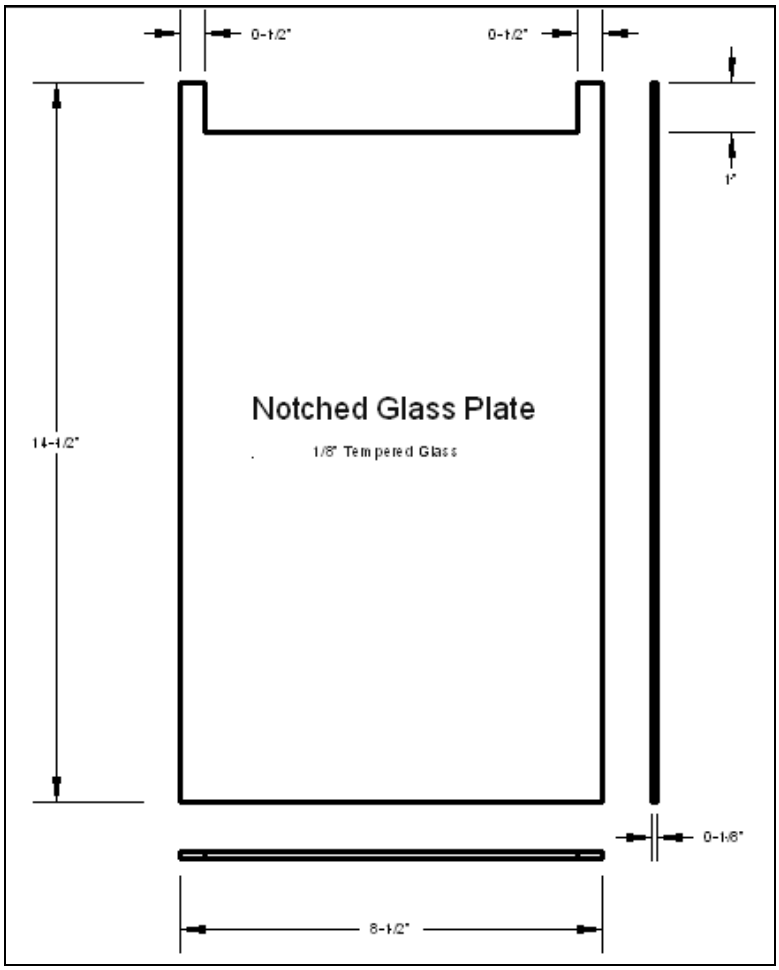


Figure A5.10: Dimensions of the notched tempered glass plates. The notch was required to allow for electrical contact between the running buffer in the upper buffer reservoir and the polyacrylamide gel between the two glass plates. A rubber gasket was used to seal the junction between the notched glass plate and the same notch cut into the front wall of the upper buffer reservoir. The glass plate, gasket, and upper buffer reservoir were held tightly together by two woodworking clamps to prevent buffer leakage.

