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Title: Molecular Characterization of Bacterial Populations Implicated in the Anaerobic Metabolism of Toxic Plant Alkaloids from Two Different Experimental and Environmental Sources.

Abstract approved:

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A. Morrie Craig

There have been many studies that describe the protective degradation or metabolism of potentially harmful plant toxins, such as, mimosine from *Leucaena leuconcephala,* pyrrolizidine alkaloids from tansy ragwort (*Senecio jacobaea*), oxalate and some mycotoxins by rumen microbes. There are many cases of plant-related toxicoses suffered by ruminant animals where there are no microbes known to offer protection. Ergot alkaloids produced by the endophytic fungus *Neotyphodium coenophialum* have been shown to cause the syndromes of fescue foot and summer slump in animals grazing tall fescue (*Festuca arundinacea*) grass varieties that carry the endophyte. Economic losses due to these diseases have been estimated to be about $1 billion per year. For this reason, it is economically desirable to find one or more microbes

that could be introduced into the rumen environment to protect animals from fescue toxicosis and pyrrolizidine alkaloid toxicosis. Studies that have found protective microbes focused on identification of those microbes using mainly phenotyping and enrichment or isolation techniques. In the studies presented here, two different experimental and environmental systems were examined. Culture-independent molecular biology techniques were used to take advantage of differences found between bacteria in the 16S small ribosomal subunit gene as a means of characterizing diversity. Analytical chemistry techniques were used to place diversity observations in the context of function.

Microbes within a consortia culture derived from the rumen of a sheep were previously shown to metabolize pyrrolizidine alkaloids found in the invasive weed, tansy ragwort. Thin layer chromatography was used to monitor microbial activity. The bacterial enrichment was characterized by molecular cloning techniques and by the molecular fingerprinting technique of denaturing gradient gel electrophoresis (DGGE). Phylogenetic analysis of the enrichment revealed that the consortium is composed of no more than five putative bacterial species which associated to the *Anaerovibrio, Desulfovibrio, Megasphaera, Prevotella,* and *Synergistes* generas. The DGGE results were directly compared to the cloning data by amplifying eight phylogenetically representative clones by polymerase chain reaction (PCR) and analyzing them by DGGE. Direct DGGE analysis of the enrichment displayed greater 16S diversity than the clone library used in this study, suggesting that at least one of the organisms present in the enrichment comprises less than 1% of the total

cell population.

Earthworms of the species *Eisenia fetida* are commonly used in the practice of verimicomposting. This is the process by which worms are used in a closed bin or pit, to aid in the breakdown of manure and other agricultural waste in order to generate highly fertile soil from the worm's digestive waste products, or cast. The worms were used in a two-treatment vermicompost experiment. One treatment was given tall fescue seed assayed to contain over 10,000 parts per billion of the endophyte toxin ergovaline (E+ treatment). The other treatment was used as a control and was given tall fescue seed with ergovaline amounts that fell below analytical levels of detection (E- treatment). Many of the worms originally introduced into the E+ treatment died off before a population of worms established itself as a productive vermicompost system. The success of this population of worms may be due to the presence of beneficial or protective microbes within their digestive tract. Digestive tracts were dissected and collected from worms of each treatment in February of 2007. The guts were homogenized and anaerobically cultured with and without added ergovaline. The disappearance of ergovaline and the stereo-isomer ergovalinine from worm-gut cultures was monitored daily by high-performance liquid chromatography (HPLC). DNA was purified from the same gut homogenate samples used for incubation experiments. Cloning of the 16S gene was applied to determine the identity of the bacteria present in the treatments during the month of February. The computational program Mr. Bayes was used to group restriction-fragment length polymorphism (RFLP)

patterns based on their uniqueness. The program DOTUR was used to estimate the level of diversity based on ribotype distances. With the aim of encompassing the fullest amount of represented sequence diversity, these data were used to specifically select 192 clones from each 960 clone library. Selected clones were analyzed by sequencing the 16S insert. Clone sequences from both treatments made associations to bacterial 16S reference sequences in the phyla *Actinobacteria*, *Bacteroidetes, Chloroflexi, Firmicutes, Planctomycetes, Alpha, Beta, Gamma, Delta* and *Epsilon proteobacteria, Verrucomicrobia* and unclassifed bacterial sequences. Four clones associated best with a plastid sequence derived from a green algae species. The global similarity between libraries indicated that the populations were not significantly different at the time of sampling. HPLC data showed no observable loss of ergovaline from incubations of E+ or E- worm treatment samples taken in February. Incubation experiments in December of 2006 using only E+ treatment worm guts had demonstrated the potential for microbial metabolism of ergovaline; however, no molecular work was done at that time.

An integrative approach was taken to describe the function and bacterial composition of the two systems studied. The use of analytical chemistry techniques placed the observations of microbial diversity into context. Thin-layer chromatography was used to check that the ability of the pyrrolizidine alkaloid-degrading enrichment was still intact after being revived from long-term storage. This allowed for direct comparisons to earlier data published about the identity of microbes within these enrichments. HPLC data

indicated that worm-gut homogenates characterized in February did not demonstrate the same metabolic activity as was observed during an incubation of gut material taken from E+ treatment worms in December. Since there was no previous molecular data to compare potential differences in bacterial diversity from these two time points, the molecular data collected in February will be used as a reference of the background population at a time when there was no metabolism of ergovaline. It is intended that the results of these studies will further the understanding of which microbes are involved in the metabolism of the particular compound of interest. In this way, steps may be made toward refining the culturing and propagation of desirable bacteria that may potentially serve as a treatment for ruminant animals challenged by the presence of either pyrrolizidine or ergot alkaloids in their diets.

Molecular Characterization of Bacterial Populations Implicated in the Anaerobic Metabolism of Toxic Plant Alkaloids from Two Different Experimental and Environmental Sources.

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Rogan MacKay Rattray

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I understand that my thesis will become part of the permanent collection of Oregon Sate University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Rogan MacKay Rattray, Author

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1.1. Introduction.

The concept of bacterial degradation of compounds that are deemed pollutants or toxicants has been widely accepted by microbiologists [1; 9]. For example, the explosive, trinitrotoluene (TNT), was one of the most prevalently produced munitions during the World War I and II eras. Clean-up costs for large TNT contaminated sites are estimated to be upwards of $800 per ton of soil [40]. Early work demonstrated that microbes could potentially be used as a means for TNT degradation [14; 68]. This ability may be used as a biotechnology application to alleviate environmental contamination problems due to this compound. The nitro groups of TNT give the molecule a fairly high redox potential. This property of TNT makes aerobic metabolism an inefficient means of bioremediation. More recent work has shown that microbial metabolism of TNT is best achieved under anaerobic conditions [21; 40; 61]. These researchers examined anaerobic cultures for reduced metabolites of TNT, i.e., the nitro groups of TNT had been converted into amino moieties by reduction and hydrolysis. Another study isolated a *Clostridium bifermentans* species capable of degrading TNT to triaminotoluene (TAT)[62]. The reduction of TNT by anaerobic microbes from the rumen has also been studied [39]. Fleischmann et al. (2004) could not detect any metabolites of TNT more reduced or degraded than TAT and no microbes were isolated or characterized. The reduction of TNT under anaerobic conditions has been attributed to microbes from many different ecologies. The current consensus of opinion is that it is a very ubiquitous ability. Much of the current evidence

available indicates that the activity may be transiently attributed to the production of enzymes in the nitroreductase family [13; 35; 44; 62; 94].

1.2. Degradation of mimosine, oxalate, and mycotoxins by rumen microbes.

Some metabolic abilities of bacteria have been shown to improve human and animal health. There have been studies that describe the protective degradation or metabolism of potentially harmful plant toxins by rumen microbes [3; 17; 23; 24; 27; 54; 59; 82] . The toxin mimosine is found in the tropical legume *Leucaena leuconcephala*. Mimosine is converted to the more toxic forms, 3-hydroxy-2(1H)-pyridone (2, 3 DHP) and 3-hydroxy-4(1H)-pyridone in the rumen. *Leucaena* has been investigated for its practical use as forage for ruminant animals in place of grains or straws, which are more expensive and/or difficult to acquire by animal producers in regions where *Leucaena* grows naturally. The observation was made that goats native to places where *Leucaena* grew were able to eat the plant whereas domestic animals introduced to the same areas became ill and in some cases died [54]. Since it is commonly accepted that the bacteria that inhabit the rumen are responsible for the metabolism of the plant matter consumed by the animal, investigations were performed to determine the observed difference between native and domestic livestock and possible solutions to the problem. In work by Jones and Megarrity (1986)[54], successfully conveyed resistance to *Leucaena* toxicosis by transferring whole rumen fluid from Hawaiian goats to Australian cattle. Later work by Dominguez-Bello (1997) demonstrated that Venezuelan sheep appeared to have a native population of rumen bacteria adapted to the metabolism of mimosine as well as 2,3 DHP and 3,4 DHP. In this study, she also made the observation that feeding *Leucaena* to sheep increased the number of both Gram-positive and Gram-negative rods observed in the rumen fluid. A number of isolates were also obtained, many of

which could degrade 2, 3 DHP but only a few of which could also degrade 3, 4 DHP. After work was reported on the development of a better cultivation media for mimosine-degrading bacteria, Allison et al. (1992) reported the isolation of a single species of bacteria which he called *Synergistes jonesii.* They reported that this newly isolated rumen bacteria was capable of converting 3, 4 DHP into 2, 3 DHP before clearing it from culture [3]. Dominguez-Bello later reported on the 2,3 DHP degradation kinetics of this species and made progress toward characterizing the pathway this organism used to metabolize these toxins [27; 82].

Similar cases for the implication of rumen bacteria in the degradation of harmful natural toxins have been shown. These include the plant toxin oxalate. Unlike *S. jonesii,* which was isolated from enrichments made directly from sheep and goats adapted to *Leucaena*, oxalate-degrading rumen bacteria were isolated from populations derived from sheep, but enriched for in a chemostat. Further refinements of this chemostat enrichment led to the isolation and classification of the bacterium named *Oxalobacter formigenes* [17; 23; 24]. Later work with this organism revealed that it uses oxalate hydrolysis to generate a negative electrical potential between its inner and outer membranes, which it then uses in an indirect manner, to generate ATP through proton motive force [59].

The metabolism of different mycotoxins potentially encountered by ruminants has also been investigated. Kiessling et al. (1984) found that the mycotoxins zearalenone, T-2 toxin, diacetoxyscirpenol and deoxynivalenol were well metabolized by whole rumen fluid; whereas aflatoxin B1 and ochratoxin A were not [57]. Westlake et al. (1987) investigated the effects of these mycotoxins in addition to Verrucarin A, on the growth rate of *Butyrivibrio fibrisolvens* specifically. They found that this organism was able to degrade all but aflatoxin B1 and that none of the toxins tested inhibited *B. fibrisolvens* growth [106].

1.3. Metabolism of Pyrrolizidine Alkaloids by Ruminal Microbes.

The presence of hepatotoxins in the pyrrolizidine alkaloid (PA) family is a challenge to many domestic animal species and humans world-wide [66; 96; 110]. These toxins elicit a condition known as seneciosis, with clinical signs that range from acute to chronic, and that often manifest as veno-occlusive liver disease [95]. The toxicity of these molecules occurs through the covalent binding of dehydroalkaloid moieties produced by the liver to negatively charged biomolecules such as amino and nucleic acids. The binding of PA metabolites to biomolecules can lead to potential disruption of cell function or cell death [109].

In South Africa, 5 – 15% of all plant poisonings of livestock is seen as seneciosis. Thus, it is the third most prevalent cause of plant poisoning or mycotoxicoses in that region [56; 72]. In the Northwest United States, plants containing these toxins are grow along side other livestock range forage. In the past, economic loss to Northwest livestock producers has been as high as $20 million annually because of PA poisoning [19]. Although many developed agricultural countries appear to be controlling the presence of the plants that contain PAs, there are still apparent hot-spots and outbreaks of these plants world-wide. Therefore, seneciosis poses a potentially serious and significant problem for livestock producers now and in the future.

Research has shown that not all livestock animals are affected equally by PAs [31; 48]. In studies where sheep or goats were challenged by the presence of PA-containing tansy ragwort (*Senecio jacobaea*) in their forage, it was observed that levels as high as 200% of the animal's body-weight could be consumed with no observable clinical effects to these animals [18; 47]. Other studies have shown that cattle and horses can only consume 5% – 10% of their body weight in tansy before manifesting clinical signs of PA toxicosis [18; 47]. Because the toxins produced by tansy have a very similar chemistry

and toxicology to mimosine, this lab has investigated rumen microbes that might be responsible for the resistance that local sheep tended to exhibit toward the consumption of these plants. Initially investigators examined the rate of disappearance of PAs incubated with whole rumen fluid from sheep, goats and cattle. For those experiments, live and sterilized rumen fluid from different animals was spiked with pure PA extracts from tansy. The disappearance of PAs was assayed using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). It was demonstrated that the rumen fluid from sheep and goats had microbes capable of metabolizing a solution of mixed PAs from tansy beyond the detection limits of the assays in less than four hours of incubation [102]. Work to cultivate only the microbe(s) responsible for the metabolism of tansy PAs was performed *in vitro* using an enrichment media with the addition of tansy PAs. This cultivation method was similar to those used to cultivate rumen microbes that could degrade the plant and mycotoxins discussed above. The result of the tansy microbe enrichment study was a transferable bacterial culture that could metabolize PAs; however, the rate of metabolism was slightly slower than incubation of PAs in whole rumen fluid (six hours of incubation compared to four). The isolation of this consortium of bacteria was the follow-up to the original observation that tansy PAs were metabolized faster in sheep rumen fluid compared to rumen fluid from cattle (four hours and twenty-four hours respectively). The isolation was achieved using established anaerobic media making methods, with trial and error culturing of whole rumen fluid dilutions [64].

Once the least diverse enrichment of bacteria that could still metabolize PAs was established, work was done to identify the microbes in the enrichment. In that work, the 16S rRNA gene was used as the marker for molecular analysis. The techniques of length heterogeneity PCR (now called T-RFLP), traditional RFLP and PCR product cloning with sequencing analysis were performed. The results of the T-RFLP analysis indicated that there may

be as many as seven unique species of bacteria within the consortia [64]. RFLP analysis indicated that there may be as few as five species. Cloning, sequencing and phylogenetic analysis of 16S PCR products demonstrated that the bacteria of the PA degrading consortia were diverse and that there were representative sequences from five different phyla of bacteria, all of which had cultivated representatives from the rumen. Because the lengths of the sequence data used in this analysis were small, definitive identities for the bacteria were not established.

1.4. Metabolism of ergot alkaloids by worm-gut microbes.

In contrast to examples of microbial protection from toxicosis in ruminants, there are many cases of plant-related toxicosis where no microbes are known to offer protection [76]. The phenomenon of mycotoxicosis arising from the consumption of tainted foods by animals and humans is not uncommon. Although many of these toxins are produced by fungi that grow on the exterior of the plant and feed material to be consumed, there is a class of toxins that are produced and delivered *in plantae*. The knowledge of grasses that are noxious and harmful to domestic animals has been known by mankind for hundreds of years. Only recent research has brought us to the point of knowing the causative agents of the toxicity. Tall fescue grasses of the species *Festuca arundinacea* have been known to carry an endophytic fungus *Neotyphodium coenophialum* (historically *Acremonium coenophialum*) since around the year 1940. However, it has only been within the last 25 years that science has accepted that the endophyte is the primary cause of the clinical syndromes of summer slump and fescue foot in livestock. Economic losses due to these diseases have been estimated to be about $1 billion per year [75].

The presence of the endophyte in tall fescue (E+) is deemed beneficial

to the plant, and probably developed early as a mutual relationship between the two organisms [74; 75]. The production of the endophyte alkaloids imparts desirable traits to the grass such as drought tolerance and the ability to thwart predation by destructive insects. Strains of tall fescue have been developed without the presence of the endophyte (E-), and research has shown that animals that feed on these varieties are healthier and do not suffer from toxicosis, compared to animals feeding on E+ grasses. Consequently, even though E- grasses are a better food source, they are observably more susceptible to insect predation and are not as heat or drought tolerant. This is why E+ varieties of tall fescue are more persistent when grown [10].

The negative aspect of the presence of endophyte in forage, toxicosis, comes from the production of a number of alkaloids by the endophyte itself. This battery of chemical defenses gives grasses harboring the fungus a distinct advantage over E- grasses and causes clinical toxicosis in animals feeding on E+ tall fescue. The alkaloids produced by the endophyte in tall fescue fall into four main categories and are displayed in Table 1 [86].

Table 1.1. Ergot alkaloids produced in tall fescue by the endophyte *N. coenophialum*.

Ergopeptides	***Ergolines***	***Pyrrolizidine alkaloids***	***Clavines***
ergovaline	ergonovine	loline	chanoclavine
ergovalinine	lysergol	*N* -acetyl loline	agroclavine
ergosine	lysergic acid amide	*N*- formyl loline	penniclavine
ergonine		*N* -acetyl norloline	elymoclavine
ergotamine		perlolidine	6,7-secoagroclavine
ergocristine			
α-ergocryptine			
β-ergocryptine			

The PA compounds present in tall fescue are traditionally believed to be responsible for insect deterrence and not responsible for animal toxicosis. The

clavines are believed to be precursors for the ergopeptide and ergoline molecules [80]. It is the molecules of these latter two categories that are believed to be the primary animal toxicants found in tall fescue, with ergovaline as the main cause of toxicosis. The physiological activities of ergovaline and the other ergot alkaloids have been studied with no definitive conclusions being reached. The research that has been done does suggest some possible modes of activity. Vasoconstriction is the main physiological problem encountered by animals suffering from fescue toxicosis. In warmer climates such as the southeastern United States, the classical manifestation of this is referred to as 'slump' where the animals tend to gain less weight during the summer months, presumably because they are spending more time trying to regulate their body temperature than eating. In cooler climates, fescue toxicosis manifests as the syndrome called 'fescue foot', where animals suffer from the loss of extremities such as ears, tails and hooves. Larson et al. (1995)[60], found that ergovaline binds to the D2-dopamine receptors of rat tissue culture cells. It is this potential dopamine receptor binding that may correlate to the inhibition of prolactin release observed in animals with clinically diagnosed fescue toxicosis [77]. Other physiological effects leading to vasoconstriction to which the ergot alkaloids have been tied are α-andrenergic and dopamine receptor binding [73; 77; 86]. Though some research has suggested that there may be evidence for partial metabolism of the ergopeptides by the rumen microbiota, there is no direct evidence that this metabolism imparts any protection to the animal [25; 98].