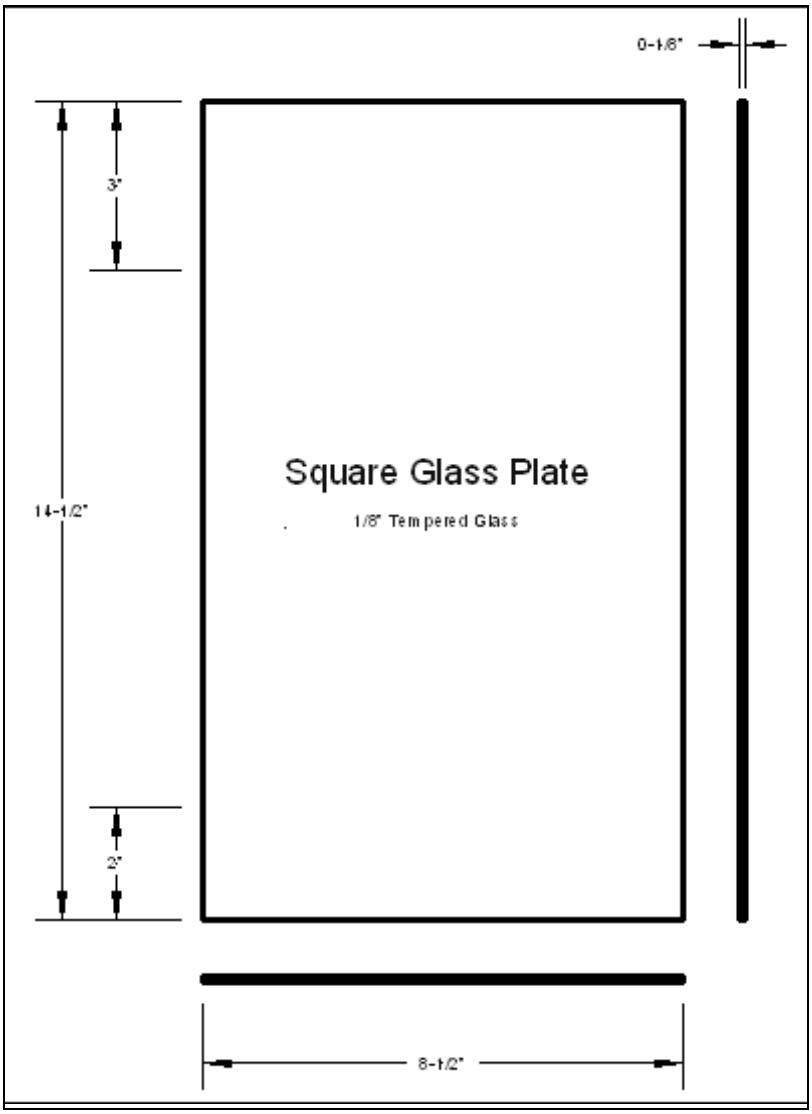


Figure A5.11: Dimensions of the square tempered glass plates. These plates had the same overall dimensions as the notched plates. Each polyacrylamide gel was poured between one notched plate and one square plate, separated by 1/16" Teflon® spacers.

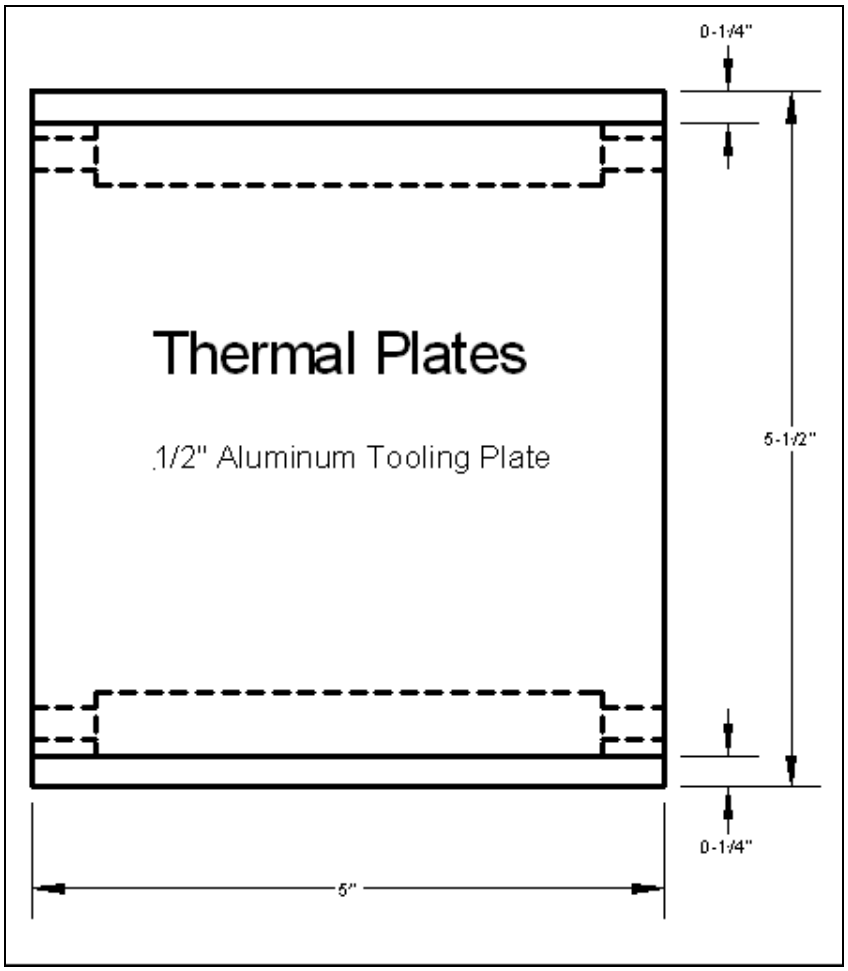


Figure A5.12: Dimensions of the thermal plates used for the TGGE apparatus. Due to the long width of these plates, it was not possible to bore a hole straight through from one side to the other. Instead, the plates were machined by routing channels along the top and bottom edges. Holes were then drilled from the sides into the channels and tapped for insertion of threaded hose barbs. The open tops of the channels were then sealed to create tubes by attaching ¼" thick aluminum caps secured with bolts. Gaskets were used between the caps and the main body of the thermal plates to prevent leakage. Except for the side in direct contact with the glass plate in the assembled cassette "sandwich", the entire surface of the thermal plate was insulated to prevent heat loss using ½" thick Rboard®.

Appendix 6. The following figures depict the electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. Dotted lines surrounding the major products denote the bands that were excised and eluted using a QBioGene® GeneClean® *Turbo* kit. Lane titles correspond to identifying numbers assigned to the bands appearing on the TGGE gel (see Figure 56). Two PCR amplifications were performed for each stabbed band; these like reactions are indicated by the designations of "PCR1" or "PCR2" following the band identification number in the lane heading. Each 100 µL PCR product volume was divided equally across three consecutive lanes. Abbreviations used for lanes containing products from known bacterial species are: *Bacillus megaterium* (BM), *Escherichia coli* (EC), *Agrobacterium rhizogenes* (AR), and *Agrobacterium tumefaciens* (AT). Abbreviations for soils used in the lane headings are as follows: Woodward (WDW), Kansas (KNS), Stillwater (STW), Pawhuska (PAW), and Goodwell (GDW). "UNK" lanes contain amplification products from TGGE bands representing the unknown bacteria which survived the surface disinfection procedure. Lanes containing PCR control reaction products are seen on the gels and designated as: *B. megaterium* positive control (BM+), no-template PCR negative control (PCR -), and a band stab negative control (Stab -), using template obtained by stabbing the TGGE gel well outside the sample lanes. Standard lanes contained GeneChoice® DNA Ladder I. Electrophoresis was performed at 200 V for 25 min using 1.2% agarose, 1X sodium borate (pH 8.5) gels containing 0.5 µg/mL EtBr.

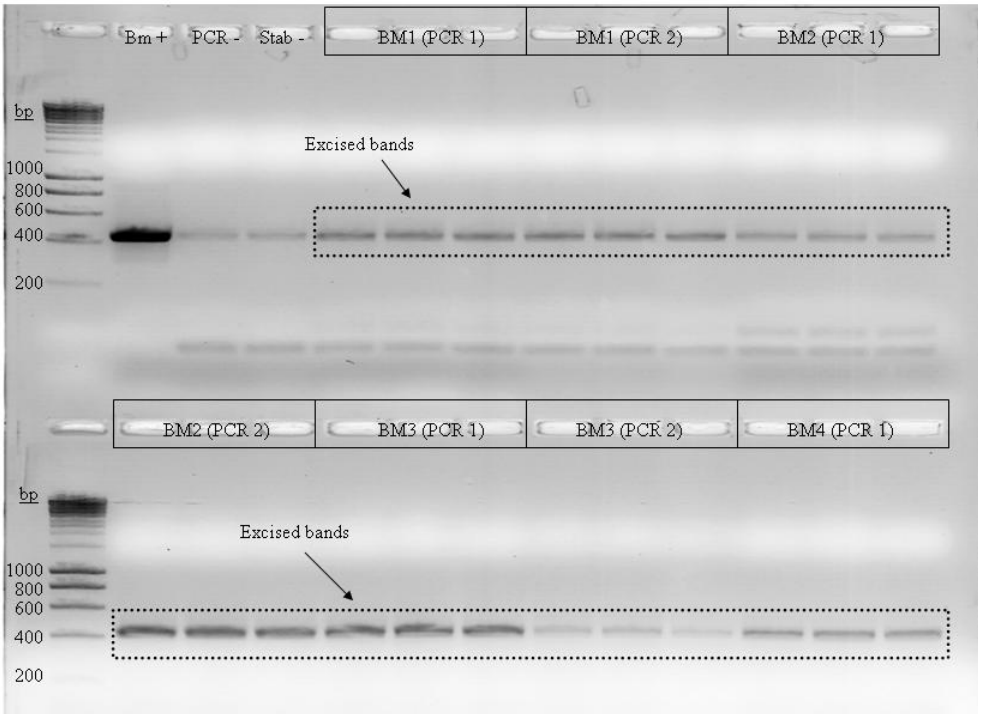


Figure A6.1: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from *B. megaterium* bands 1 through 4. Bands excised and eluted are indicated by dotted lines.

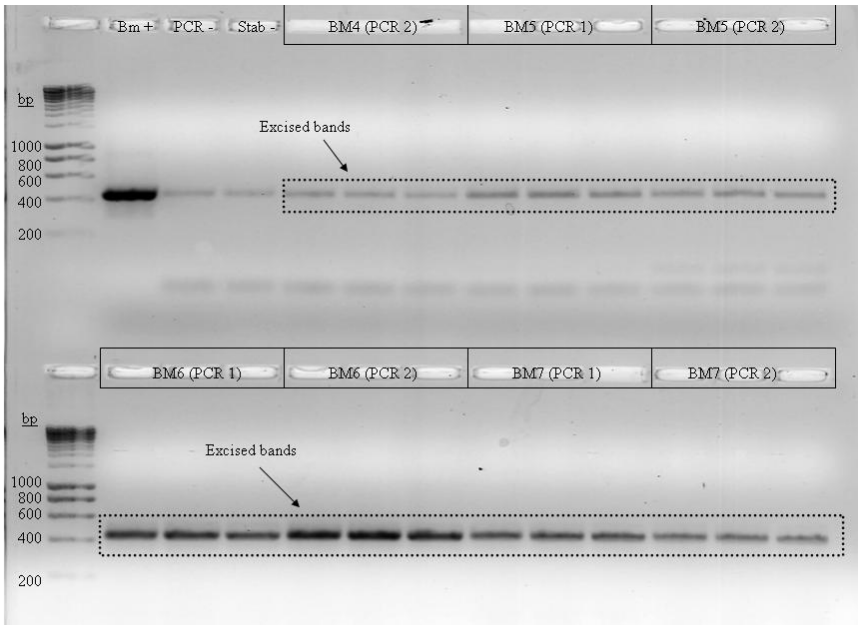


Figure A6.2: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from *B. megaterium* bands 4 through 7. Bands excised and eluted are indicated by dotted lines.