1.5. Thesis statement.

Traditionally, metabolism of plant alkaloids by rumen-microbes is a phenomenon that has been demonstrated by incubating filtered rumen digesta *in vitro* with the alkaloid to be studied. This method has been employed by

researchers to describe degradation of the toxic alkaloid mimosine from the *Leucaena* plant [2; 7; 26; 54]. Work from this lab has used whole rumen fluid incubations to describe the metabolism of pyrrolizidine alkaloids from tansy ragwort [102]. All of these studies used analytical chemistry techniques such as thin-layer chromatography, high-performance liquid chromatography or colorimetric assays to detect loss of the alkaloid compounds from *in vitro* culture. These observations led to the knowledge that anaerobic rumen microbes were responsible for the observed metabolism of alkaloids of interest. However, these studies were not able to produce a greater understanding of which microbes were potentially involved in such metabolic activities.

Conversely, some studies focus only on characterization of the microbial diversity of a system. These analyses usually yield little insight into the discovery of potentially novel microorganisms that may perform a subtle metabolic role of interest. Studying alkaloid metabolism by pure cultures of cultivated bacteria can lead to a better understanding of the metabolic role these organisms may play in the larger system from which they were derived. This is the case in studies on the mimosine degraders by Allison [3] and Dominguez-Bello [27]; however, the cultivation of pure-culture bacteria is difficult and time-consuming and requires much knowledge about the biology of the target organism in order to derive specific culturing media to grow them in or on. For the most complete microbial ecological studies, the elucidation of microbes potentially involved in novel metabolic processes should come down to the balanced use of both sets of techniques.

The primary hypothesis of this thesis is that there are anaerobic microorganisms which are associated to the gut of higher eukaryotic animals. The exposure of gut microbes to alkaloid toxins encountered in an animal's diet will select and enrich for microbes capable of metabolizing those toxins. In the case of the PA degrading bacteria, this has already been demonstrated [64; 102]. The secondary hypothesis of this thesis is that the identity of

microbes from the gut environment can be determined using molecular techniques to amplify and detect bacterial DNA markers. A comparison can then be made between the bacterial populations of experimental treatments to deduce which group of bacteria may be responsible for toxin metabolism. The molecular evidence is supported by sensitive analytical chemistry techniques to demonstrate the metabolic activities simply by observing the disappearance of plant alkaloid toxins from *in vitro* cultures of anaerobic bacteria. Comparisons between experimental and control treatments are made in order to elucidate factors contributing to the observed function of the system.

Molecular characterization of sheep ruminal enrichments that detoxify pyrrolizidine alkaloids by denaturing gradient gel electrophoresis and cloning.

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2.1. Abstract.

An enrichment of strictly anaerobic bacteria from ovine rumen fluid, which has previously been named L4M2, is known to detoxify animal hepatotoxins from the pyrrolizidine alkaloid family. These toxins are present in the tansy ragwort plant (*Senecio jacobaea*). These plants have been described in livestock animals' range forages in regions of the world such as the Northwest United States and South Africa. The bacterial enrichment was characterized by molecular cloning techniques and by the molecular fingerprinting technique of denaturing gradient gel electrophoresis (DGGE). Phylogenetic analysis of the enrichment revealed that the consortium is composed of no more than five putative bacterial species which associated to the *Anaerovibrio, Desulfovibrio, Megasphaera, Prevotella,* and *Synergistes* generas. These are all known to exist in the upper gastrointestinal tract of ruminant animals. This work improved upon previous attempts to characterize the consortium by obtaining nearly full-length ribosomal 16S rDNA sequences through cloning. The DGGE results were directly compared to the cloning data by PCR amplifying eight phylogenetically representative clones and analyzing them by DGGE. Direct DGGE analysis of the enrichment displayed greater 16S diversity than the clone library used in this study, suggesting that at least one of the organisms present in the enrichment comprises less than 1% of the total cell population. These data will be used to further refine the enrichment in hopes of future use as a probiotic, which could be administered to animals challenged by the presence of tansy ragwort in their forage.

2.2. Introduction

The presence of hepatotoxins in the pyrrolizidine alkaloid (PA) family is a challenge to many domestic animal species and humans world-wide [66; 95; 110]. These toxins elicit a condition known as seneciosis, which ranges in effects from chronic to acute, and often manifests as veno-occlusive liver disease [95]. In the Northwest United States, plants containing these toxins are present amongst other livestock range forage. In the past, economic loss to Northwest livestock producers has been as high as $20 million due to PA poisoning [19]. In South Africa, seneciosis has been attributed to 5 – 15% of all plant poisonings of livestock making it the third most important cause of plant poisoning or mycotoxicoses in that region [56; 72]. Though many developed agricultural countries appear to be controlling the presence of the plants that contain PAs, there are still apparent hotspots and outbreaks of these plants world-wide. Therefore, seneciosis poses a potentially serious and significant problem for livestock producers now and in the future.

Research has shown that not all livestock animals are affected equally by PAs. In studies where sheep or goats were challenged by the presence of PA-containing Tansy ragwort (*Senecio jacobaea*) in their forage, it was observed that levels as high as 200% of the animal's bodyweight could be consumed with no observable clinical effects to these animals [18; 47]. Other studies have shown that cattle and horses can only consume 5% – 10% of their body weight in tansy before manifesting clinical signs of PA toxicosis [19; 47].

In earlier work by Craig et al. (1986), it was demonstrated that the rumen environment was largely responsible for the resistance sheep exhibited to tansy toxicosis [18; 64]. Work from this group produced an enrichment of strictly anaerobic sheep ruminal bacteria. This enrichment was named L4M2, and was shown to metabolize PAs from tansy *in vitro* [64]. The earliest efforts to characterize the bacterial composition of the L4M2 consortia used

traditional staining and microscopy to describe observed cell morphology. The 16S small ribosomal gene (16S rDNA) was also used as a means of characterization. The molecular techniques of restriction fragment length polymorphism (RFLP), length heterogeneity PCR (LH-PCR), as well as the partial sequencing of 16S clone insert fragments, yielded results that resolved the putative representatives of L4M2 to the most likely class of bacteria [64]. It is desirable to determine a more specific characterization of the bacteria of L4M2 in order to develop minimal culturing conditions necessary to elicit the degradation of PAs by the fewest organisms possible.

Denaturing gradient gel electrophoresis (DGGE) is a well-established technique to separate double-stranded DNA fragments of similar size that have been amplified by the polymerase chain reaction (PCR). Separations are based on differences in the melting characteristics of the amplicons as a result of their primary and secondary structure [38; 71]. By taking advantage of the melting characteristics between DNA of similar size and differing sequences, DGGE has been shown to be a useful tool for assaying complex microbial populations in the rumen [58] and other environmental systems [70]. Also, 16S rDNA-DGGE has been used to detect microbial population changes temporally as well as spatially [46] using 16S rDNA. Limitations to the exclusive use of DGGE for observing microbial diversity as well as making phylogenetic inferences have been discussed by Muyzer and others [70]. However, the use of DGGE as a relatively fast, high-throughput method for detection of microbial diversity was not discounted.

In this study, we used DGGE as well as the more traditional method of cloning PCR amplified fragments into an *Escherichia coli* (*E. coli*) vector in order to better elucidate the composition of the PA-degrading enrichment L4M2. DNA sequencing was used following both techniques for phylogenetic comparisons. Where universal bacterial primers encompassing a large portion of the 16S rRNA gene were selected for the generation of a clone library [105], the V3 hyper-variable region of 16S rRNA gene was the target of amplification

for DGGE analysis [69]. The sequence data were used to make phylogenetic relationships to sequences in GenBank [11]. For direct comparison of the two methods, the sequences obtained from the reamplification of DGGE bands were computationally aligned with the clone sequences. In addition, clone representatives from each unique phylotype were amplified using the DGGE primers and compared to the banding profile of L4M2.

2.3. Methods

2.3.1. Anaerobic bacterial cultures and media preparation.

Bacteria of the L4M2 enrichment consortia were revived from cryogenic storage at -140°C in liquid nitrogen. One milliliter of cryogenically stored cells were added to 2.5 mL of culture media E for pyrrolizidine alkaloid degraders (HK_2PO_4, 1.4 mM; $C_6H_5Na_3O_7 \cdot 2H_2O$, 0.3 mM; NaCl, 8.2 mM; NH_4SO_4, 3.6 mM; H_2KPO_4, 1.8 mM; $CaCl_2 \cdot 2H_20$, 0.4 mM; $MnSO_4 \cdot 7H_2O$, 0.4 mM; $Na_3C_6H_5O_7 \cdot 2H_20$, 2.7 mM; Sodium acetate, 5.7 mM; Sodium propionate, 0.5 mM; Sodium butyrate, 1.1 mM; Isobutyric acid, 1.3 mM; 2-methylbutyric acid, 1.3 mM; Isovaleric acid, 1.1 mM; valeric acid, 1.1 mM; $EDTA \cdot Na_2 \cdot 2H_2O$, 46.2 µM; $FeSO_4 \cdot 7H_2O$, 28.8 µM; $MnSO_4 \cdot H_2O$, 40 µM; BH_3O_3, 19.4 µM; $CoCl_2 \cdot 6H_2O$, 2.0 µM; $ZnSO_4 \cdot 7H_2O$, 1.5 µM; $MoNa_2O_4$, 0.6 µM; $NiCl_2 \cdot 6H_2O$, 0.3 µM; $CuCl_2 \cdot 2H_2O$, 0.2 µM; Hemin, 0.2 µM; Clarified rumen fluid, 10% v/v; pH 7.0) [64]. Media components were combined in a round bottom flask, brought to a pH of 7.0 using 10N NaOH, then boiled for 5 minutes and purged of oxygen by sparging with a N_2-CO_2 (75:25) gas mixture for 40 minutes. The media was then stoppered and immediately placed into an anaerobic glove box (Coy, Grass Lake, MI) with a mixed atmosphere of CO_2 and H_2 (9:1 respectively), and aliquotted to serum bottles, which were sealed with butyl rubber stoppers and aluminum crimp caps. Anaerobically prepared reducing agents (3.8 mM Na_2CO_3, 0.3 mM Na_2S, 0.2 mM Cystine HCl; final

concentrations) were added to each bottle of media prior to use. The sealed media and reducing agents were autoclaved for 30 minutes at 120°C and stored at 39°C in the anaerobic glove box until used. One milliliter of overnight bacterial cultures was used to inoculate 2.5 mL of new media with 40 µL of pyrrolizidine alkaloids purified from tansy ragwort [18; 63] in a phosphate buffered solution prepared using standard anaerobic techniques. The final concentration of PA in the cultures immediately after transfer was 60 µg mL^{-1}. The PA solution was added to every transfer in order to maintain the cultures' ability to metabolize the alkaloids.

2.3.2. TLC detection of PA detoxification.

PA detoxification was monitored by thin layer chromatography as described previously by Lodge-Ivey et al. (2005) [64]. Briefly, 500 µL of culture was removed per sampling time, which was usually zero and 24 hours post inoculation. Organic extraction and concentrations of samples was performed prior to spotting the samples onto a plate. Plates were developed in PropA solution (Chloroform:Methanol:Propionate; 36:9:5) and visualized by aerosol application of Dragendorf's reagent (Sigma chemicals) and 5.0% w/v sodium nitrate solution. Plates were photographed for archiving.

2.3.3. Nucleic acid extraction.

Due to the low cell density of overnight cultures, four days worth of overnight cultures were pooled together to achieve an adequate amount of biomass from which to extract DNA. Genomic DNA was extracted using a Gentra PureGene DNA Isolation kit (Gentra Systems, Minneapolis, MN) and a modified protocol for extraction from Gram-negative cultures was employed. In brief, four overnight cultures were pooled to a volume of about 10 mL and then concentrated by centrifugation at 650*g* for 10 minutes in a Beckman TJ-6 centrifuge (Beckman Coulter, Fullerton, CA). The supernatant was removed

and the cell pellet was resuspended in 300 µL of Gentra Cell Lysis solution. Further DNA isolation was followed according to the manufacturer's recommendations.

2.3.4. 16S rDNA-based L4M2 clone library construction.

PCR primers were used to amplify the 16S rDNA from genomic DNA extracted from L4M2 cultures. For clone library construction, primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′, positions 7 to 26 of E. coli) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′, positions 1513 to 1494 of E. coli) were used [64]. All PCR thermocycling was carried out using the TripleMaster PCR system (Eppendorf, Hamburg, Germany) in a PTC-200 thermocycler (MJ Research Inc., Watertown, MA). Each 25 µL PCR reaction contained: about 60 ng of purified genomic DNA, 200 µmol of each dNTP, 2 µL of 10x HighFidelity Buffer with 25 mM Mg2+, 200 nM of each primer, 1.25 U TripleMaster polymerase mix and 17.25 µL sterile 18 MΩ water. Amplification of 16S rDNA using the fD1/rP2 primer set was achieved with a thermocycling program that consisted of a 2 min denaturation cycle at 95˚C followed by 25 cycles of 20 seconds at 95˚C, 20 seconds at 57˚C and 45 seconds at 72˚C, with a final elongation cycle of 72˚C for 10 minutes. PCR products were purified using the QIAquick PCR purification kit according to the manufacturer's recommendations (Qiagen Inc., Valencia, CA). Concentrations of purified products were determined using a NanoDrop 1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Cloning and transformation of competent E. coli cells using PCR products from the fD1/rP2 reactions was performed using the TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's recommendations. The pCR 4-TOPO® vector was used in conjunction with TOP-10 chemically competent cell of E. coli (Invitrogen Corporation, Carlsbad, CA). Ten µL of transformed cells with 20 µL of S.O.C. media (Invitrogen Corporation, Carlsbad, CA) was spread onto an LB agar

plate (EMD chemicals Inc., Gibbstown, NJ) which contained 50 µg mL^{-1} ampicillin (EMD) as the selective agent and was incubated overnight at 37˚C. Ninety-six colonies were randomly selected for library construction and incubated overnight at 37˚C with shaking at 250 rpm in 1.25 mL LB broth, Miller salt concentration (Becton Dickinson and Co., Sparks, MD), with 80 µg mL^{-1} ampicillin. Plasmids were harvested from overnight sub-clone cultures using the Qiagen DirectPrep 96 Miniprep kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol.

2.3.5. DGGE analysis of 16S-V3 rDNA from L4M2 genomic and clone library samples.

For DGGE, the primer set described by Muyzer et al. (1993) [69] was used to amplify the hypervariable region, V3, of 16S rDNA corresponding to positions 341 – 534 in E. coli. The forward primer was named V3fDGGE for this study (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′. The first 40 nucleotides 5′ to the beginning of the forward primer were added as a 'GC-clamp' (underlined) [69]. The reverse primer was named V3r for this study (5′-ATTACCGCGGCTGCTGG-3′). PCR reactions were set-up as described before when using genomic or plasmid templates. Template concentrations for DGGE band re-amplification reactions were not determined. Thermocycling conditions for genomic DNA template reactions consisted of a 2 min denaturation cycle at 95˚C followed by 25 cycles of 20 seconds at 95˚C, 20 seconds at 63˚C and 30 seconds at 72˚C, with a final elongation cycle of 72˚C for 7 minutes. Thermocycling for the re-amplification of extracted DGGE bands was similar to the steps used for genomic V3 amplification except that the 72˚C elongation steps during the 25 cycles and final elongation were shortened to 17 seconds and 5 minutes respectively. Thermocycling for the amplification of the V3 region of 16S clone inserts from plasmid preparations

were the same as for DGGE band re-amplification except for the initial 95˚C denaturation step which was 5 minutes in length.

The DCode system for DGGE (BioRad, Hercules, CA) was used according to the manufacturer's recommendations. For the separation of 16S-V3-DGGE products comparison and analysis, a 16×16 cm, 8% polyacrylamide (37.5:1, acrylamide/bis-acrylamide, BioRad, Hercules, CA) gel with a linear denaturing gradient of 40% – 65% (100% denaturing is 7M urea with 40% formamide; Mallinckrodt Baker Inc., Phillipsberg, PA) was polymerized using a final concentration each of 0.09% (v/v) ammonium persulfate (Mallinckrodt Baker Inc. Phillipsberg, PA) and TEMED (Promega Corp., Madison, WI) solutions. All purified PCR products were mixed with 10 µL of 2×loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 70% glycerol, 29.9% 18 MΩ water) and brought up to a final volume of 20 µL in TE pH 8.0 (10 mM Tris·Cl, 1 mM EDTA) before loading. Seventy nanograms of 16S-V3 PCR products were loaded in lanes where mixed L4M2 fragments were separated. Twelve to fifteen nanograms of PCR products were loaded into each lane where only one major product was expected. Gels were run for 16 hours at 60V in 1× TAE (0.4M Tris base, 0.2M acetic acid, glacial, 50mM EDTA, pH 8.0) at 60˚C.

Gels were stained using the SilverStain Plus kit (BioRad, Hercules, CA) according to the manufacturer's instructions. Gels were visualized on a fluorescent light box (Star X-ray, Amityville, NY). Gel images were captured using Kodak DC120 and CX4230 digital cameras. Kodak 1D image analysis software v.3.6.3 (Eastman Kodak Scientific Imaging Systems, Rochester, NY) was used to analyze the resulting bands.

2.3.6. DGGE band excision and re-amplification.

DGGE bands of interest from the mixed L4M2 profiles were stabbed with a sterile 200 µL pipette similar to the method described by Wilton et al. (1997) [108] . For this experiment, the tip was placed into 10 µL of PCR

reaction solution with water and primers only (see PCR conditions above) in a 200 µL PCR tube. The solution was gently pipetted up and down to deposit any DNA from the tip into this solution. Fifteen microliters of PCR reaction solution containing the remaining components (see PCR conditions above) were then aliquotted to each reaction for a final volume of 25 µL. Thermocycling was performed as described above for extracted DGGE bands. Re-amplified band products were then subjected to further DGGE analysis as described above to qualitatively check for product purity.

2.3.7. Sequencing and phylogenetic analysis of clone inserts and DGGE-PCR products.

Cycle sequencing was performed using the ABI Big Dye Terminator system (Applied Biosystems, Foster City, CA) and the resultant products were analyzed with an Applied Biosystems capillary 3730 Genetic Analyzer. DGGE-PCR products were submitted for sequencing with 6 picomoles of the V3r primer. Sequencing of clone inserts was accomplished by elongating from T7 promoter and T3 primer sites on the TOPO-TA vector to obtain overlapping reads of the whole 16S insert. Partial sequencing reads of the clone inserts were made into contiguous sequences using the CAP3 tool available on the in-house bioinformatics website of the Oregon State University Center for Gene Research and Bioinformatics (http://bioinfo.cgrb.oregonstate.edu). 16S insert contigs were checked for chimeric sequences using the software program PINTAIL [8] as well as the chimera detection tool from the RDB II website (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU) [16]. The SDSC Biology Workbench website (http://workbench.sdsc.edu) was used to search sequence databases and to manage sequence data. Phylogenetic analysis was performed using the PHYLIP phylogeny inference package v.3.6 [36; 37]. Full-length clone sequences as well as DGGE band sequences were compared to sequences in published databases using NCBI BLAST [5] and

the SDSC Biology Workbench which extracted information from the GenBank bacterial sequence database. Only reference sequences of named organisms from the databases with the highest homology scores to the query sequences were chosen for phylogenetic comparison. GenBank reference sequences and clone library sequences selected for comparison to DGGE band sequences were aligned to query sequences using the Smith-Waterman algorithm of the 'Align 2' tool found on the in-house bioinformatics website [78; 93]. These alignments were performed to normalize these longer sequence lengths to the portion of the reference sequences that best represented the V3 regions in order to make direct phylogenetic comparisons to the sequences of the observed DGGE bands. Full-length 16S rDNA clone and reference sequences were aligned using the Clustal W multiple sequence alignment algorithm [100]. The IUB/Bestfit weight matrix was used with a gap open penalty of 15.0 and a gap extension penalty of 6.66. Bootstrapping of the alignment was performed 100 times using the 'seqboot' tool of PHYLIP [36; 37]. Distance matrices of the bootstrap replicates were produced by the DNADIST tool of PHYLIP using the 2 parameter model of Kimura with a transition: transversion ratio of 2.0. These matrices were used to build un-rooted neighbor-joining trees in PHYLIP. A consensus tree was found using the 'consense' tool of PHYLIP and branch distances were inferred using a maximum likelihood analysis of the bootstrap alignments with a transition: transversion ratio of 2.0 with constant rate variation between sites. All sequences used for phylogenetic analysis have been deposited in GenBank. Sequences from band-stab samples were assigned accession numbers DQ308563 – DQ308569. Sequences from clones were assigned accession numbers DQ308570 – DQ308605.