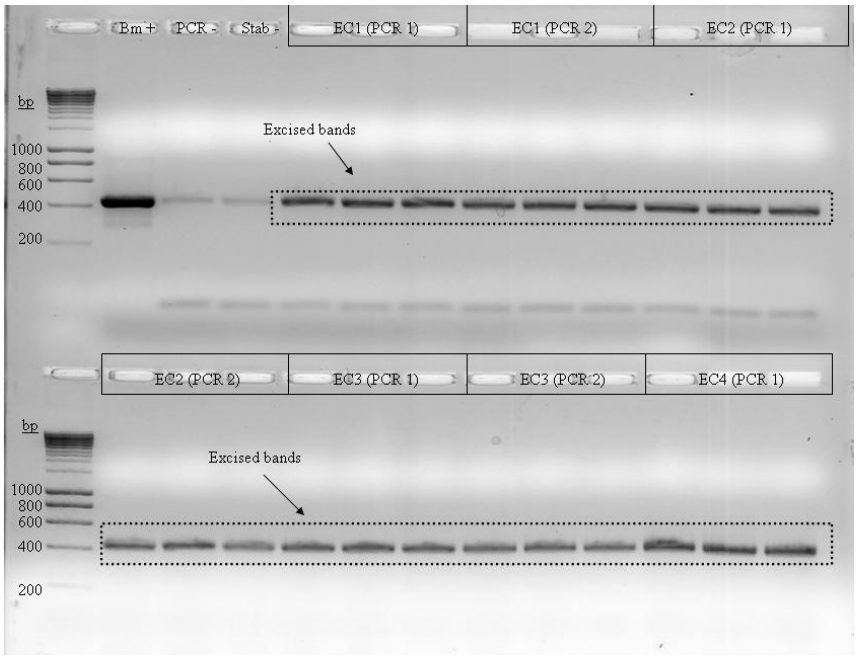


Figure A6.3: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from *E. coli* bands 1 through 4. Bands excised and eluted are indicated by dotted lines.

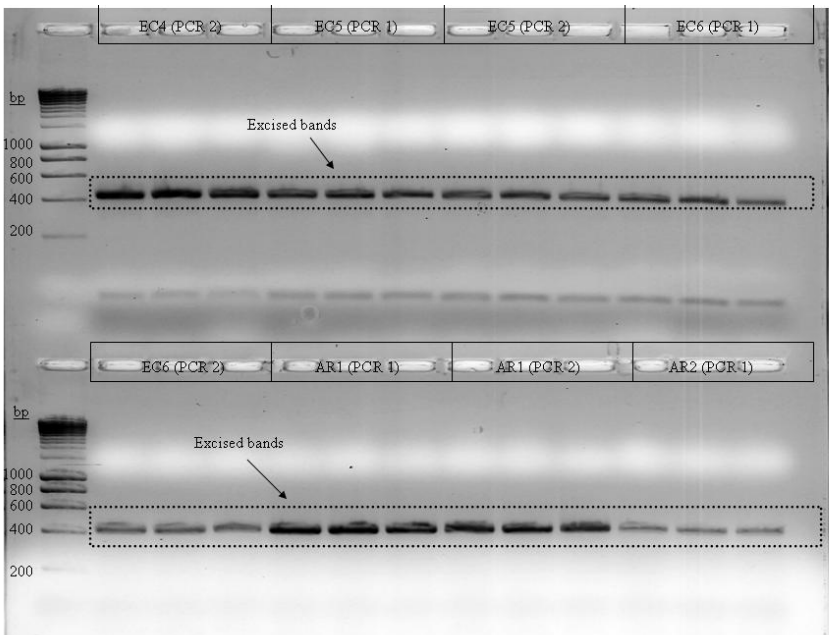


Figure A6.4: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from *E. coli* bands 4 through 6 and *A. rhizogenes* bands 1 and 2. Bands excised and eluted are indicated by dotted lines.

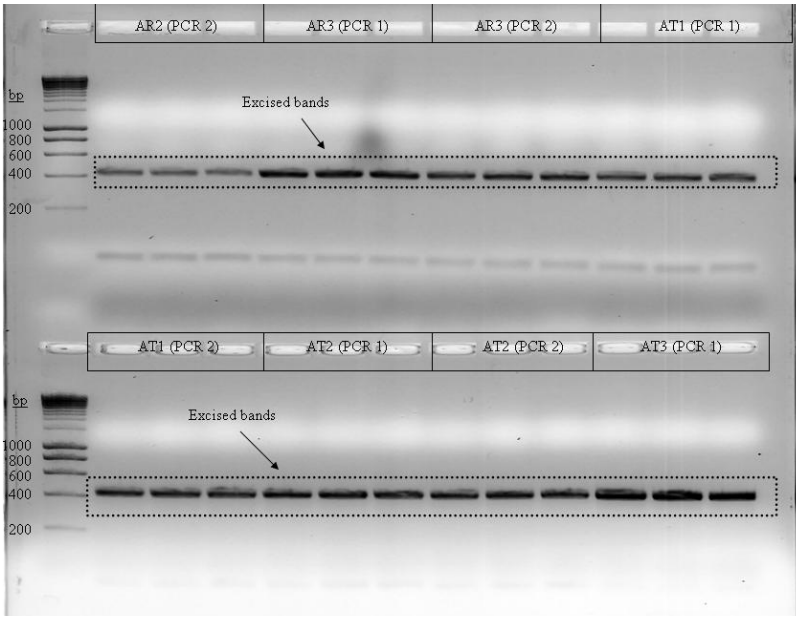


Figure A6.5: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from *A. rhizogenes* bands 2 and 3 as well as from *A. tumefaciens* bands 1 through 3. Bands excised and eluted are indicated by dotted lines.

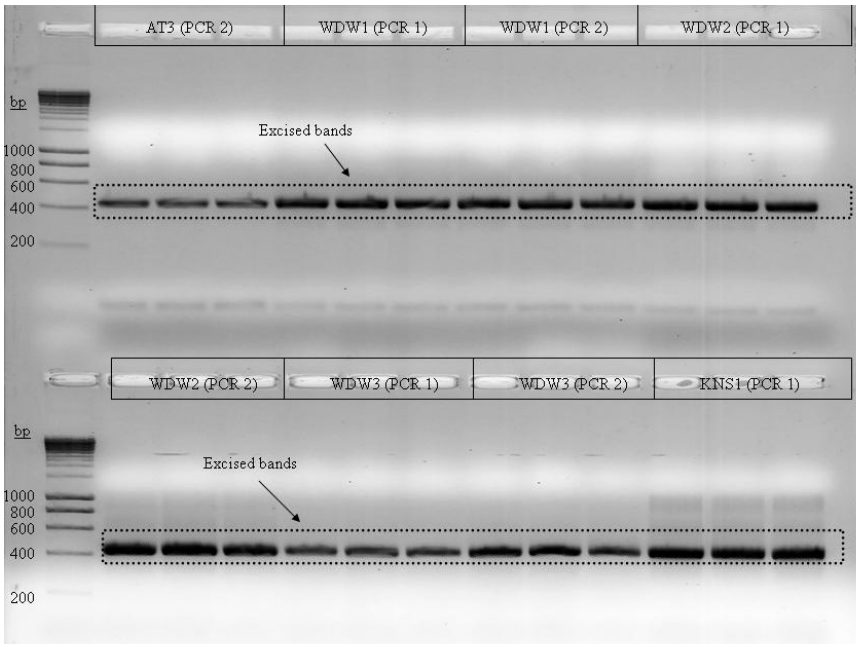


Figure A6.6: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from *A. tumefaciens* band 3, Woodward bands 1 through 3, and Kansas band 1. Bands excised and eluted are indicated by dotted lines.

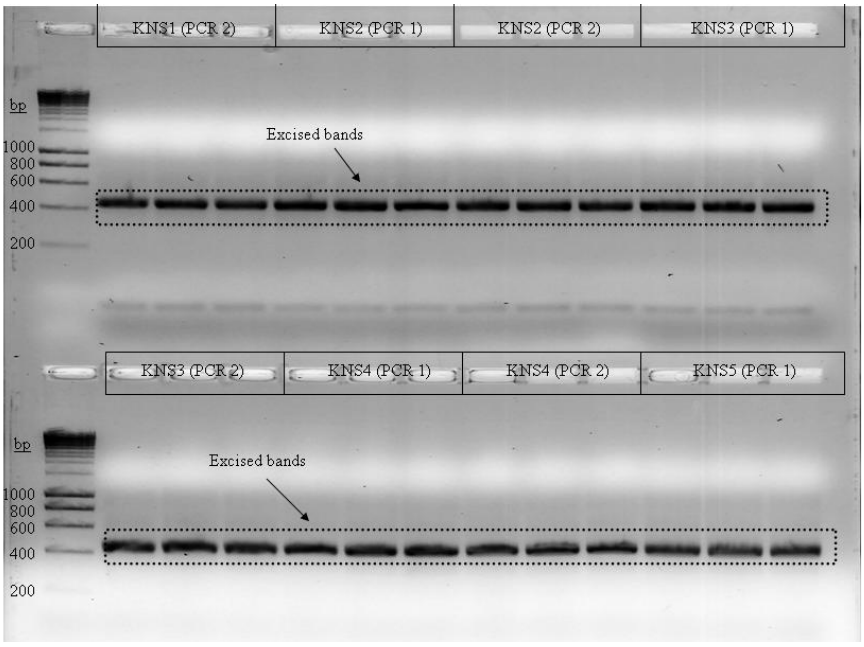


Figure A6.7: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from Kansas bands 1 through 5. Bands excised and eluted are indicated by dotted lines.