2.4. Results

2.4.1. L4M2 clone library analysis.

Forty-four clone plasmid preparations were initially sequenced. The remaining fifty-two plasmid preparations of the library were sorted into five distinct ribotypes by RFLP analysis (data not shown). Eighteen of these preps were sequenced. Five out of eighteen were determined to be chimeras by the methods described above. The remaining thirteen sequences made no new phylogenetic associations when compared to the first set of sequences; therefore, they were not used in the phylogenetic analysis. Thirty-six non-ambiguous, non-chimeric cloned sequences were used in the final phylogenetic analysis. Clone sequences that were ≥99.5% similar to one another were arranged into the same phylogenetic group (Table 2.1). This analysis yielded a tree that clearly displays four major phylotypes (Fig. 2.1).

Table 2.1. L4M2 16S rDNA clones as grouped by phylogenetic analysis. Clones in italics were used as group or subgroup representative in the phylogenetic analysis. Percent similarity was determined using the Smith–Waterman algorithm as described in the Methods section. The sequences in each group were compared to the representative clone for that group (NA=not applicable since clone would be compared to itself). Clone B09 was included in the phylogenetic analysis but excluded from the table as there were no other homologous sequences to group it with.

Group	*Subgroup*	*Clone*	*Percent similarity to representative clone (%)*
Group 1	Subgroup A	*F01*	NA
	Subgroup B	*E05*; F08; D10; E04; G02; H01	NA; 99.8; 99.8; 99.8; 99.7; 99.9
	Subgroup C	*G05*; H02; B04; E01; E11; H11; G12; G06; H03	NA; 99.8; 99.9; 99.8; 99.9; 99.9; 99.9; 100; 99.7
	Subgroup D	*C02*	NA
	Subgroup E	*E12*; F03; C12; B10; D01; D02; D04	NA; 99.9; 99.9; 99.6; 99.8; 99.8; 99.8
Group 2		*C01*; F06; C07; E08; E02	NA; 99.7; 99.5; 99.7; 99.6
Group 3		*G09*; H12; G01; A09; F10; F11	NA; 100; 99.8; 99.9; 99.9; 99.8

Since clones within a given group displayed greater than 99% similarity to each other, many of the phylogenetic subgroups had very short evolutionary distances between representatives which resulted in branches of virtually no length and could not be visually resolved. Therefore, a consensus bootstrap

tree having branch lengths not related to evolutionary distance was used in order to see the sub-groupings between the thirty-six clone sequences used in the analysis (data not shown). We then used one clone from each respective group or sub-group as a representative operational taxonomic unit (OTU) for phylogenetic comparison between groups. Table 2.2 shows the percent similarity between the clones used as group representatives when examining only the normalized V3 sequence of the clone insert. Only one clone from Group 1 (196.G05) was 99.5% similar to three other clones in the same group (clones 196.C02, 196.E05 and 196.E12). All other clones were ≤99% similar to other clone group representatives. This simplified the display of evolutionary distance between OTUs so that the global trends of each tree were not lost. Pair wise alignments were performed on each clone and its nearest neighbor reference sequence using the Smith-Waterman algorithm tool.

Table 2.2. Un–gapped sequence homology (% similarity) between the computed 16S rDNA V3 regions of clone group representatives. Percent similarity was determined using the Smith–Waterman algorithm as described in the Methods section. Similarity between group and subgroup representatives was ≤99.5%.

CLONE	*G09*	*G05*	*F01*	*E12*	*E05*	*C02*	*C01*	*B09*
G09	100.0							
G05	75.0	100.0						
F01	73.0	96.4	100.0					
E12	75.0	99.5	95.9	100.0				
E05	75.0	99.5	95.9	99.0	100.0			
C02	75.0	99.5	95.9	99.0	99.0	100.0		
C01	75.1	80.0	84.0	80.5	81.4	80.5	100.0	
B09	80.3	86.7	83.2	86.7	86.7	87.3	86.7	100.0

Table 2.3 summarizes the associations made by clone group representative sequences to GenBank reference sequences used in the phylogenetic analysis. Clones of Group 1 formed five sub-groups despite their high similarity to each other (Table 2.2). Each sub-group displayed about 90% and 91% un-gapped sequence homology to reference sequences of *Desulfovibrio intestinalis* and *Desulfovibrio desulfuricans* respectively. The exception was clone 196.F01, which made its closest associations to

sequences of this same group, but only displayed about 95% un-gapped sequence similarity to other clone representatives of Group 1 and less than 90% un-gapped homology to the *Desulfovibrio* reference sequences. Clones which clustered into Group 2 displayed about 94% un-gapped sequence homology to the reference sequence of *Megasphaera elsdenii* YJ-4. Sequences from Group 3 clones displayed about 95% un-gapped sequence homology to the reference sequence from *Prevotella ruminicola* ATCC 19189. The clone 196.B09 displayed just over 97% un-gapped sequence homology to the reference sequence for a human oral isolate classified as a *Synergistes* species, designated P4G-18 P1. GenBank accession numbers for all reference sequences used in the phylogenetic analysis are listed in Table 2.3.

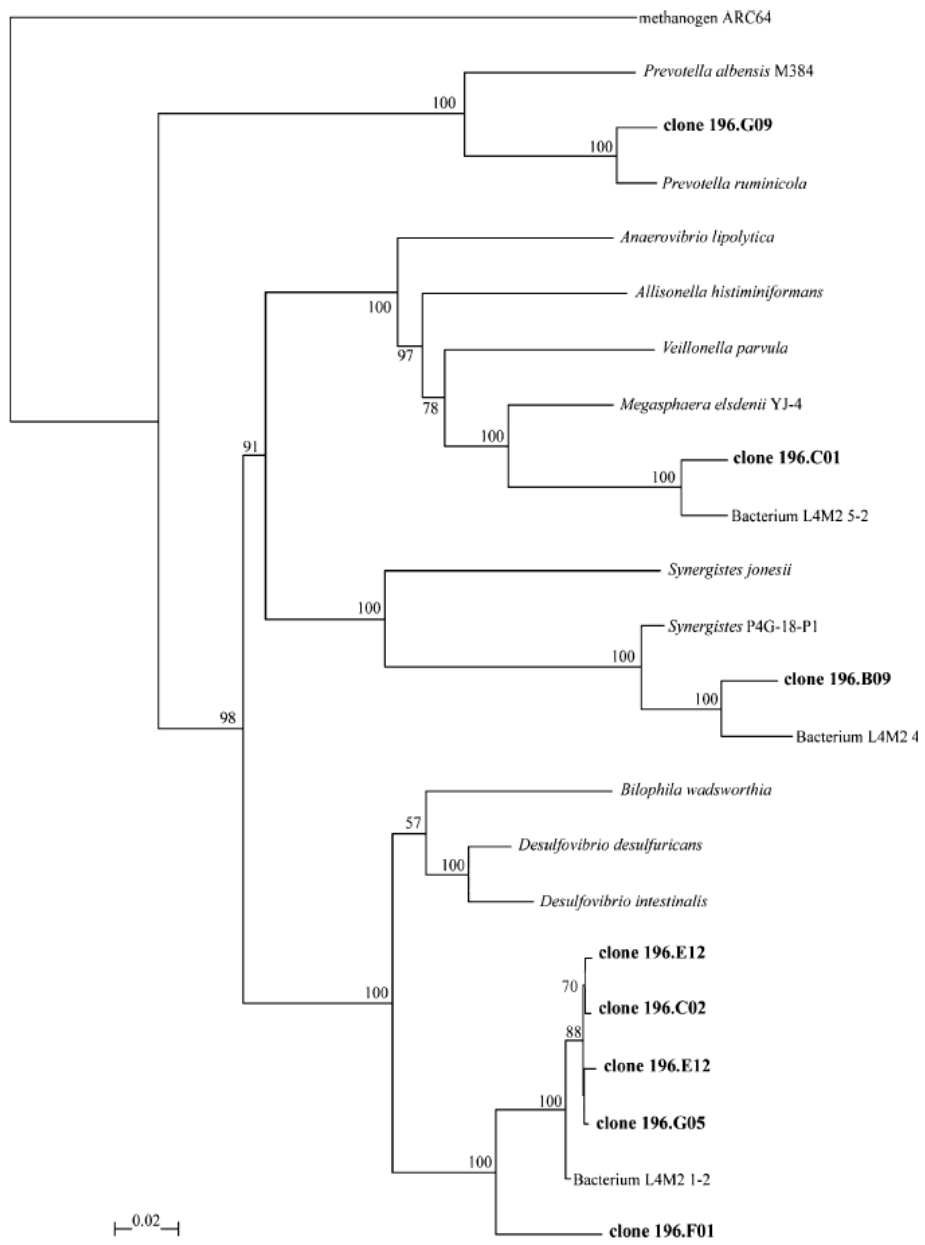

Figure 2.1. Phylogenetic analysis of L4M2 16S rDNA clone library. An un-rooted neighbor-joining tree of OTUs representing 16S rDNA from L4M2 clones and GenBank reference sequences. L4M2 clones are designated by **bold** characters**.** Bootstrap values for 100 trees were placed at the nodes. Branch lengths were generated using the maximum likelihood method as described in the materials and methods. The scale bar represents 0.02 mutations per site. 1535 nucleotides were used in the analysis. The sequence for GenBank reference AF029211, Unidentified Methanogen ARC64, was used as the out-group in the analysis.

L4M2 sequence: GenBank accession number	*Fragment length (bp)*	*Genus of best association*	*Nearest phylogenetic neighbor(s) (GenBank accession number)*	*Percent homology*	*Nucleotide overlap*
DGGE band 196L-b1: DQ308563	156	*Desulfovibrio*	Bacterium L4M2 1-2 (AY862592); *D. intestinalis* strain KMS2 (Y12254)	95.5; 89.5	157; 144
DGGE band 196L-b2: DQ308564	158	*Desulfovibrio*	Bacterium L4M2 1-2 (AY862592); *D. intestinalis* strain KMS2 (Y12254)	97.2; 89.8	145; 147
DGGE band 196L-b3: DQ308565	161	*Desulfovibrio*	Bacterium L4M2 1-2 (AY862592); *D. desulfuricans* strain MB (AF192154)	91.1; 88.4	146; 147
DGGE band 196L-b4: DQ308566	155	*Desulfovibrio*	Bacterium L4M2 1-2 (AY862592); *D. desulfuricans* strain MB (AF192154)	94.9; 89.7	157; 146
DGGE band 196L-b5: DQ308567	159	*Anaerovibrio*	Bacterium L4M2 1-7 (AY862595); *A. lipolytica* (AB034191)	93.8; 93.1	145; 145
DGGE band 196L-b6: DQ308568	163	*Veillonella*	Bacterium L4M2 5-2 (AY862596); *V. parvula* (AF192154)	95.1; 95.7	163; 116
DGGE band 196L-b7: DQ308569	167	*Synergistes*	*Synergistes* sp. PG4_18 P1 (AY207056)	62.4	125
Clone 196.F01: DQ308589	1527	*Desulfovibrio*	*D. desulfuricans* strain MB (AF192154)	89.2	1532
Clone 196.E05: DQ308585	1512	*Desulfovibrio*	Bacterium L4M2 1-2 (AY862592); *D. desulfuricans* strain MB (AF192154)	98.7; 90.8	387; 1514
Clone 196.G05: DQ308597	1513	*Desulfovibrio*	Bacterium L4M2 1-2 (AY862592); *D. desulfuricans* strain MB (AF192154)	98.9; 91.1	387; 1514
Clone 196.C02: DQ308575	1513	*Desulfovibrio*	Bacterium L4M2 1-2 (AY862592); *D. desulfuricans* strain MB (AF192154)	98.7; 91.0	387; 1514
Clone 196.E12: DQ308588	1513	*Desulfovibrio*	Bacterium L4M2 1-2 (AY862592); *D. desulfuricans* strain MB (AF192154)	98.7; 91.0	387; 1514
Clone 196.C01: DQ308574	1530	*Megasphaera*	Bacterium L4M2 5-2 (AY862596); *M. elsdenii* strain YJ-4 (AY038996);	100.0; 93.9;	636; 1535
Clone 196.G09: DQ308599	1493	*Prevotella*	*P. ruminicola* ATCC 19189 (L16482)	95.5	1465
Clone 196.B09: DQ308572	1488	*Synergistes*	Bacterium L4M2 4-15 (AY862597); *Synergistes* sp. PG4_18 P1 (AY207056)	99.5; 97.2	613; 1468

Table 2.3. Names and GenBank accession numbers for L4M2 DGGE band sequences and clone group representatives used in the phylogenetic analysis. Each L4M2 sequence was listed with its closest neighboring reference sequence(s) as well as the similarity metrics between the L4M2 and reference sequences. Reference sequences used for phylogenetic analysis but not included in this table were *Allisonella histaminiformans* (GenBank #AF548373), *Bilophila wadsworthia* (GenBank #U82813), *Prevotella albensis* strain M384 (GenBank #AJ011683) and *Synergistes jonesii* (GenBank #L08066). All L4M2 sequences from this study were deposited in GenBank under the sequential accession numbers DQ308563 – DQ308605.

2.4.2. DGGE band analysis.

DGGE bands representing the amplified V3 region of the 16S rDNA genes present within the L4M2 enrichment are shown in Figure 2.2, lane 8. Bands picked for sequence analysis are present in lanes 1 – 7 of Figure 2.2.

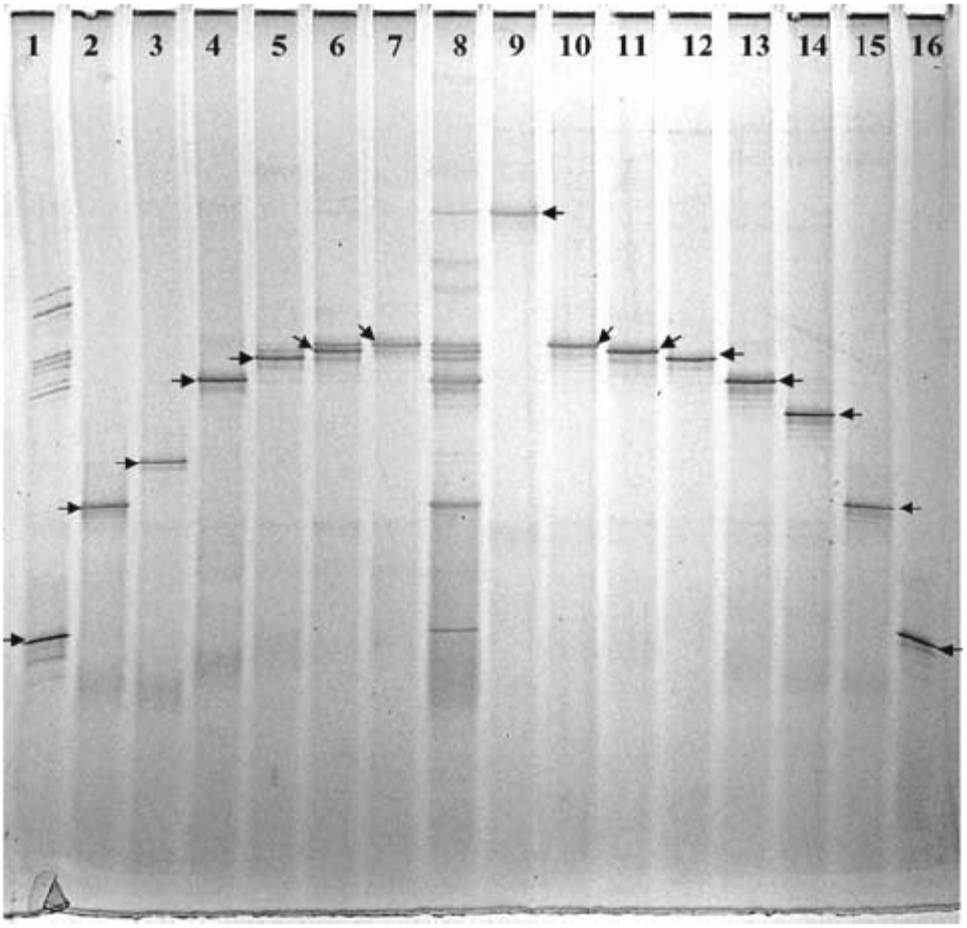

Figure 2.2. DGGE gel images of 16S-V3 rDNA PCR products representing L4M2. Band-stab and clone V3-DGGE products were ran in parallel with a sample of mixed 16S-V3 products amplified from L4M2 genomic DNA (Lane 8). Lanes 1 – 7 represent band-stab re-amplification products from the mixed sample of L4M2. Lanes 9 – 16 represent 16S-V3 DNA amplified from sub-group representatives of the L4M2 clone library. Arrow heads indicate the intended band in each lane which best represents a band of relative mobility in lane 8.