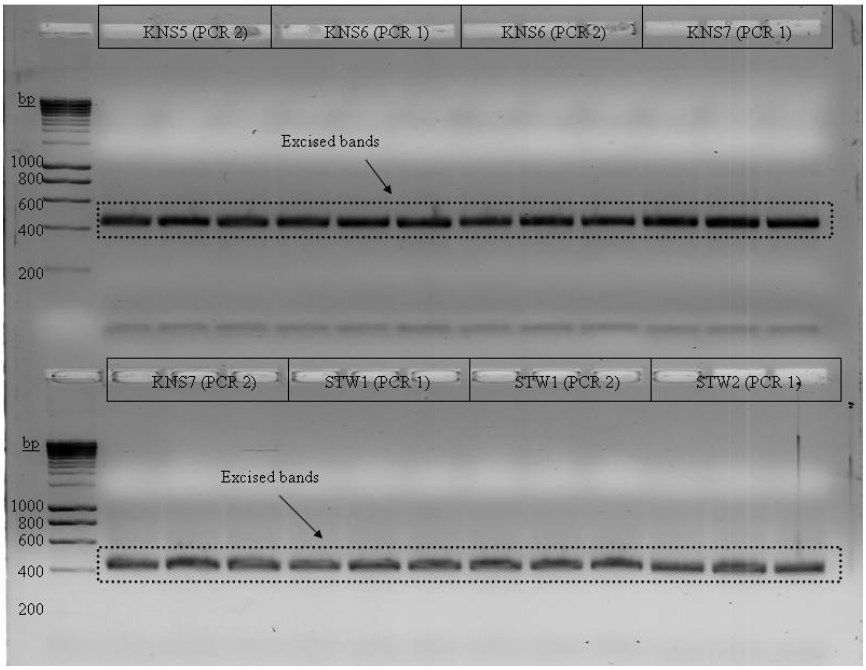


Figure A6.8: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from Kansas bands 5 through 7 and Stillwater bands 1 and 2. Bands excised and eluted are indicated by dotted lines.

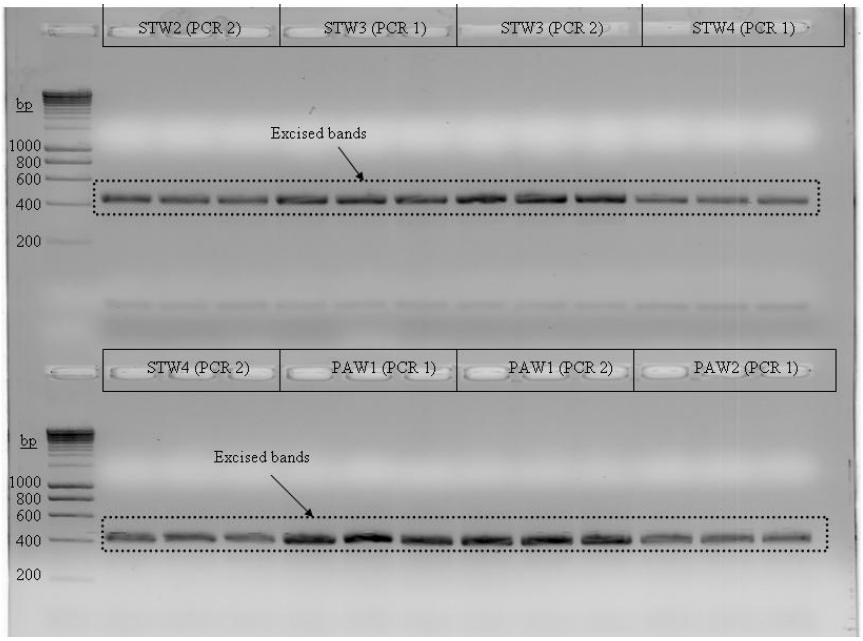


Figure A6.9: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from Stillwater bands 2 through 4 and Pawhuska bands 1 and 2. Bands excised and eluted are indicated by dotted lines.

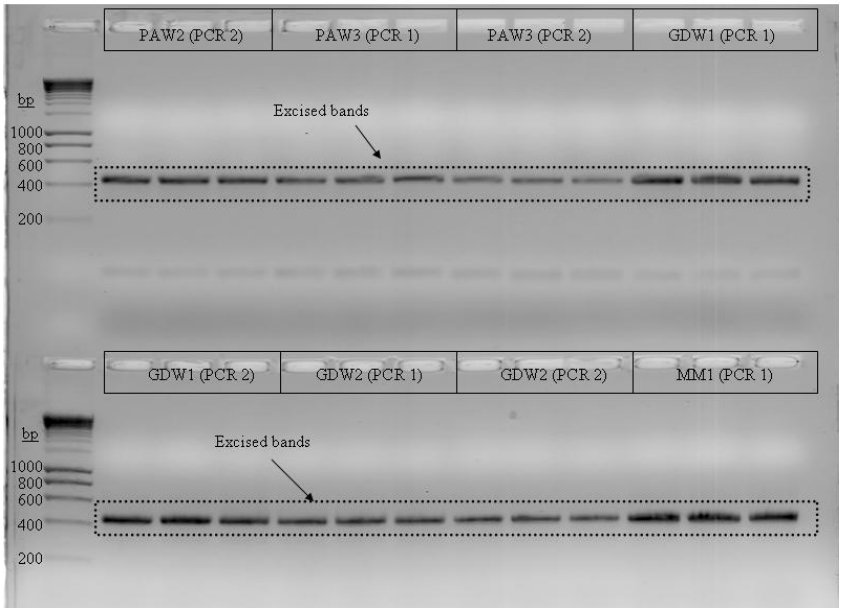


Figure A6.10: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from Pawhuska bands 2 and 3, Goodwell bands 1 and 2, and MetroMix® 366 band 1. Bands excised and eluted are indicated by dotted lines.

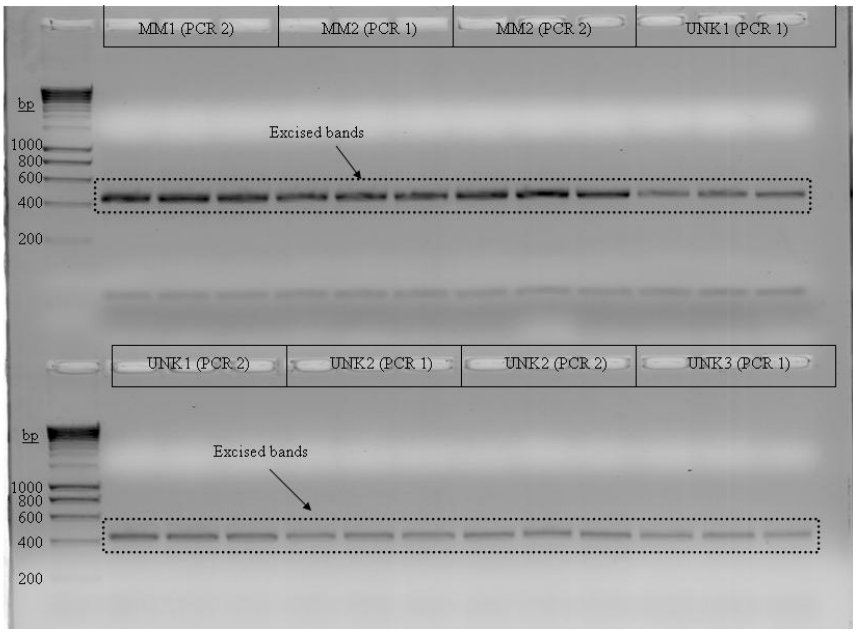


Figure A6.11: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from MetroMix® 366 bands 1 and 2, and bands 1 through 3 from the unknown bacteria surviving surface disinfection. Bands excised and eluted are indicated by dotted lines.

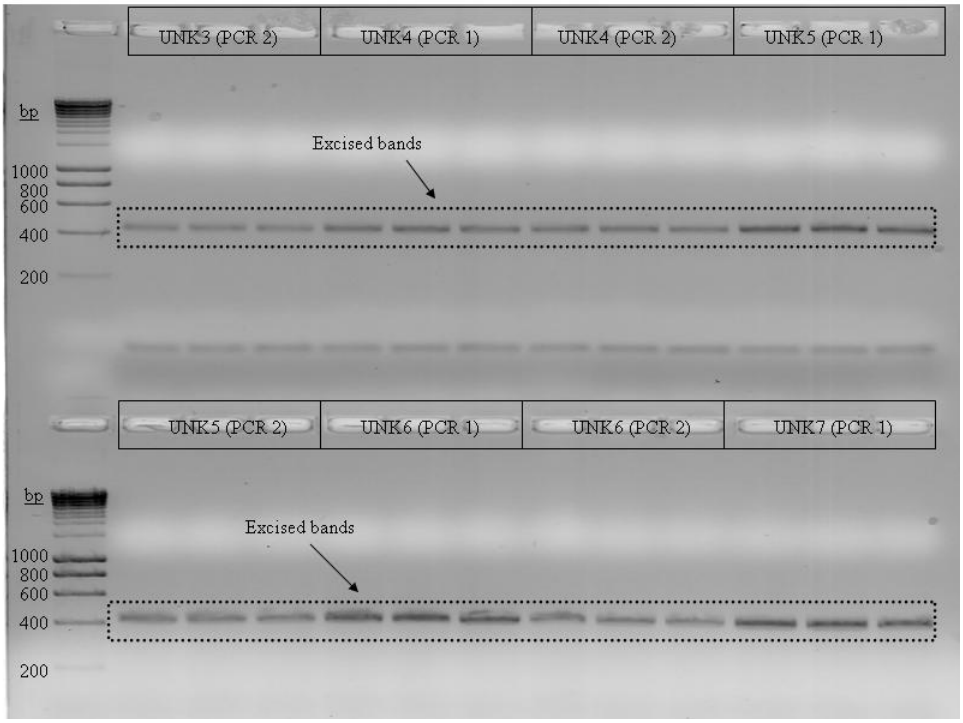


Figure A6.12: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from bands 3 through 7 from the unknown bacteria surviving surface disinfection. Bands excised and eluted are indicated by dotted lines.