The sequences of these bands were less than 168 bases in length and did not include the portion of the sequence to which the reverse primer would have annealed. The tree in Figure 2.3 was produced when a phylogenetic comparison of the DGGE bands were made to clone and reference sequences that had been normalized to represent the portion of the V3 region of the 16S gene that was available from the band sequences. The sequences from bands 196L-b1 – b4 (Fig. 2.2, lanes 7, 6, 5 and 4 respectively) clustered with sequences in the *Desulfovibrio* group as well as clone sequences from Group 1. The band sequences were not different enough from each other to make specific phylogenetic associations with any clones. Band 196L-b5 (Fig. 2.2, lane3) made a close association to a reference sequence of *Anaerovibrio lipolytica*. Band 196L-b6 (Fig. 2.2, lane 2) associated with sequences of *M. elsdenii* YJ-4 and *Veillonella parvula* as well as clone 196.C01 and a partial 16S reference sequence from a bacterial isolate of an earlier cultivation of this enrichment named Bacterium L4M2 5-2 [64]. As shown in lane 1 of Figure 2.2, DGGE band 196L-b7 was the predominant fragment in the sample, but it was not the only one. This was the result even after 3 subsequent stabbing and reamplification steps. It would seem that since band b7 is the last band of the profile, co-migration of other fragments in the profile is unavoidable and a limitation of DGGE [42; 91]. Band b7 produced ambiguous sequencing results that were illustrated by a low quality ABI chromatogram (data not shown). This yielded very poor BLAST hits which resulted in very weak and distant associations to the clones and reference sequences used in the phylogenetic analysis. All of the re-amplified DGGE bands displayed lower percent similarity to the reference sequences when compared to clones making similar phylogenetic associations (Table 2.3).

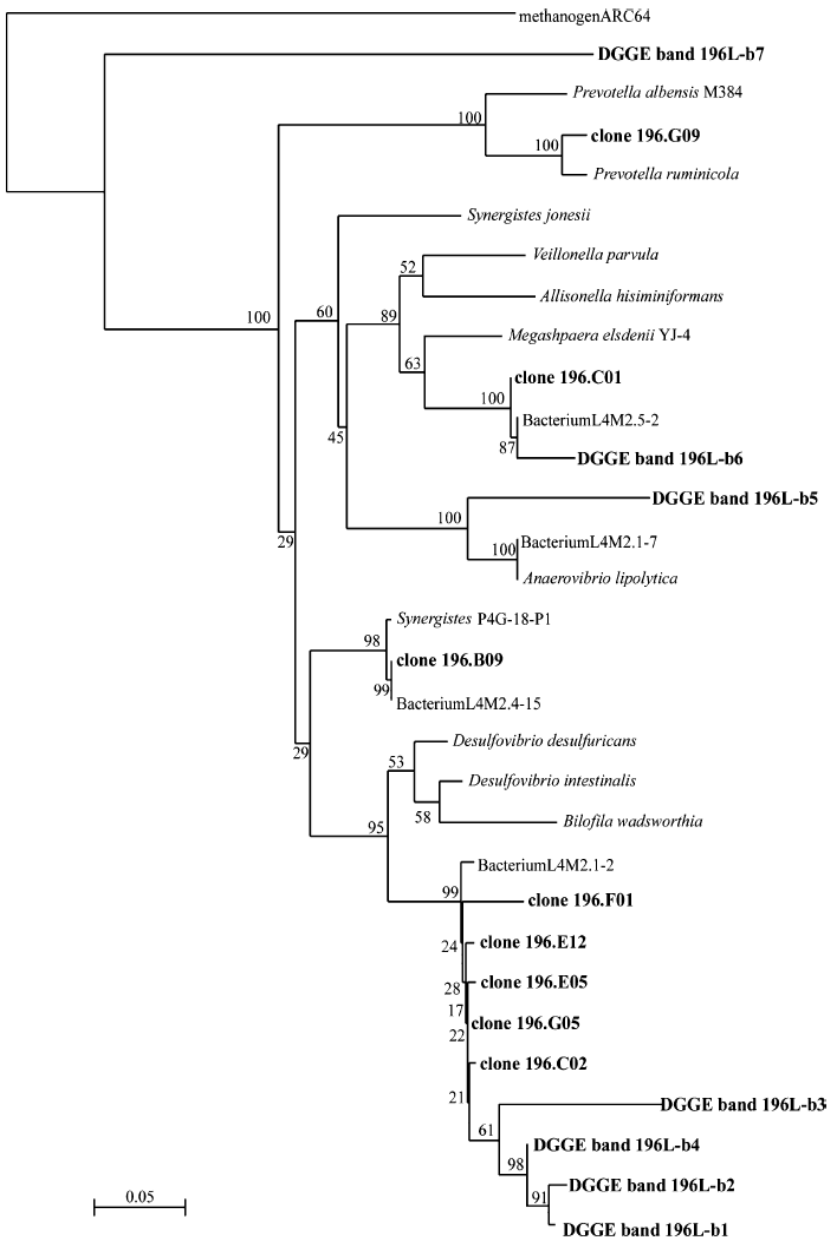

Figure 2.3. Phylogenetic analysis of 16S rDNA V3 region sequences. An un-rooted neighbor-joining tree of OTUs representing the 16S rDNA V3 region from L4M2 clones, DGGE bands and GenBank reference sequences. Sequences used for clone and references were computed from full-length 16S sequences. DGGE band sequences were used as published in GenBank. Bootstrap values for 100 trees were placed at the nodes. Branch lengths were generated using the maximum likelihood method as described in materials and methods. The scale represents 0.05 mutations per site. 167 nucleotides were used in the analysis. The sequence for GenBank reference AF029211, Unidentified Methanogen ARC64, was used as the out-group in the analysis.

2.4.3. DGGE analysis of clones.

DGGE analysis of phylogenetic group representatives from the clone library were compared to the mixed DGGE products from L4M2. The results are displayed in Figure 2.2, lanes 9 – 16. Eight unique bands of DGGE-PCR amplified products were observed from individual clones representing the eight unique phylotypes in the clone library (Fig. 2.1). The clone fragments were compared to a lane of mixed L4M2 V3 products used in the DGGE band analysis described above.

Group 3 clone 196.G09 produced a band which traveled a distance in the gel relative to a very faint band in the mixed lane that was not originally picked for DGGE sequencing analysis. Clone 196.E12, 196.E05 and 196.F01 produced bands relative to DGGE bands b1, b2 and b3 respectively. Clone 196.G05 produced a band relative to DGGE band b4. The band from Clone 196.C02 traveled a distance relative to a very faint band that was not picked for DGGE sequence analysis. Clone C01 was relative to DGGE band b6. Clone 196.B09 produced a band that traveled a distance relative to DGGE band b7.

2.5. Discussion

The L4M2 consortium of bacteria that detoxify pyrrolizidine alkaloids from tansy ragwort were characterized in this report using two molecular techniques. Cloning has been used to gain information from pure-culture, as well as, environmental bacterial samples. Even though cloning of L4M2 PCR products representing 16S rDNA has been previously employed, this technique was used here to improve the classification of the bacteria of L4M2. The size of the cloned 16S amplicons in this study were 1,513 bases on average compared to about 1,470 bases as determined by the 16S primers used [64]. Though this is not a significant improvement in product length, the

resulting sequences from clones in this study were the full-length of each insert, whereas the previous work only reported half of the amplicon sequence. This work has yielded a more complete set of data from which we have confidently classified the 16S sequences of L4M2 represented in this clone library to the *Desulfovibrio, Megasphaera, Prevotella* and *Synergistes* generas of bacteria (Fig. 2.1; Table 2.2). Three of these four phylotypes were represented by multiple clones. This was an anticipated result from this enrichment, as we did not expect a high amount of diversity in the consortia [64].

The production of a DGGE fingerprint as well as identifying the constituent bacteria of L4M2 by sequencing observed DGGE bands were new methods used to elucidate this enrichment. The results from the previous study of L4M2 were inconclusive with regard to the potential number of constituent bacteria. The purpose of utilizing this technique to analyze a simple bacterial consortium like L4M2 was to check that the diversity of sequences represented by our clone library was comprehensive. We were also interested in determining whether the data obtained by sequencing re-amplified DGGE bands would be strong enough to characterize a mixed bacterial sample without going through the step of cloning. The DGGE-PCR amplicons from the V3 region of the 16S rRNA gene were about 200bp in size as determined by agarose electrophoresis (data not shown), yet sequencing of these products yielded no more than 167 nucleotides of data. This length of sequence is not usually considered sufficient to make succinct phylogenetic associations [6]. Despite the short length, the phylogenetic affiliations of the DGGE band sequences are remarkable when compared to clone and reference sequences normalized to represent the V3 region of 16S rDNA (Fig. 2.3). Except for DGGE band b7, all of the band sequences made strong associations to the same reference sequences that were used for clone comparisons as well as the clones chosen as group representatives (Fig. 2.3). To confirm that the observed phylogenetic associations between the clone

library and DGGE bands produced from the enrichment were real and not purely computational, a DGGE analysis of the eight clone OTUs was performed. The relative travel distance between clone inserts amplified by the V3 primers and the V3 amplicons from the consortia, were observed. This bench work supported the computational results to confirm that phylogenetic associations between bands and clones were justified.

Clones of Group 1 were most closely related to reference sequences of the *Desulfovibrio* genus. Five subgroup affiliations between clones of this major group were produced. We believe that this is most likely the result of the presence of multiple 16S genes. As was reported by Heidelberg [45], the genome of *Desulfovibrio vulgaris* Hildenborough, was found to have five ribosomal operons when it was sequenced. Alignments of these sequences displayed about 99.8% similarity between the operons (data not shown). While this amount of variation is lower than that observed between the clones of Group 1, it supports the idea that there can be variation copies of the 16S gene. None of the sequences generated by this study shared high homology to this species of *Desulfovibrio*; however, the fact that we observed five phylogenetic sub-groups related to the *Desulfovibrio* genus, which displayed virtually no evolutionary distance between sequences, is most likely indicative of multiple 16S operons from one species present in the L4M2 consortia. As illustrated, the computed V3 sequences from the representative clones corresponding to phylogenetic Group 1 (clones F01, E05, G05, C02 and E12) displayed as little as 95.9% homology to each other and as much as 99.5% homology (table 4). When the V3 region of the clone inserts were amplified by PCR and analyzed by DGGE, we saw that each representative from the respective sub-groups had a unique band travel distance that directly corresponded to bands of the mixed profile (Fig. 2.2B). That each of these clones represent a unique 16S gene fragment despite the extremely small sequence differences between them is significant, especially coupled to the fact that they represent actual sequence diversity as illustrated by the bands

from the mix they correspond to. These physical observations have allowed us to correlate the phylogenetic associations being made by both the clone of Group 1 and DGGE bands 196L-b1 – b4.

Though the number of clones that comprised Group 2 and 3 were far fewer, the same trend where DGGE bands generated from group representative clones corresponded to observed bands from the L4M2 mixed product lane held true. The Group 2 clones represented by clone 196.C01 told us that there are sequences representing a *Megasphaera* species. The Group 3 clones represented by clone 196.G09 were indicative of a *Prevotella* species (Table 2.3). In truth, it was the phylogenetic analysis of clone sequences leading back to DGGE analysis which showed us how we had missed picking and sequencing phylogenetically significant bands from the mixed profile that represents a *Prevotella* species present in L4M2. It was therefore quite useful for us to compare the mixed profile against the library in order to not only check that the library was representative of the diversity within L4M2, but to show that the exercise of DGGE band excision and reamplification was incomplete. Clone 196.B09 made its best phylogenetic association to a human oral bacterial isolate that had been classified as a *Synergistes* species and was another example of a clone sequence that did not correspond to a sequenced DGGE band after phylogenetic analysis (Fig. 2.3). DGGE analysis revealed that this clone was actually representative of DGGE band 196L-b7. Since the sequence data that was produced by band b7 was of poor quality, it made phylogenetic assignment of this sequence difficult and rather ambiguous. Only when compared to the DGGE profile of clone B09, were we able to see that this band represented the putative *Synergistes* species in L4M2. The converse to these examples of clones elucidating some of the diversity observed in the DGGE profile of L4M2, is the example of DGGE band b5 which made its best phylogenetic affiliations to reference sequences for *Anaerovibrio lipolytica* and a previously submitted L4M2 sequence of bacterium L4M2 1-7 (Fig. 2.3; Table 2.2). Yet, upon phylogenetic comparison

to the clone library, it was determined that none of the sequenced clones were a match to this band. This was confirmed by the fact that no clones produced BLAST hits similar to those observed for band b5. DGGE analysis also confirmed that we did not have a clone from our library that represented band b5 due to the absence of clone fragments that could produce a band that exhibited a travel distance relative to DGGE band b5.

The fact that a potentially representative organism from this enrichment was missed by cloning analysis is disappointing. We assumed that a clone library of 96 sequences would contain a comprehensive collection of 16S rDNA sequence information about the diversity of this relatively simple enrichment, and yet this was not the case. Since 1% is the lower limit of detection by cloning analysis [6] it is not unreasonable to have missed rare amplicons in a 96-clone library such as the putative *Anaerovibrio* species in L4M2. The sensitivity limits of DGGE has been shown to be similar to cloning [70] though the detection of bacteria using group-specific primer sets can potentially allow detection of bacteria that represent less than one-millionth of the total community population [104]. We had anticipated that DGGE analysis may not have been able to completely represent the diversity of the mixed group of organisms, especially considering how DGGE relies heavily on the use of PCR which carries the potential for problems associated with PCR bias and primer specificity [70]. However, in this case, DGGE analysis displayed greater diversity than cloning. It would appear that because DGGE has allowed us to “see” all of the PCR products that were formed while by-passing the step of capturing them in a vector prior to visualization, less information was lost. DGGE could therefore be considered a more sensitive detection technique than cloning in this case. Using DGGE, we were able to detect the presence of organisms that probably represent less than 1% of the bacterial biomass of this enrichment. Previous studies of L4M2 have determined that these organisms may represent about 0.3% of all bacteria found in the ovine rumen as determined by most probable number analysis [102; 103].

DGGE has traditionally been unable to utilize DNA fragments large enough to be considered useful in producing enough phylogenetic information for making specific classification of bacteria; however, this study has shown that the DGGE band sequences that were obtained made the same associations to the references as representatives from the clone library. This would suggest that the use of DGGE or other analyses such as quantitative real-time PCR (qPCR) may be more practical than cloning when used to detect the presence and relative abundance of L4M2 bacteria *in vivo*. We have also shown that for this type of *in vitro* culture, DGGE can be a useful tool for confirming the relative amount of microbial diversity observed when coupled with other methods that generate larger pieces of sequence data.

The work presented here has been the best characterization of L4M2 thus far. We would like to be able to assign species designations to the organisms of L4M2, but most of the pair-wise alignments indicated homologies of less that 95% similarity between full-length clone sequences and those of their most closely related reference sequences. Since clone 196-B09 was the only sequence that made a >97% match to a reference sequence, it is very likely that the organisms that constitute L4M2 are unique strains if not new, uncharacterized species of bacteria that exist in the rumen ecosystem. If we are to classify the organisms of L4M2 at the species level, we will need to rely on classic anaerobic culturing techniques of isolating and maintaining pure cultures of the organisms represented by the phylogenetic analyses presented here. Ultimately, we plan to use the knowledge we have gained here to reduce L4M2 to the most essential bacteria needed for the metabolism of pyrrolizidine alkaloids.

With greater knowledge about these organisms, we will be able to prepare a natural probiotic to administer to agriculturally important ruminant animals that are not resistant to the toxicosis caused by the ingestion of plants containing PAs. It has been previously demonstrated that whole rumen fluid transferred from resistant ruminants to susceptible ones conveys resistance

[15; 23; 53; 54]. This is not an efficient method of inoculating a large number of animals as it takes a great deal of rumen fluid to create this effect. Since the genera represented in L4M2 are traditionally considered secondary metabolite utilizers in the rumen ecosystem, we believe that the organism(s) involved in the break-down process naturally exist at a very low concentration in the rumen ecosystem. Having a pure culture or consortia will be the most desirable and efficient way to administer a high enough concentration of the L4M2 microbes necessary to convey resistance to PA toxicosis.

2.6. Acknowledgements

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The molecular characterization of bacterial populations found in the intestine of the earthworm *Eisenia fetida* from two experimental vermicompost treatments.

3.1. Introduction

Tall fescue (*Festuca arundinacea*) is an important pasture grass used to sustain grazing cattle, sheep and horses. The straw is often used as a forage source in place of fresh grass in dairies, and during the winter in cooler climates such as the Northwest United States. Tall fescue has been known to carry the mutualistic endophyte fungus *Neotyphodium coenophialum* since around the year 1940. The presence of the endophyte in Tall fescue (E+) has been deemed beneficial to the plant, and probably developed early as a mutual relationship between the two organisms. It is the production of the endophyte alkaloids which impart desirable traits to the grass such as drought tolerance and insect resistance. It has only been within the last twenty-five years that the endophyte has been accepted as the primary cause for the presence and production of toxins that are attributed to the syndromes of fescue slump and fescue foot in livestock [10]. Fescue toxicosis and ergot alkaloids are estimated by USDA to cost U.S. livestock producers $1 billion per year [75]. Because the endophyte is a desired trait for this grass species to have, a solution must be found that will allow animals to consume this feed source without the livestock producer worrying about harming the animal.

There are documented examples of anaerobic bacteria from the rumen that confer a level of protection against certain plant toxicoses to host animals through the metabolism of those compounds into non-toxic moieties [3; 27; 102]. It has also been demonstrated that resistance to some plant toxins can be transferred to susceptible ruminants using inoculants containing bacteria capable of metabolizing the plant toxin [54; 102]. The detoxification process appears to be a result of the chemical reduction of the toxin as a result of the microbes using these compounds as an electron acceptor during energy metabolism. There is no direct evidence that anaerobic bacteria from the rumen of sheep or cattle are capable of using a similar mechanism to metabolize the endophyte toxin ergovaline or any other compound of

endophyte origin from Tall fescue. This has led to the search for other potentially novel, anaerobic, bacteria from sources other than the rumen that can metabolize ergovaline.

Soils are highly diverse ecosystems which harbor a wide range of microorganisms that are capable of a many metabolic activities. The bulk of soil, however, is considered aerobic with only small anoxic microcosms interspersed throughout. This fact does not fit well into the model of transferring metabolically important bacteria to a strictly anaerobic environment such as the rumen. A more feasible source for anaerobic bacteria would be from another organism that harbors and selects for anaerobic bacteria to aid in food conversion and metabolism. *Eisenia fetida* is an Oligochaete worm that is one of the most common species used in an agricultural practice called verimicomposting. This is the process in which worms are used in a closed system to aid in the breakdown of manure and other agricultural waste in order to generate highly fertile soil from the worm's digestive waste products, also called cast [92].

Studies have shown that the population of microbes ingested by worms are mainly transient and reflects the bacteria found in the surrounding soil [28; 33; 41]. Other studies have found that bacterial numbers are higher in the gut [87] and that there are more culturable anaerobes obtained from the worm-gut than from the soil [51; 55]. Research in this field has not reached a consensus as to whether bacteria found in the earthworm gut are indigenous to the gut only. Some evidence suggests that there may be bacteria that proliferate better in the gut than the soil. Other bacteria appear to have co-evolved a symbiotic relationship within the earthworm-gut [22; 88; 101]. To-date, only two studies have been published about the effects of endophyte infected Tall fescue on soil microbes or earthworms. In one study, the focus was the determination of whether the state of the grass (E+ versus E-) affected groups of soil microbes in the rhizosphere around the plants compared to the bulk soil in two different soil types. The researchers concluded that E+ grass appeared

to suppress the growth of archaea and the high G+C gram positive bacteria for which they had probed. However the authors acknowledged the possibility that RNA probing may have limited the amount of information gained about the diversity of the microbes found within each treatment [52]. In the second study, work was done to asses the effects on endophyte infected Tall fescue on *E. fetida*. This study only focused on the earthworm. Though they found that the worms feeding on E+ plant leaves performed better that worms consuming E- leaves, there was no assertion as to the potential reason for the difference [50].

This study describes a comparative analysis of the difference between the prokaryotic populations found within the gut of the earthworm *E. fetida*. The worms were part of a two-treatment vermicompost system where, in addition to other domestic plant and fiber material, one treatment was given Tall fescue seed containing over 10,000 parts per billion of the endophyte toxin ergovaline (E+ treatment). The other treatment was used as a control and was given Tall fescue seed with ergovaline amounts that fell below analytical levels of detection (E- treatment). Many of the worms originally introduced into the E+ treatment died off before a population of worms established itself as a productive, vermicompost system. Preliminary incubation experiments with only the E+ treatment worms has indicated that they may harbor some bacteria capable of metabolizing ergovaline to some extent. The goal of this work was to determine whether the success of the population of E+ treatment worms is due to the presence of beneficial or protective microbes within their digestive tract. Worms from each of the treatments described above were collected and dissected for their digestive tracts. The guts harvested from the worms were homogenized and added to anaerobic bacterial culture media with and without the addition of the endophyte toxin ergovaline. The disappearance of ergovaline and the stereo-isomer ergovalinine from worm-gut cultures were monitored daily by high-performance liquid chromatography (HPLC). DNA was purified from worm-gut

homogenates produced from each treatment. Molecular cloning of the 16S rRNA gene was applied in order to determine the identity of the bacteria present in the gut of the worm. Though the incubation work described here was thorough, the metabolism of ergovaline was not observed. However, this work lays the foundation for the future studies of this novel and relatively uncharacterized system. Parameters are now established for assaying the levels of ergovaline during incubation. Methodology for the detailed examination of the bacterial diversity of these experimental treatments has also been applied. Ultimately, the use of these methods and techniques will lead to a better understanding of these experimental treatments, including the isolation of the bacteria responsible for the metabolism of ergovaline.

3.2. Methods

3.2.1. Collection of worms and digestive tracts.

The flow diagram in Figure 3.1 outlines the experimental processes of sample collection from treatment worms, incubation treatment set-up and data analysis and collection detailed in the following three sections. For all treatments, worms were cultivated in round vermicompost bins that had the same mixture of household and garden vegetable scraps and shredded office waste paper added. For the E+ treatment, the Titan Ltd. variety of Tall fescue seed was added. These seeds were determined to contain >10,000ppb ergovaline (EV) as analyzed by standard practices for determining EV concentration from plant material [20]. The E- treatment, received the Fawn Fescue variety of seeds, which were tested to have no detectable EV. Worms were collected from each bin in early February of 2007 by taking random grab samples from three different locations within each bin. Each grab sample consisted of both worms and bin material. Each bin’s grab sample was individually sorted to separate out the worms.

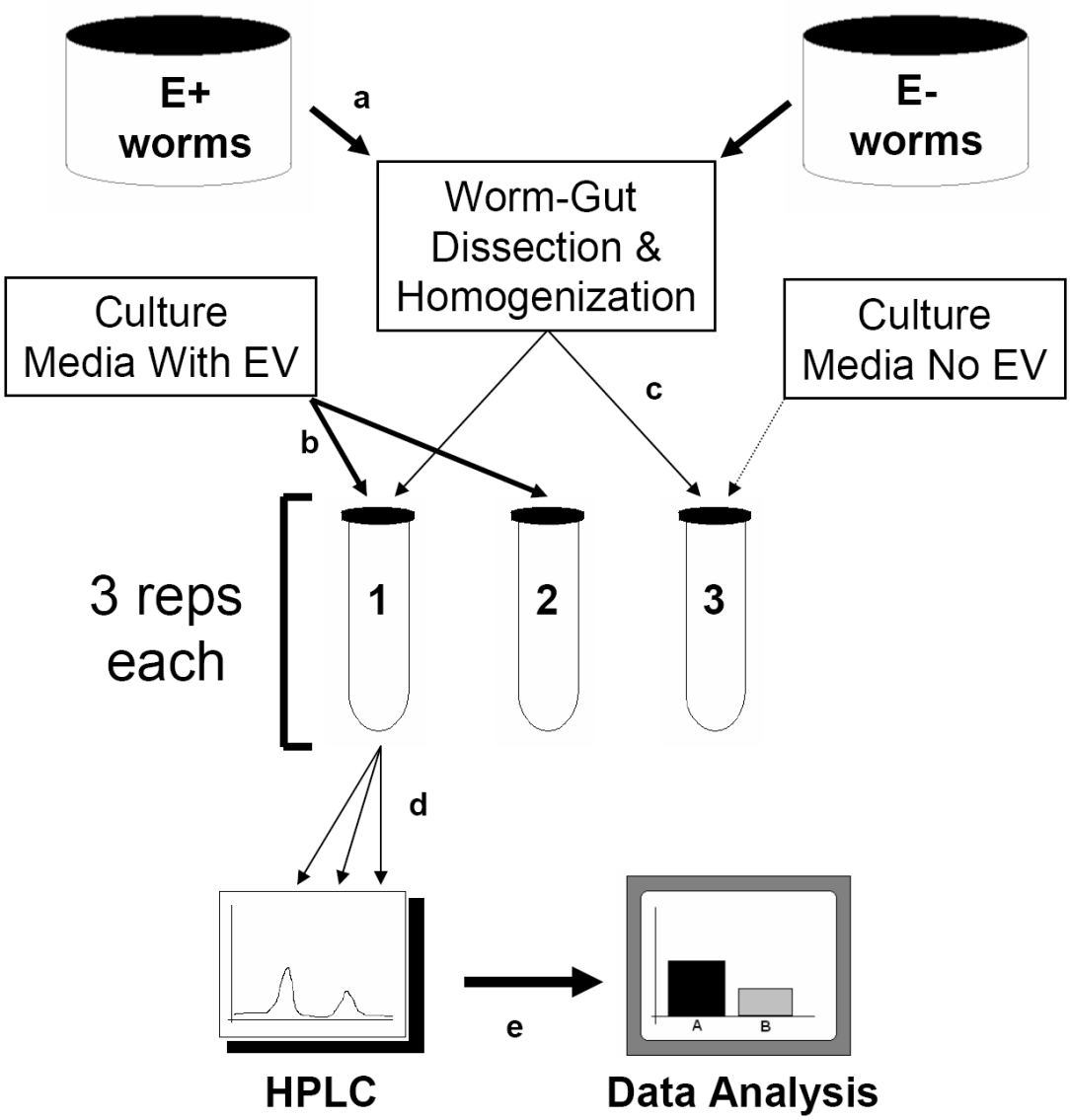

Figure 3.1. Flow diagram for the analysis of the metabolism of ergovaline by the anaerobic incubation of worm-gut homogenates. Worms were taken from each treatment and the guts were dissected and homogenized (a). Culturing treatments had 3 experimental replicates each. Anaerobic culture media without ergovaline was added to experimental treatment **3**. Anaerobic culture media was prepared with the sterile addition of ergovaline and distributed between experimental treatments **1** and **2** (b). Worm gut homogenates were added to the tubes of treatment **1** and **3** (c). Three sample replicates from each experimental replicate were taken per time point for HPLC analysis (d). HPLC results were analyzed and compared (e).

Collected worms were cleaned by soaking with ddH2O then sterilized by immersing in 100% ethanol for five minutes. Sterilized worms were transferred to a 50mL Falcon tube which was then flushed with O_2-free gas for 10 min and sealed tightly before bringing into the anaerobic glove tent (Coy, Grass Lake MI). Eight (8) worms from each treatment bin were dissected using a #20 scalpel and dissecting pins on an ethanol wiped, polystyrene board. Dissections were made through a length-wise incision from which the entire digestive tract of each worm, posterior to the gizzard, was collected. Harvested worm guts were placed in a 2.0 mL micro centrifuge tube and then homogenized with a battery-operated, hand-held tissue grinder and 0.7 mL of culture media.

3.2.2. Bacterial Culturing.

The anaerobic bacterial culture media used was modified from one described for the cultivation of rumen bacteria that metabolize the PAs from tansy ragwort [64; 81]. The formula for the media is the same except here we substituted the clarified rumen fluid component, for the same volume of a solution that was derived from the worm cast of the E+ treatment bin. This clarified soil fluid was created by adding 200 g of worm cast to 1000 mL of HPLC grade water. The mixture was mechanically stirred for one hour at about 120 rpm. The solution was allowed to settle for an additional hour. The supernatant was then collected and centrifuged at 16,000*g* for 30 minutes. This clarified supernatant was collected in plastic bottles and stored at -20° C until needed for media preparation. This anaerobic soil media was prepared and used in the same manner as described previously [81]. The worm samples from each treatment bin were treated as separate experiments. Each experiment consisted of three culturing treatments, and each treatment had three (3) replicates. One treatment had only worm-gut homogenate (WG) added to each replicate as a negative control. The positive control treatment

was un-inoculated culture media with the addition of EV. The experimental treatment had both WG and EV.

One anaerobic serum bottles fitted with a butyl stopper and crimp-cap containing one hundred seven (107) mL of reduced media was used per experiment. Because EV is heat labile, we did not have a positive control treatment of autoclaved WG tubes with the addition of EV. We also found that it was too difficult to make sterile additions of EV to individual tubes and get the same starting concentration between all experimental tubes. Therefore, we opted for the method of transferring ten (10) mL of reduced media to each of the three tubes of the negative control treatment prior to the addition of EV. This left a total of 77 mL of reduced media without EV in the bottle. Two (2) vials of crystalline EV were taken from cryo-storage at -140° C under liquid nitrogen and reconstituted in methanol aerobically. The tubes were combined by adding 0.2 mL methanol to one tube, vortexing, then a short centrifugation before transferring the volume to the other tube and repeating the process before being taken into the anaerobic tent where the volume was added to the remaining 77 mL of media by syringe. The final concentration of EV in the media was expected to be 450 ng mL^{-1}. Ten (10) mL of media + EV was dispensed by syringe into Balch tubes fitted with a butyl septum and crimp-cap for the WG+EV and EV only treatments. Transfer 0.15 ml of worm solution into tubes of the WG+EV and WG only treatments was performed using a 1.0 mL syringe with 18 ga. needle and aseptic technique. Tubes of all treatments were incubated at 38° C with rocking and under low light in the anaerobic tent.

3.2.3. Experimental sampling and ergovaline quantitation.

A 1.0 mL syringe fitted with a 22ga needle was used for triplicate removal of 0.5 mL samples from each tube in the experiment, per day. These produced twenty-seven (27) samples for time-point analysis of alkaloid

concentration. All sampling was performed in the anaerobic tent. Samples were placed in labeled 1.5 mL microcentrifuge tubes. Samples were removed from the tent and centrifuged for 2 min at 10,000*g*. 0.3 mL of sample supernatant were placed in 0.5 mL HPLC vial inserts in amber HPLC vials and sealed with a sampling crimp cap. Analytes were separated using a Luna 150 × 4 mm, C18 column (Phenomenex, Torrance, CA) attached to a Perkin Elmer Series 200 autosampler and quaternary biopump. The method used to separate ergovaline (EV) and its stereo-epimer (ergovalinine; EV*), involved a 40 µl injection volume followed by a mobile phase gradient program where 60%:40% (A:B) was pumped for a 1 minute equilibrium step followed by a linear ramp to 5%:95% (A:B) over 2 minutes, then holding at that ratio for an additional minute after which the solvents were linearly ramped back down to the starting ratio over 2 minutes and held at that ratio for 1 minute before the next sample was injected. Solvent A was an aqueous 0.1 M ammonium carbonate solution, while solvent B was acetonitrile. The flow-rate was 1.0 ml per minute. The analytes were detected using a Perkin Elmer Series 200 fluorometric detector with the excitation wavelength set at 240 nm and the emission wavelength set at 420 nm. Peak areas for EV and EV* were integrated using the Perkin Elmer TotalChrom software. Data was exported to Excel (Microsoft, Redmond, WA) for analysis. The areas of both isomers were combined and used for concentration calculations in order to take into account the natural epimer shift that occurs with ergovaline. In this manner, the positive control did not falsely appear to be decreasing when there was only a shift toward equilibrium between the isomeric forms of the compound.

3.2.4. Cell collection and DNA purification.

Worm-gut homogenates produced for culturing inoculation as described above were used as the material from which DNA was extracted. The MoBio UltraClean Soil DNA Kit (MoBio, Carlsbad, CA) was used according to the

manufacturer's recommended protocol with modifications described as follows: Homogenate samples were centrifuged for 1 minute at 16,000*g* to pellet cells. The supernatant was removed and 60 µL of solution S1 was added to the cell pellet, which was resuspended, then transferred to the bead-beating tubes provided in the kit. Samples were briefly vortexed and then incubated at 70°C for 10 minutes. The recommended volume of 200 µL of Solution IRS was then added to each tube before being vortexed horizontally for 1 minute. Samples were then incubated for 10 minutes at 70°C. Samples were vortexed for 5 minutes horizontally and then centrifuged at 10,000*g* for a minute. The supernatant was transferred to a new tube before 250 µL of solution S2 was added. The samples were vortexed briefly before being placed on ice for 10 minutes. The samples were taken off ice and centrifuged for 2 minutes at 10,000*g*. Supernatants were placed in new tubes and 1300 µL of solution S3 was added. These samples were passed through spin filters provided with the kit in three 700 µL loads for 30 sec. at 10,000*g*. A volume of 300 µL of solution S4 was passed through each filter for 30 sec @ 10,000*g*. Filters were spun an additional minute after the elutant was poured off to insure dryness. Genomic DNA was eluted into a final volume of 30 µL using MoBio solution S5 (10mM Tris) by adding the solution to the filter, incubating at room temperature for one minute, then centrifuging for one minute at 10,000*g*. The purifications were checked for quality and size by 1% TAE agarose gel and for quantity using a NanoDrop 1000 UV spectrophotometer (A_{260}; NanoDrop Technologies, Wilmington, DE).

3.2.5. 16S rDNA-based L4M2 clone library construction.

PCR primers were used to amplify the 16S rDNA from genomic DNA extracted from worm-gut homogenates of the E+ and E- treatment bins. For clone library construction, primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′, positions 7 to 26 of *E. coli*) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′,

positions 1513 to 1494 of *E. coli*) were used [105]. All PCR thermocycling was carried out using recombinant *Taq* polymerase (Fermentas Inc., Glen Burnie, MD) in a PTC-200 thermocycler (MJ Research Inc., Watertown, MA). Each 25 µL PCR reaction contained: about 60 ng of purified genomic DNA, 200 µmol of each dNTP, 2.5 µL of 10x PCR Buffer 2 mM Mg^{2+}, 200 nM of each , 1.0 U polymerase and 17.25 µL sterile 18 MΩ water. Amplification of 16S rDNA using the fD1/rP2 primer set was achieved with a thermocycling program that consisted of a 2 min denaturation cycle at 95˚C followed by 25 cycles of 20 seconds at 95˚C, 20 seconds at 57˚C and 45 seconds at 72˚C, with a final elongation cycle of 72˚C for 10 minutes. PCR products were purified using the QIAquick PCR purification kit according to the manufacturer's recommendations (Qiagen Inc., Valencia, CA). Concentrations of purified products were determined by NanoDrop. Cloning and transformation of competent E. coli cells using PCR products from the fD1/rP2 reactions was performed with the TOPO TA Cloning Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's recommendations. The pCR 4-TOPO® vector was used in conjunction with TOP-10 chemically competent cell of *E. coli* (Invitrogen Corporation, Carlsbad, CA). After transformation, cells were pelleted by centrifugation at 1500*g* for 30 seconds. The supernatant was removed and the cells were resuspended in 400 µL of new S.O.C. media (Invitrogen Corporation, Carlsbad, CA). After resuspension, 200 µL from each transformation was spread onto two separate 24 x 24 cm Falcon bioassay plates, each containing LB agar (EMD chemicals Inc., Gibbstown, NJ) supplemented with 50 µg mL^{-1} kanamycin (EMD). Plates were incubated overnight at 37˚C. The Qpix 2 colony picking robot (Genetix, Boston, MA) was used to pick 960 clone colonies from each library. Picked clones were archived into individual wells of a 96-well microtiter plate containing 200 µL LB broth with a 1X concentration of Hogness buffer [89] and supplemented with 50 µg mL^{-1} kanamycin. After overnight incubation at 37°C with shaking, the archive plates were stored at -20°C. Archive plates were

labeled according to treatment: E- treatment clone plates were set “A” followed by the number of the plate in the group. (e.g. A01 – A10). E+ treatment clone plates were set “B” followed by the number of the plate in the group (e.g. B01 – B10).

3.2.6. Plasmid preparation and library screening.

Plasmid DNA preparation from clones was similar to the method described in section 1.6 of Current Protocols in Molecular Biology [90] and was as follows. Archived cell libraries were used to inoculate corresponding deep-well blocks containing 300 µL of media TYGPN [34] supplemented with 50 µg mL^{-1} kanamycin. Each block was covered with porous sealing tape and incubated for ~24 hours at 38°C with shaking at 300 rpm. Cells were pelleted by centrifugation at 4000 rpm for 20 min. The supernatant was then removed. 50 µL of GTE solution with Rnase A (50 µg mL^{-1}) was added and the cells were resuspended by vortexing. A solution of 0.2N NaOH, 1% SDS was made from 1N NaOH and 10% SDS stock solutions. 100 µL was added to each well of the block. The wells were mixed by tapping and then incubated at room temperature for 5 minutes. 50 µL of 5M potassium acetate was added and mixed by tapping before being placed on ice for 5 minutes. Blocks were then centrifuged for 20 minutes at 4000 rpm. 150 µL of supernatant was transferred to a new tube, to which 150 µL of cold isopropanol was added. The tubes were incubated at -20°C for 20 min and then centrifuged for 20 minutes at 4,000 rpm. The supernatant was decanted off and the pellet was washed with 100 µL of 70% ethanol by vortexing. The pellet was spun again for 10 minutes at 4,000 rpm. The supernatant was poured off and the pellet was re-washed and re-spun. Plates were air dried at 38 °C for 30 minutes. DNA was resuspended in 30 µL of TE (pH 8.0) and stored at -20°C until further use.

About 250 ng of plasmid preparation from each clone were digested using the restriction endonuclease BstN1 (New England Biolabs, Ipswich, MA). Reactions were set-up in 96-well PCR plates as 20µL final volumes

using 1 unit of enzyme, 1X final buffer concentration, 100 μg mL^{-1} BSA. Plates were incubated for at least one hour at 60°C followed by separation of fragments using a 2.5% TBE agarose gel. Samples were run on a 24 x 40 cm gel at 150V for 30 min. The voltage was then increased to 200V for two additional hours. Gels were stained with ethidium bromide and visualized by UV trans-illumination. Images of gels were captured using a Kodak DC120 digital camera connected to a computer running Kodak 1D image analysis software, version 3.6 (Eastman Kodak, Rochester, NY). Sample lanes were compared to a 100 bp size ladder (Fermentas Inc., Glen Burnie, MD) in order to calculate fragment size. Only fragments between 240bp and 1031bp were used for analysis of RFLP patterns.