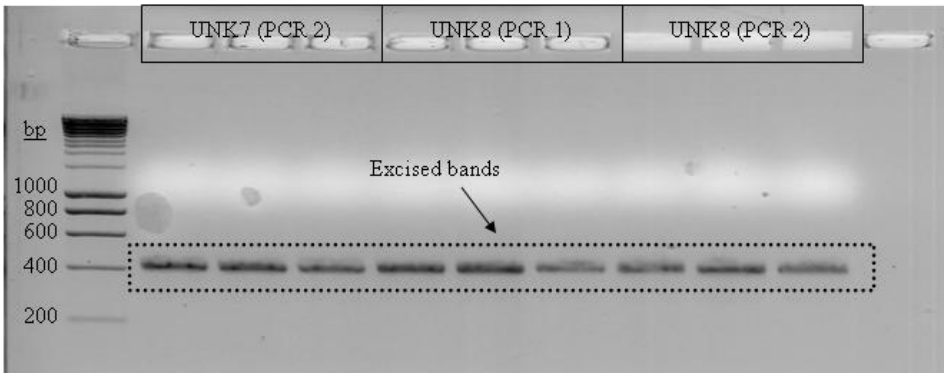


Figure A6.13: Electrophoresis and excision of products produced by PCR amplification of individual TGGE gel bands. This gel contained amplification products from bands 7 and 8 from the unknown bacteria surviving surface disinfection. Bands excised and eluted are indicated by dotted lines.

VITA

James Neil Enis

Candidate for the Degree of

Master of Science

Thesis: COMPOSITION AND DIVERSITY OF *MEDICAGO TRUNCATULA* ROOT BACTERIAL ENDOPHYTE POPULATIONS RESULTING FROM GROWTH IN DIFFERENT OKLAHOMA SOILS

Major Field: Plant and Soil Sciences

Biographical:

Personal Data: Born in McAlester, Oklahoma, USA, on May 5, 1975, to Jim D. and Linda S. Enis.

Education: Associate of Science from Eastern Oklahoma State College, Wilburton, Oklahoma, 1999; Bachelor of Science from Oklahoma State University, Stillwater, Oklahoma, 2001. Completed the requirements for the Master of Science degree with a major in Plant and Soil Sciences at Oklahoma State University, Stillwater, Oklahoma in July, 2008.

Experience: Tutor, Student Support Services, Eastern Oklahoma State College 1997-1999; Undergraduate Research Assistant, Department of Plant and Soil Sciences, Oklahoma State University 1999-2001, Lew Wentz Fellow, Oklahoma State University 2000-2001; Internship, Samuel Roberts Noble Foundation, Plant Biology Division 2001; Research Assistant, Department of Plant and Soil Sciences, Oklahoma State University 2001-2003; Research Specialist, Department of Entomology and Plant Pathology, Oklahoma State University 2003-2005; Chair, Department of Horticulture, Eastern Oklahoma State College 2006-2008; Chair, Department of Chemistry and Microbiology, Eastern Oklahoma State College 2008-Current.

Professional Memberships: North American Colleges and Teachers of Agriculture

Name: James Neil Enis Date of Degree: July, 2008

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: COMPOSITION AND DIVERSITY OF *MEDICAGO TRUNCATULA* ROOT BACTERIAL ENDOPHYTE POPULATIONS RESULTING FROM GROWTH IN DIFFERENT OKLAHOMA SOILS

Pages in Study: 238 Candidate for the Degree of Master of Science

Major Field: Plant and Soil Sciences

Scope and Method of Study: Differences in composition and diversity of the *Medicago truncatula* root bacterial endophyte population resulting from growth in six diverse soils and one commercial growing medium were evaluated by cloning and sequencing of 16S rDNA amplification products and temperature gradient gel electrophoresis (TGGE) of 16S rDNA fragments.

Findings and Conclusions: Cloning and sequencing of 16S rDNA revealed 36 genera of bacteria encompassing five phyla as putative *M. truncatula* root endophytes. Plants grown in two acidic soils with a forest background and a commercial growing medium possessed the highest endophyte diversity. Moderate diversity was observed in plants grown in three managed agricultural soils. Endophyte diversity was lowest in plants grown in soil collected from an undisturbed native tallgrass prairie. TGGE failed to adequately resolve the endophytic bacterial 16S rDNA fragments and 16S rDNA bands on the silver-stained TGGE gel could not be sequenced or cloned into plasmid vectors for identification.

ADVISER'S APPROVAL: Michael P. Anderson