The restriction pattern from each clone was converted into a data string using a script developed for use in the R package for statistics [99]. Fragment size data was binned into a 'present-or-absent' string of 0's and 1's given a user-defined fragment size range of 0 – 1031 and string length of 30, which was then applied to each clone's fragmentation pattern. The RFLP data strings were imported into a NEXUS based character matrix generated by the Mesquite software package [65]. The RFLP data from each clone library was place into an independent matrix. The NEXUS files produced by Mesquite were input into Mr. Bayes version 3.1.2 [4; 49; 83]. Markov Chain Monte Carlo was performed on the RFLP data using the F81 two-state model. The data was analyzed for 100,000 generations. Consensus trees displaying each library's clone names were formed from the best 100 trees from each library's analysis.

An interleaved PHYLIP format of the Mesquite character matrix from each library was also converted to a maximum likelihood distance matrix using The program Treepuzzle 5.2 [85; 97] by performing a likelihood mapping analysis of the binary state data using the F81 model of substitution. The distance matrix served as the input for an analysis of RFLP pattern richness using DOTUR [84]. Rarefaction values for a 3% difference between taxa were

examined and used as a guide for picking the number of clones that would be sequenced from each library. It was determined that 192 clones from each library would be chosen for sequence analysis. Clones were chosen based on the Bayesian consensus tree topology. Where clusters of two or more clones were found on the tree, only one clone from each cluster was chosen as an example for sequencing. The desired number of clones was not reached by the first selection method. Therefore, some clones were chosen that fell into distinct branches, not in a cluster. Random clone choices were also made in order to make up the difference in number of clones for sequencing from each library.

3.2.7 Sequencing and phylogenetic analysis of clone inserts.

Cycle sequencing was performed using the ABI Big Dye Terminator system (Applied Biosystems, Foster City, CA) and the resultant products were analyzed with an Applied Biosystems capillary 3730 Genetic Analyzer. Partial sequencing of clone inserts was accomplished by elongating from T7 promoter and T3 primer sites on the TOPO-TA vector. To obtain overlapping reads of the whole 16S insert, ABI chromatograms of the partial reads were imported in the Geneious software package [30] and made into contiguous sequences using the 'Contig' tool. Only sequence contigs that were found to have the original D1f and P2r primer sequences on their terminal ends were used for analysis. 16S insert contigs were checked for chimeric sequences using the chimera detection tool from the RDB II website (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU) [16]. Sequenced clones were checked for redundancy by nearest neighbor analysis. Groups of clone sequences with a similarity score of 97% or more were collapsed to one representative sequence for downstream analysis. The remaining clone sequences were compared to sequences in published databases using NCBI BLAST [5]. Reference sequences of named organisms from the databases with the highest homology scores to the query sequences were chosen for

phylogenetic comparison when possible. A multiple alignment of full-length 16S rDNA clone and reference sequences for each library was performed using the Clustal W multiple sequence alignment algorithm [100]. The IUB/Bestfit weight matrix was used with a gap open penalty of 10.0 and a gap extension penalty of 0.1. The aligned sequences were used to construct phylogenetic trees by neighbor-joining analysis using 100 bootstrapping iterations. These were used to construct consensus trees representing the 16S rDNA associations made by clones of each treatment library to reference sequences pulled from GenBank [11]. Consensus trees were viewed using Njplot [79] and edited using The Gimp 2.2 graphic editing software in Linux (http://www.gimp.org/unix/). All cloned 16S sequences produced for phylogenetic analysis will be deposited in GenBank.

3.3. Results

3.3.1. Anaerobic incubation of ergovaline.

Anaerobic incubation experiments were performed for the duration of three days. Examination of samples from the E- and E+ vermicompost treatments were performed separately but within one week of each other. The results from both incubation experiments' WG+EV treatments displayed no greater loss of EV+EV* from that of the positive control (EV only). For the E- sample incubation, the starting and ending concentrations of combined EV+EV* were 238.3 ng mL^{-1} and 211.9 ng mL^{-1}, respectively, for the treatment incubated with worm-gut homogenate. These values were 212.3 ng mL^{-1} and 209.6 ng mL^{-1} for the EV only treatment. For the E+ sample incubation, the starting and ending concentrations of combined EV+EV* were 423.5 ng mL^{-1} and 367.9 ng mL^{-1} for the WG+EV treatment. Starting and ending concentrations for the EV only treatment were 380.2 ng mL^{-1} and 368.2 ng mL^{-1} respectively (Fig. 3.2). Data for the WG only treatments of both

incubation experiments are displayed in Figure 3.2; however, the values are extrapolated because they fell below the lowest value of the calibration curve used to calculate EV+EV* concentrations. With the exception of the first two time points of the E+ incubation experiment (Fig. 3.2B), all within experiment time point values for the WG+EV and EV only treatments had overlapping standard deviations and could not be considered significantly different. Preliminary work performed in December, 2006, before the incubations described in Figure 3.2 demonstrated that metabolism of EV/EV* can occur using the experimental conditions described (Fig. 3.3). That work also demonstrated that the observed metabolism of EV/EV* is dependant on bacteria from the worm-gut homogenate. The antibiotic treatment experiment was performed using the same duplication methods as described for the other worm-gut incubations. In that experiment, only worms from the E+ treatment bin were used and so there are no incubation data for the same type of experiment using E- worms from the same period in time.

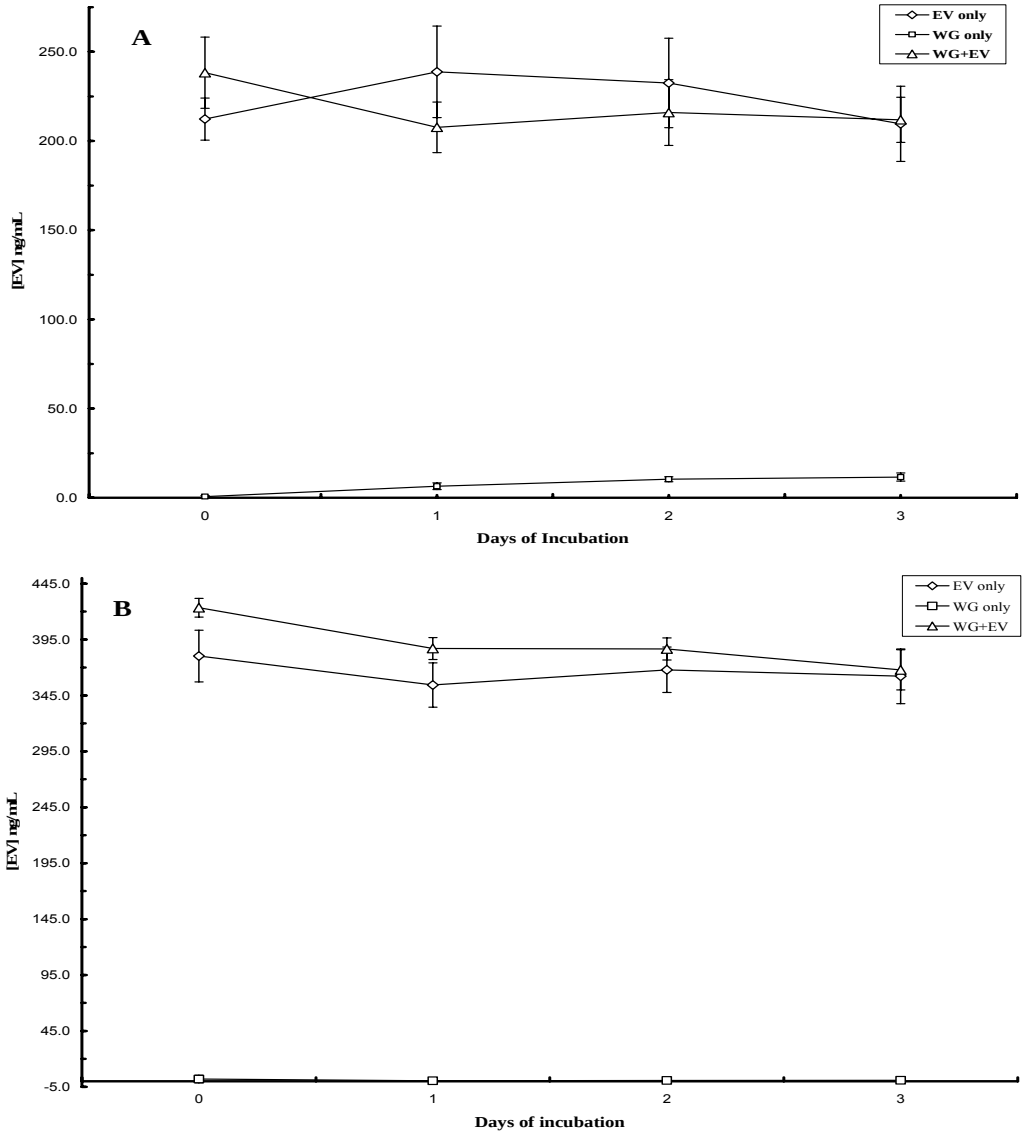

Figure 3.2. Results of WG incubations with and without ergovaline. Concentrations of detectable ergovaline and its epimer ergovalinine during incubation with worm-gut homogenates (WG+EV) and experimental controls (WG only; EV only) from E- treatment bin (A) and E+ treatment bin (B). Error bars indicate standard deviation.

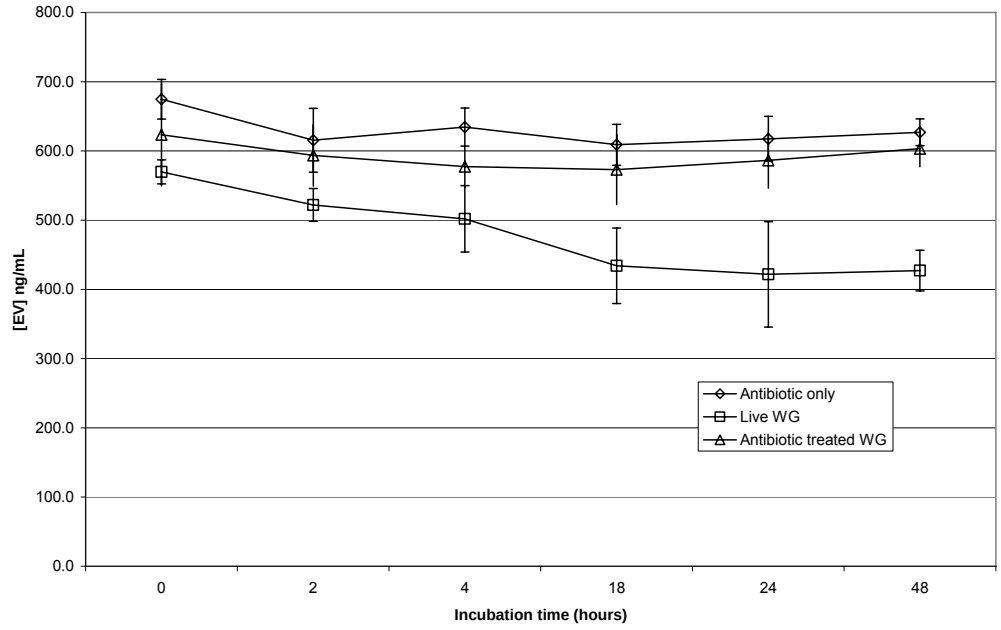

Figure 3.3. Metabolism of ergovaline and its epimer by E+ treatment worm-gut homogenates with and without the addition of antibiotics. Total ergot alkaloid (EV/EV*) concentration displayed on the y-axis in ng mL^{-1}. The antibiotics erythromycin and ampicillin were added to bacterial culture media S to a final concentration of 100 ng mL^{-1} each. Treatments were incubated at 38°C with shaking at 25 rpm.

3.3.2. Ribotype Analysis.

Two 960 clone libraries were constructed using bacterial 16S rDNA-PCR products amplified from genomic DNA purified from the gut homogenates of the E- treatment (library A) and the E+ treatment (library B). The libraries were screened by restriction digestion and RFLP analysis of plasmid preparations from the clones. This process yielded 736 usable restriction patterns from library A and 811 patterns from library B. The restriction data was converted into distance data, which was then analyzed by DOTUR as described in the methods section (3.2.6). The results of the rarefaction analysis are shown in Figure 3.4. This analysis determined that there were 237 ribotypes within library A, and 311 ribotypes in B. These values represent the number of unique ribotypes with a 3% or greater difference. The restriction

data from each clone was also converted into a data string as described in the methods section and clustered using Bayesian analysis. One Bayesian consensus tree was produced for each clone library with the same number of taxa as there were usable restriction profiles from each library. From each library's tree, 192 clones were picked for sequencing according to the criteria stated in the methods section of this chapter.

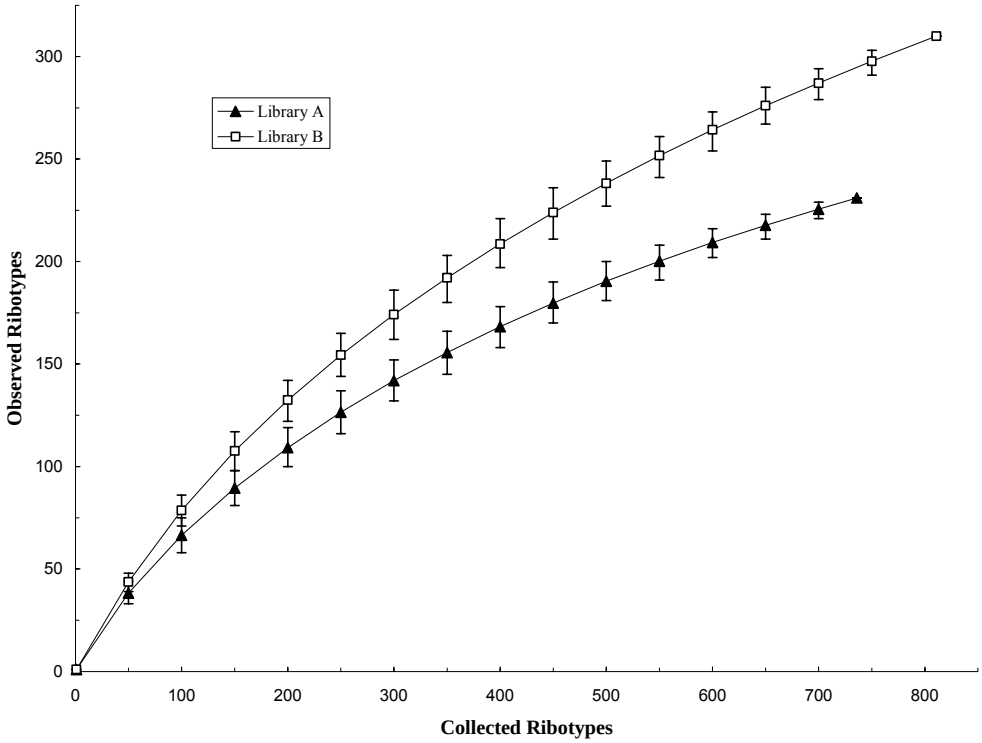

Figure 3.4. Rarefaction curves produced from DOTUR analysis. The collected number of ribotypes from each library was plotted against the number of observed of ribotypes at the 97% similarity level. Each point represents the maximum number of observed ribotypes for the given number of collected ribotypes. Error bars indicate the 95% confidence interval.

3.3.3. 16S rDNA Sequencing Analysis.

Of the clones sequenced from each library, 167 non-chimeric sequences were obtained for library A (E- treatment) and 165 sequences were obtained for library B (E+ treatment). The sequences from each library were aligned and examined for redundancy. In the end analysis, 105 sequences having 97% or less similarity to other clones in library A were used and 96 clones from library B. Clone sequences from both libraries were compared to 98 reference sequences obtained from GenBank using the alignment and phylogeny techniques described. Phylogenetically similar clades of sequences were broken down into seven sub trees according the phyla to which the reference sequences fell. Phyla represented in the libraries were *Actinobacteria* (Fig. 3.5), *Bacteroidetes* and unclassified sequences (Fig. 3.6), *Chloroflexi* and *Verrucomicrobia* (Fig. 3.7), *Firmicutes* (Fig. 3.8), *Planctomycetes* (Fig. 3.9), *Alpha proteobacteria* (Fig. 3.10) as well as *Beta, Gamma, Delta* and *Epsilon proteobacteria* (Fig. 3.11). With the exception of the *Delta proteobacteria* (Fig. 3.11), every phyla used in the analysis had clones from both libraries make some associations within them. However, not all sub-groupings found within a phylum had associations made by clones of both libraries; in fact, not all clones from one library made an association to one or more clones from the other library. There were very few closely related associations made. Most associations made between clones of each library with references and each other appeared to be rather loose, with some associations having very deeply rooted ancestry to their nearest neighbor. One example of this is that the best BLAST hit for clone A03-08H was to a plastid sequence from a pathogenic green algae species of *Helicosporidium* (Fig. 3.6).

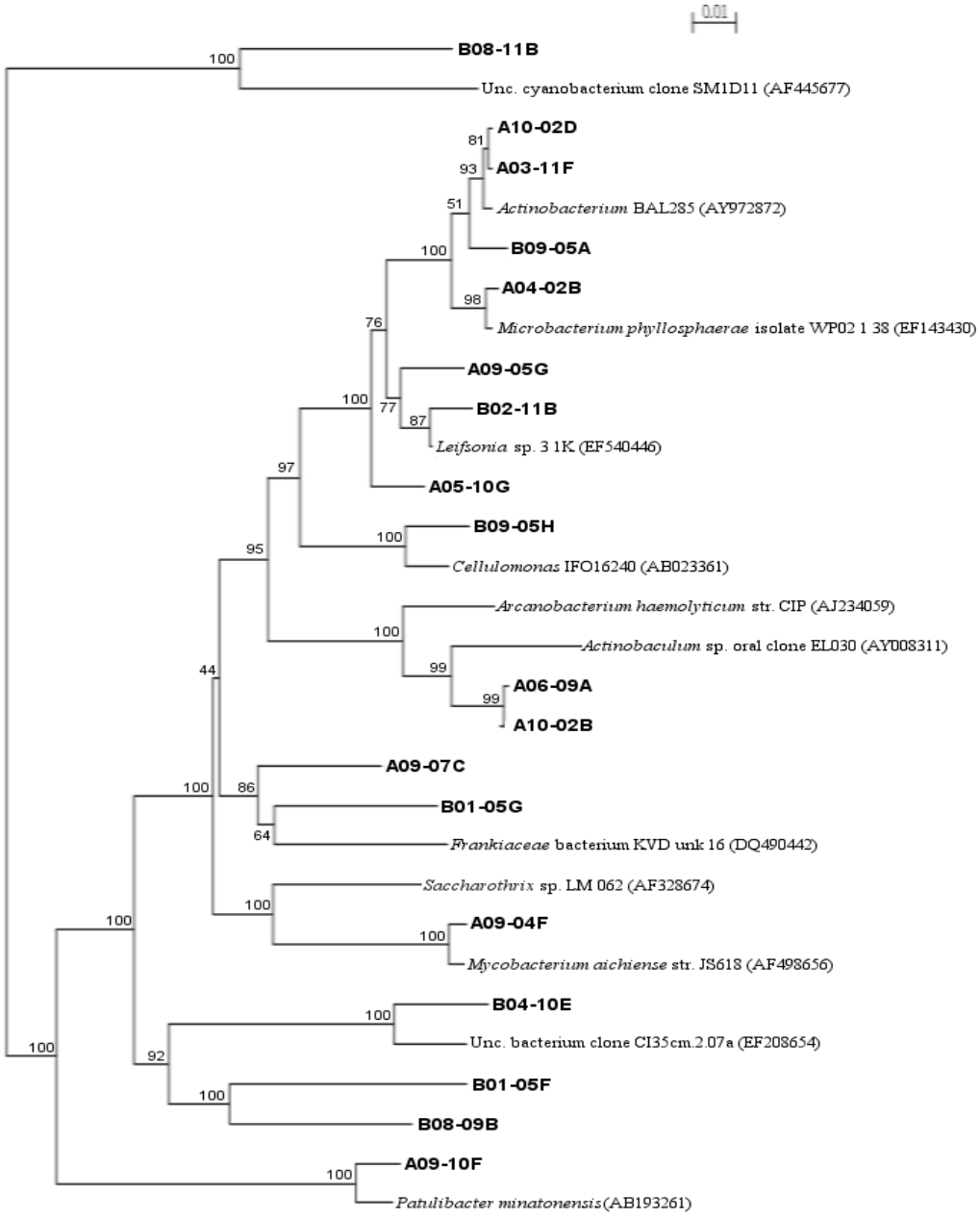

Figure 3.5. 16S rDNA phylogenetic associations within the phylum *Actinobacteria.* Neighbor-joining analysis with 100 bootstrapping generations with the corresponding values placed at the nodes of the tree. GenBank references have their accession number in parentheses. Clones are represented in **bold** type. Clone names indicate treatment. Clones beginning with **A** are from the E- treatment. Clones beginning with **B** are from the E+ treatment. Scale bar indicates 1% sequence divergence.

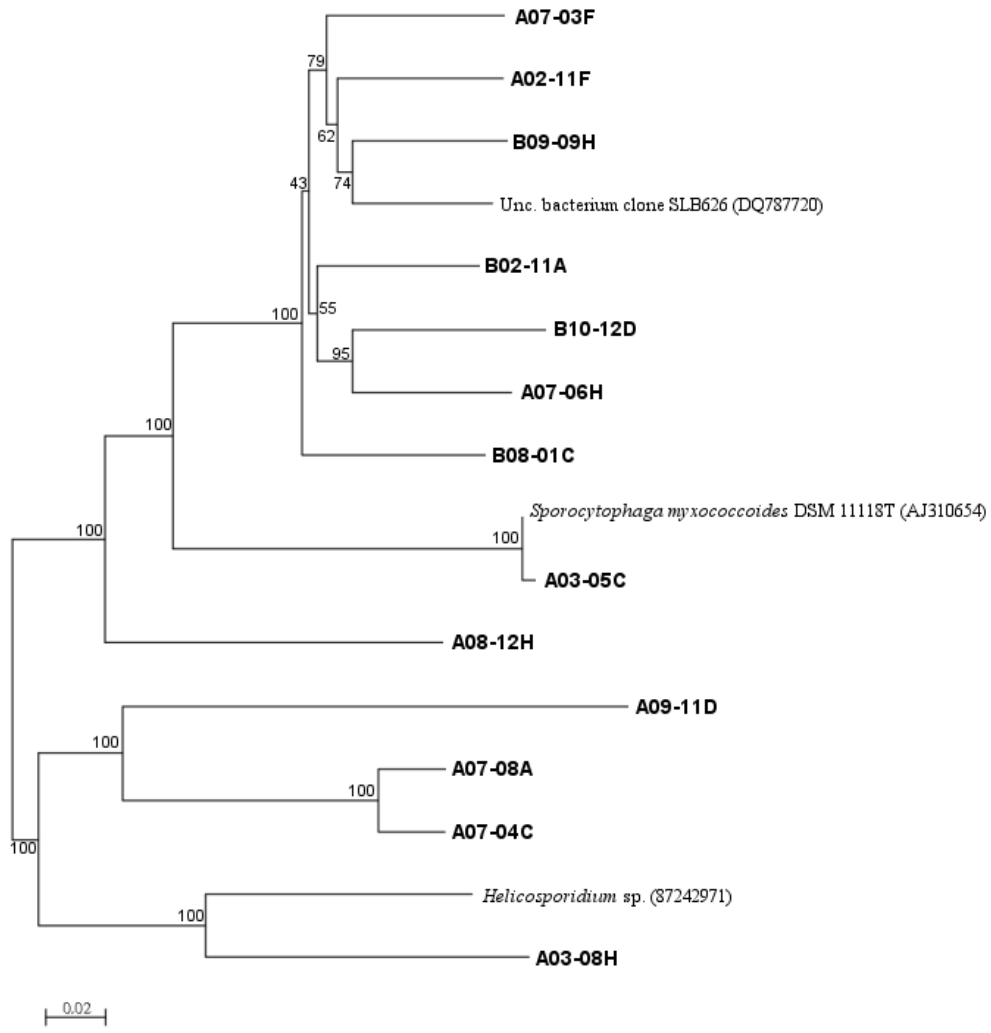

Figure 3.6. 16S rDNA phylogenetic associations within the phylum *Bacteroidetes*, other unclassified references and a green algae*.* Analysis parameters were the same as for Fig. 3.5. Scale bar indicates 2% sequence divergence.

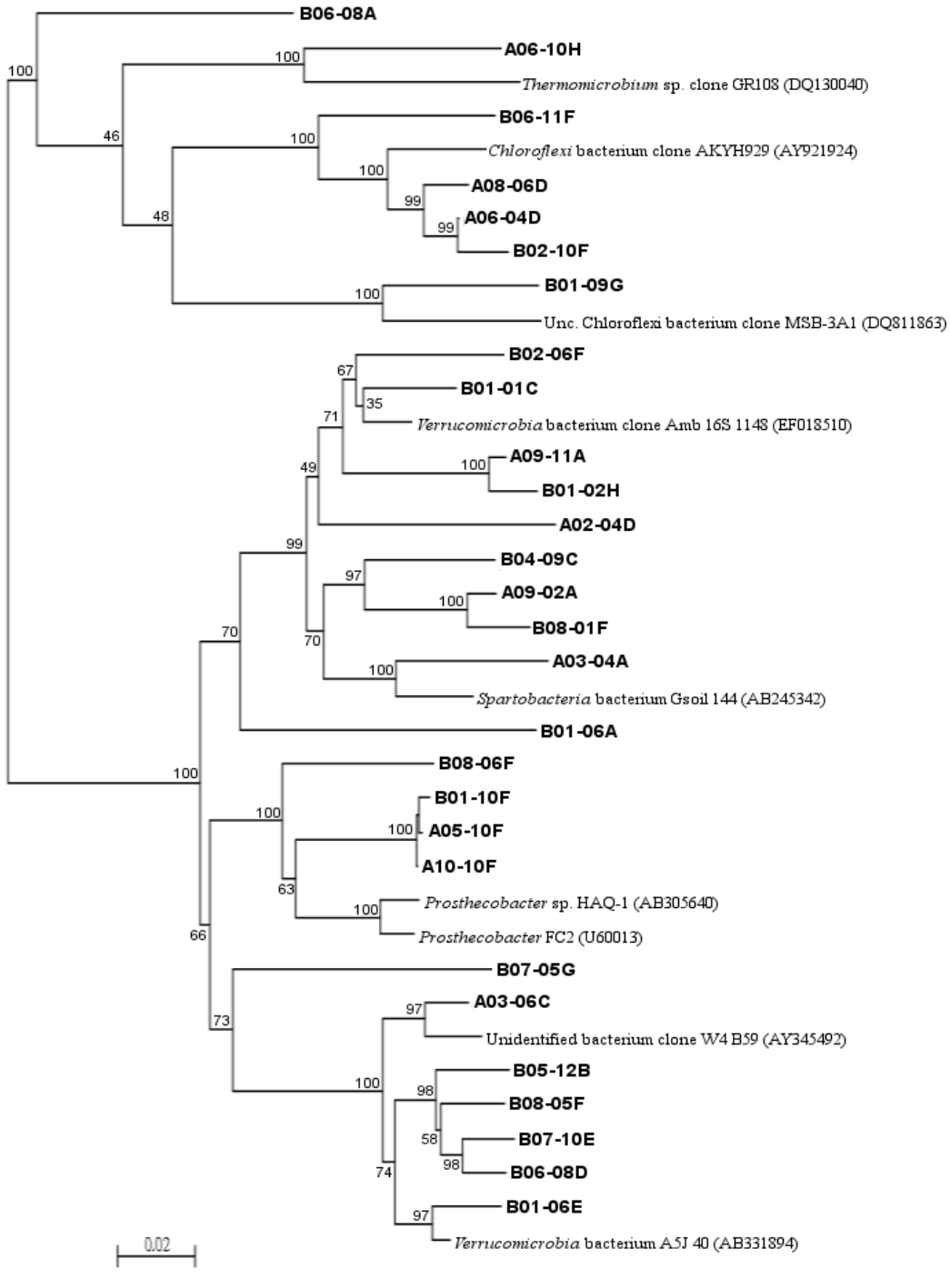

Figure 3.7. 16S rDNA phylogenetic associations within the phyla *Chloroflexi* and *Verrucomicrobia.* Analysis parameters were the same as for Fig. 3.5. Scale bar indicates 2% sequence divergence.

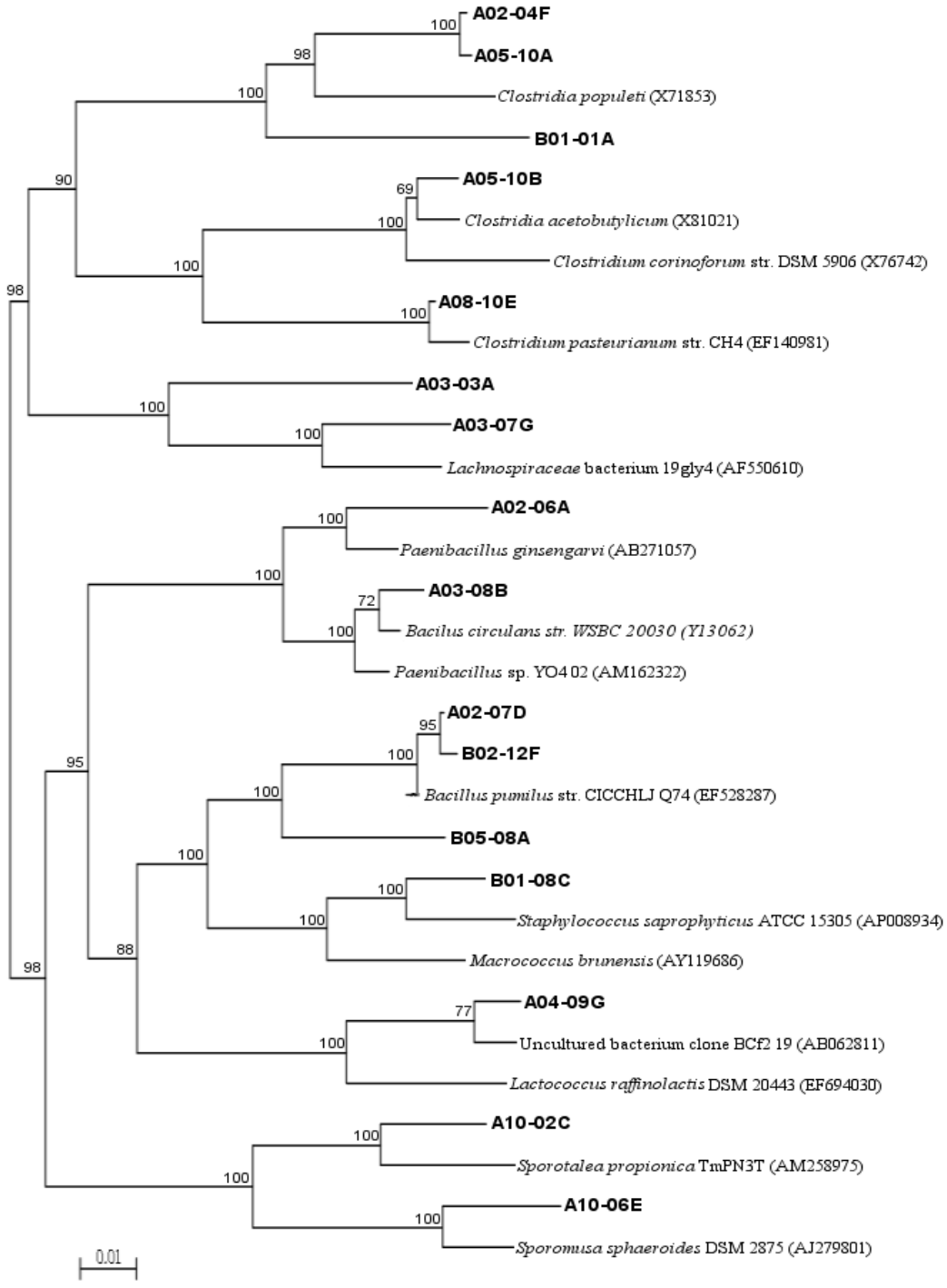

Figure 3.8. 16S rDNA phylogenetic associations within the phylum *Firmicutes.* Analysis parameters were the same as for Fig. 3.5. Scale bar indicates 1% sequence divergence.

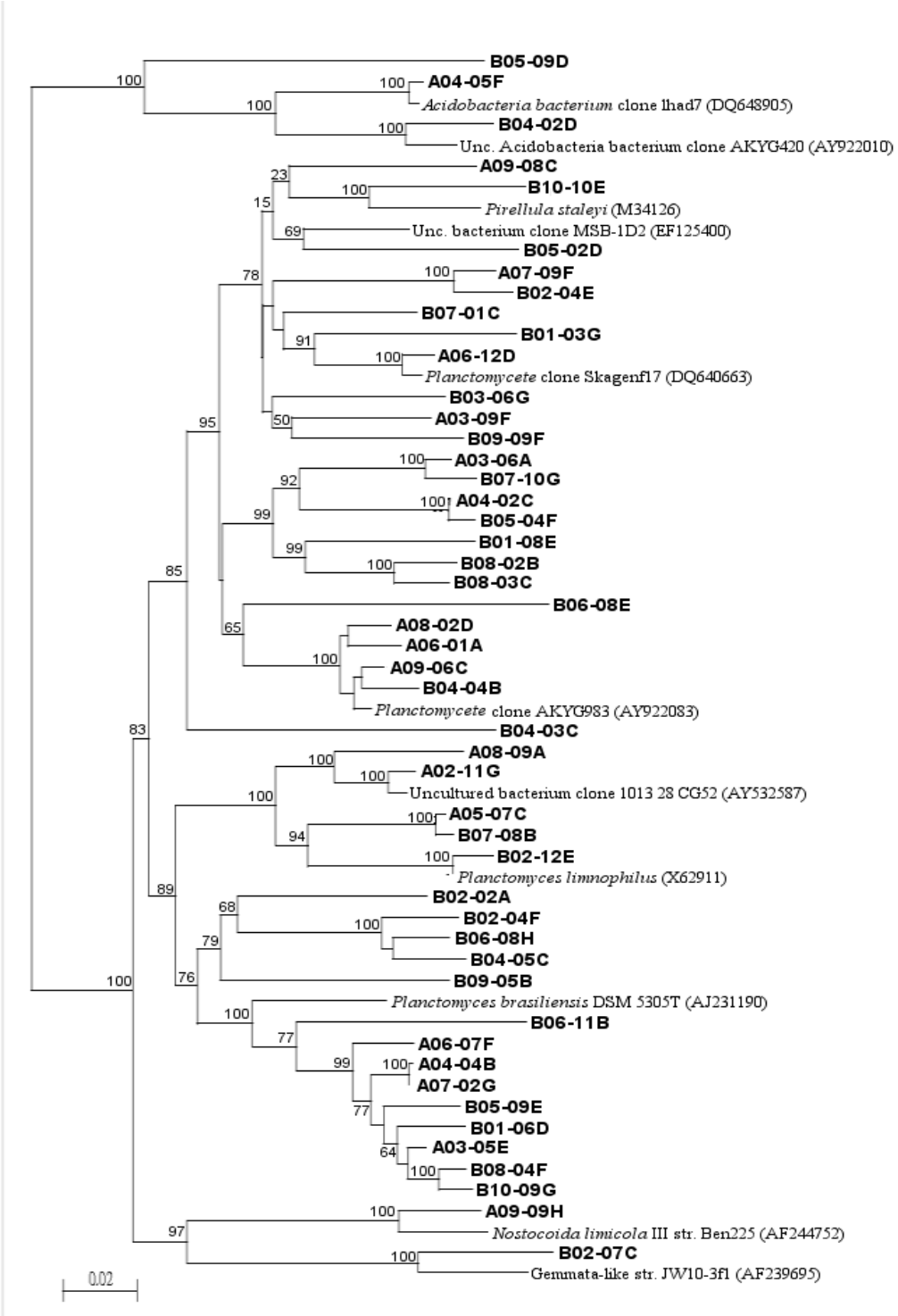

Figure 3.9. 16S rDNA phylogenetic associations within the *Planctomyces.* Analysis parameters were the same as for Fig. 3.5. Scale bar indicates 2% sequence divergence.

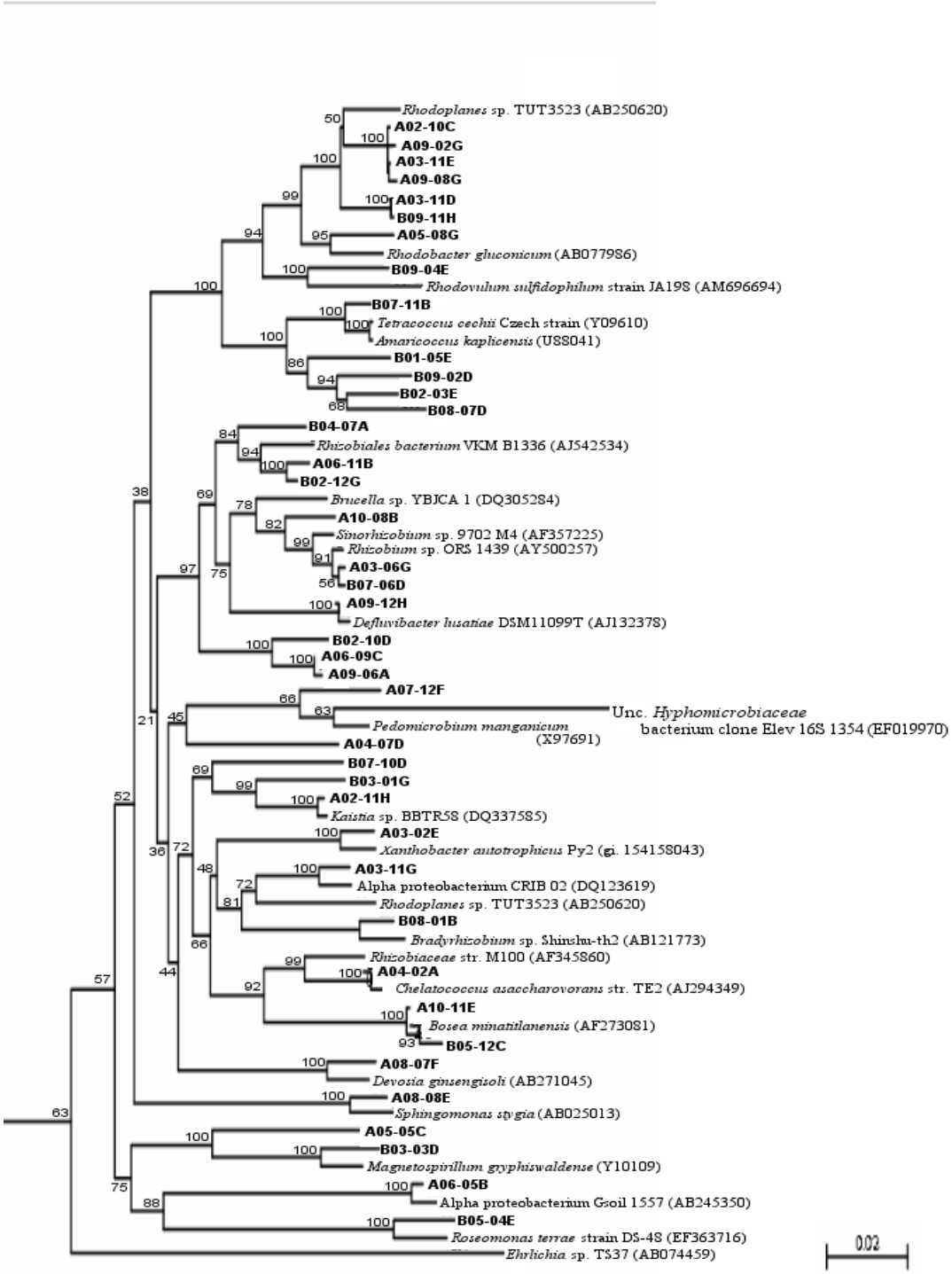

Figure 3.10. 16S rDNA phylogenetic associations within the *Alpha proteobacteria.* Analysis parameters were the same as for Fig. 3.5. The tree is rooted to the other *Proteobacteria* sub-classes (Fig. 3.11). Scale bar indicates 2% sequence divergence.

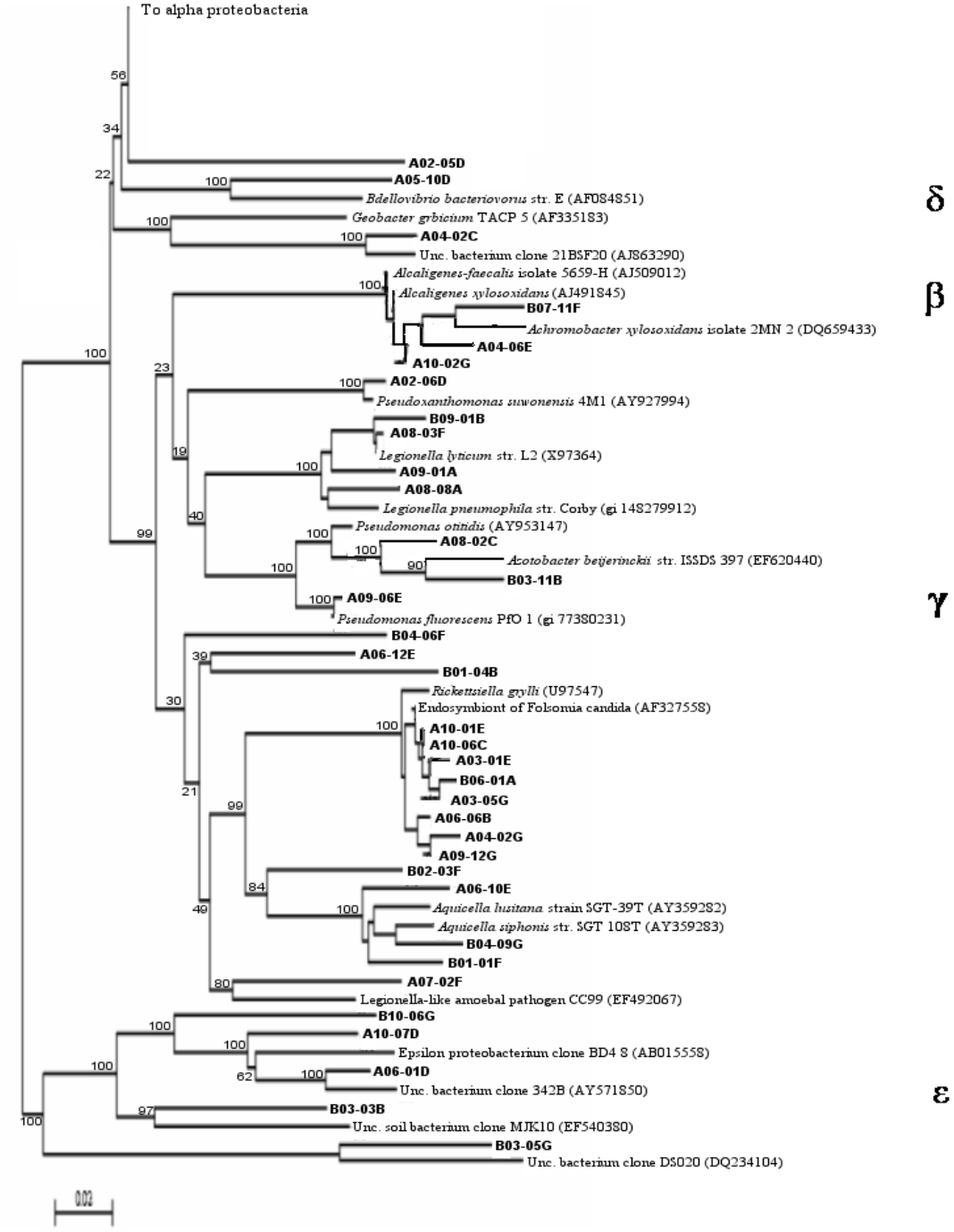

Figure 3.11. 16S rDNA phylogenetic associations within and between the *Beta, Gamma, Delta* and *Epsilon proteobacteria.* Analysis parameters were the same as for Fig. 3.5. The tree was rooted to indicate the connection to the *Alpha proteobacteria* (Fig. 3.10). Scale bar indicates 2% sequence divergence.

3.4 Discussion

I have investigated the differences in the metabolic activity toward the endophyte toxin, ergovaline, by incubations of gut microflora from the earthworm *E. fetida* vermiculturally grown under two different treatment conditions. The prokaryotic 16S diversity found within the guts of the worms used for culturing was also examined. To the author's knowledge, this has been the most comprehensive study of prokaryotic 16S diversity found solely within the gut of *E. fetida* to date and I am the only person that has examined the effects the gut contents directly have on the disappearance of ergovaline and its epimer from microbial culturing conditions.

Experimentally, worm guts were dissected from the animal and the tissues were homogenized in order to release any viable microbes. Methods for the anaerobic incubation and measurement of ergovaline were established in order to monitor the disappearance of the compound. Though there was a method available by which ergovaline is chemically extracted from plant material for quantitative analysis [20], it was too time consuming and it limited the number of samples that could be taken at experimental time points. That method also required more sample material than was practical for taking multiple time point samples from a single incubation replicate. The HPLC separation and detection method used previously was long enough that the number of experimental samples necessary for statistical significance could not be examined in a timely manner. It was found by trial and error that the anaerobic time-point samples could be examined using a very small volume and without the need for chemical extraction. This allowed samples to be ready for HPLC analysis in minutes as opposed to hours. This also allowed for smaller culture volumes to be used, which required using less of the very expensive analyte, ergovaline, to achieve the desired starting concentrations. The time of the sample analysis was reduced to about 25% of the time that was published for another HPLC analysis of ergovaline [32]. This improvement

allowed for more samples to be analyzed, which helped improve the statistical significance of each sample time point.

The incubation of gut contents from worms living in the vermicompost treatment bins during the late winter month of February did not indicate any more loss of ergovaline and its epimer from culture than that observed for positive controls (Fig. 3.2). However, E+ vermicompost treatment may potentially harbor anaerobic bacteria capable of metabolizing ergovaline as indicated by data presented in Figure 3.3. Further investigation is necessary in order to determine whether the metabolism observed in that preliminary experiment is reproducible. Antibiotic incubations should also be performed using worms from the E- treatment bin as well. It is likely that the lack of ergovaline metabolism by WG homogenates collected in February is because of the repression of the microbes responsible for the observable loss of ergovaline and epimer. As was demonstrated by earlier data (Fig 3.3), a total loss of alkaloid upwards of 25% of the beginning concentration is possible. Preliminary incubation experiments performed using WG homogenates taken from the E+ treatment bin in October, 2006, indicated that it may be possible to observe upwards of a 60% loss of ergovaline and epimer from culture over 48 hours (data not shown). The vermicompost bins used for these experiments were housed in a sheltered location. However, they were still subjected to the daily fluctuation in ambient air temperature during the seasons. At the time that samples were pulled for these experiments, the average temperature of the E+ bin was about 12°C, while the E- bin was closer to 7°C. Temperatures during the summer months were much closer to 30°C. Though the earthworm, *E. fetida*, can tolerate a temperature range of 4°C to 30°C [29], the incubation experiments indicate that the microbes potentially involved in alkaloid metabolism do not grow well at the lower temperatures of the winter months and may actually go dormant during that period of the year. More evidence that points toward the idea that the alkaloid metabolizing microbes prefer higher temperatures is that other preliminary

incubation experiments that were performed indicated that more ergovaline disappearance was observed in cultures incubated at 38°C as opposed to the more standard temperature for culturing soil microbes of 25°C (data not shown).

The results of DOTUR rarefaction analysis of the RFLP ribotypes appeared to overestimate the amount of diversity that could be expected from these libraries. Out of the 192 clones selected for sequencing from each library, about half of these (~100 sequences each library) were found to be non-redundant within a treatment. In the case of the E+ treatment library, that was one-third of the 311 taxa estimated by rarefaction and about one-half of the 237 taxa for the E- treatment library. These results indicate that rarefaction may not be an appropriate tool for the estimation of diversity using RFLP data. The results from Mr. Bayes clustering analyses proved to be much more robust. In fact, more clones were picked from the consensus trees produced using Mr. Bayes than would have been deemed necessary had the Bayesian data alone been used to pick clones. In this analysis, most of the clones in each library (76% from E- and 84% from E+) were examined by RFLP. After clustering, selection and sequencing there were no major differences in the global composition of the two treatment libraries; therefore, it is reasonable to believe that the phylogenetic trees produced are representative of the full 16S sequence diversity encapsulated by the clone libraries.

The prokaryotic diversity present in the gut of the earthworm is impressive, though not unexpected. Because the earthworm tunnels through its environment by consuming the matrix of soil and plant detritus around it, we should expect to find many of the same microbes present in the worm gut as would be found in the surrounding soil [22; 41; 55; 87]. Although the present study did not investigate the 16S diversity of the worm cast from each treatment, it did examine the diversity of the microbes found within the guts of worms from both experimental treatments. Because there was no difference in the observed metabolism of alkaloid by incubating samples from both

treatments, we expected that there would be little difference between the profiles of sequences found in the clone libraries. In fact, this was the case. Of all of the major phyla examined during the phylogenetic analysis, only the δ-*Proteobacteria* lacked clone sequences from the B library (E+ treatment). The most remarkable feature about the phylogenetic analysis was how unrelated most of the clones were to any sequences that were pulled from the databases to use as references. Very few clones in either library had a similarity score greater than or equal to 97% when compared to a reference sequence. Most clone sequences scored between 95% and 85% similarity to a reference sequence. This trend is observable upon examination of the branch lengths found between taxa of the trees produced by this study. These results would indicate that there are many potentially uncharacterized microbes that exist within the environments of both the worms used in this study, as well as the surrounding cast the worms are living in.

This study has established the base from which the discovery of ergot alkaloid metabolizing bacteria will be established. Even though the data produced from incubations of winter-time worm gut microbes did not demonstrate the presence of alkaloid metabolizing bacteria, preliminary work has shown this to be a viable model system to derive such microorganisms. The leading contribution that was put forth in this work was the establishment of reliable, reproducible, experimental methods that can and will be used to detect the bacterial dependent metabolism of ergovaline and its epimer under anaerobic culturing conditions. This work has also sought to establish the 16S profile of the worm gut environment from *E. fetida* grown under two experimental vermicompost conditions, using tall fescue seed with and without ergovaline as treatments. As the sequence libraries were produced from a moment in time when the worm-gut microbes were not capable of metabolizing the alkaloids, they will act as a good reference of the background bacterial populations found within both treatment bins. When later incubations are found to be capable of metabolizing the alkaloids again, new libraries can

be constructed and compared to the ones presented here. Ultimately, by performing anaerobic culturing experiments using worm-gut contents as an inoculum, it is hoped that the bacteria, or bacterial consortium, responsible for the metabolism of ergovaline and its epimer can be isolated and propagated for potential use as a probiotic supplement for ruminant animals challenged by the presence of ergovaline in their feed.

Chapter 4

4.1. General Conclusions

The work presented in chapters 2 and 3 explored different methods for examining the diversity of anaerobic microbes responsible for the metabolism of plant alkaloids that are deleterious to human and animal health. Both pieces of work utilized analytical chemistry methods in order to observe the metabolic activities of interest. Without such techniques, it would have been very difficult to make any conclusions about the significance of the collected molecular data, and what bearing or significance they had on the experimental system. In the case of the pyrrolizidine alkaloid metabolizing L4M2 consortium, there had been questions about how diverse it actually was. Molecular techniques had been used in a previous study [64]; however, there were still questions as to the exact number of species present. In Chapter 2, it was the independent examination of the consortium by cloning and DGGE that led to the conclusion that L4M2 is comprised of no more than five species of bacteria of rumen origin. The full-length 16S sequence data produced by the L4M2 library will be useful for additional studies. It is important to have high quality sequence data for the design and use of sequence specific probes and primers. These molecular tools can then be applied to *in vitro*, as well as, *in vivo* studies of microbes that are found in L4M2.

Oxalobacter formigenes, Synergistes jonsii and L4M2 are all examples of rumen-derived microbes with the ability to anaerobically metabolize harmful plant alkaloids. In the same interest of animal health and the prevention of economic loss, it is desirable to discover an anaerobic microbe that is capable of breaking down the endophyte toxin, ergovaline, into nontoxic metabolites. Unlike oxalate, DHP or PA, there is no documented example of a ruminant that is highly resistant to endophyte toxicosis. It was therefore necessary to

examine other environmental sources for anaerobic microbes that could carry out this task and potentially be translated into the anaerobic environment of the rumen. Chapter 3 sought to explain the observation that a vermicompost bin was given seeds containing extremely high levels of ergovaline. Initially, many of the worms in the bin had died or left the bin; however, a population of worms became established that were apparently unaffected by the continued addition of high endophyte seed to the bin.

Analytical methods for studying the disappearance of ergovaline from anaerobically incubated cultures of bacteria found in the worm-gut were improved from methods used to analyze ergovaline from plant material [20], rumen fluid [25], and liver microsome [32]. Though the previous methods were pertinent for the studies in which they were used, they were not optimal for the examination of ergovaline in worm-gut culturing experiments. The improvements allowed for a greater number of samples to be examined in a shorter period of time. The results of the anaerobic incubation of ergovaline with worm-gut homogenates from the E+ and E- treatments in the winter did not indicate that those samples had the ability to affect the concentration of ergovaline. However, preliminary work has demonstrated the potential for ergovaline metabolism from at least the microbes found in E+ worms in the fall season.

One of the goals of the study in Chapter 3 was to avoid a random examination of the diversity within the worm-guts of the two treatment conditions. Many studies of environmental diversity produce clone libraries of a limited size [12; 67] relative to the level of complexity that one may expect to find in natural environments such as soil [107] or an artificial environment such as a bioreactor [43]. There is computer software available to analyze sequence data in order to determine the level of diversity in a given sample set [84]. The limitation of this analysis is that the program can only infer diversity from the data it is given. If a selective screening method such as RFLP ribotyping is used prior to screening, the results can become skewed.

Sequencing analysis can become very expensive both monetarily and temporally when a clone library is large enough to potentially represent the full 16S diversity of an environment as diverse as soil. It is therefore necessary to be selective about how many sequences are examined. If a small clone library is produced (<100 clones), then there is the potential to not encapsulate the full range of diversity that may be expected. If a large library is produced (>500 clones), then choices may need to be made about which clones to have sequenced. This could potentially result in a loss of information even if it is contained within the library.

In Chapter 3, two large 16S clone libraries were produced, containing 960 clones for each treatment. Ribotype profiles were produced for as many clones as possible. Some clones did not produce a usable ribotype, but a large number were available from each library. Computational methods were employed to sort and group clone ribotypes without performing additional ribotyping gels. The clustering results were used as a guide to select a minimum number of clones for sequencing in order to observe the greatest amount of diversity from each library. Of the clones that were sequenced, it was observed that about one-third were redundant, and not used in the final analysis. In the final analysis, each clone library contained sequences that grouped with ten to eleven distinct prokaryotic phyla and one eukaryotic reference sequence. Because the metabolism results were inconclusive, no inferences could be made connecting the diversity observed in the libraries to bacteria potentially involved in ergovaline metabolism.

The work embodied here was a multifaceted approach to understanding the microbial ecology of bacteria that metabolize harmful plant alkaloids. Methods for the structured and directed examination of microbial diversity were used. Analytical chemistry techniques were employed for the observation of microbial metabolism of compounds of interest. The application of both chemistry and molecular biology yielded a greater understanding about the microbes of the two experimental systems described here. By taking this type

of integrated approach I have shown that more can be learned about the microbes in a given environment and their interaction with it or their effect on it. Both approaches should be applied simultaneously if an understanding is to be gained about microbes in a given environment and their interaction with it or their effect on it.

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