

BACTERIAL COMMUNITIES ASSOCIATED WITH THE HINDGUT OF *TIPULA ABDOMINALIS* LARVAE (DIPTERA: TIPULIDAE), A NATURAL BIOREFINERY

by

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ABSTRACT

Insects are the largest taxonomic group of animals on earth. Although a few thorough studies have shown insect guts host high microbial diversity, many insect-microbe associations have not been investigated. *Tipula abdominalis* is an aquatic crane fly ubiquitous in riparian environments. *T. abdominalis* larvae are shredders, a functional feeding group of insects that consume coarse particulate organic matter, primarily leaf litter. In small stream ecosystems, leaf litter comprises the majority of carbon and energy inputs; however, many organisms are unable to degrade this lignocellulosic material. By converting lignocellulose into a form that other organisms can use, *T. abdominalis* larvae influence the bioavailability of carbon and energy within the ecosystem. Evidence suggests that the bacterial community associated with the *T. abdominalis* larval hindgut facilitates the digestion of its recalcitrant lignocellulosic diet. Such lignocellulose-ecosystem-insect-microbiota interactions provide a model natural biorefinery and have become of special interest recently for the application of microbial conversion of lignocellulose to biofuels, including ethanol, butanol, and hydrogen.

Bacterial isolates from the *T. abdominalis* larval hindgut were characterized, and many had enzymatic activity against plant polymer model substrates. Several isolates had low 16S rRNA gene sequence similarity to previous described bacteria, including the proposed novel genus *Klugiella xanthotipulae* gen. nov., sp. nov. Clone libraries of the 16S rRNA gene revealed a phylogenetically diverse bacterial community associated with the larval hindgut wall epithelial and lumen material. *Clostridia* and *Bacteroidetes* dominated both hindgut wall and lumen, while *Betaproteobacteria* dominated leaf diet- and cast-associated microbiota. Although phylogenetic structure at the class level was similar between hindgut wall and lumen microbiota and between leaf diet and cast microbiota, statistical analyses suggest that these sub-communities are significantly different from one another. Enrichment cultures were constructed to cultivate a dynamic cellulolytic subpopulation from the hindgut microbiota. Although the phylogenetic structure of the cellulolytic enrichment cultures was highly variable, function remained stable over successive transfers of subcultures. The *T. abdominalis* larval hindgut hosts a novel phylogenetically diverse and dynamic microbial community.

INDEX WORDS: insect-microbe interactions, bacterial communities, lignocellulose

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## DEDICATION

For my parents, Daniel L. and Nanette C. Cook, whose determination, creativity, and calm have given me an example by which to live. Their love, support, guidance, and tireless encouragement in all my endeavors gives me the confidence and ability to succeed. Also for my brother, Ryan C. Cook, whose compassion and enthusiasm I greatly admire.

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**CHAPTER 1**  
**INTRODUCTION AND LITURATURE REVIEW**

## **Purpose**

Insects are the largest taxonomic group (Class *Insecta*) of animals on earth. Although a few thorough studies have shown insects host an environment with high microbial diversity (Brune, 2005; Breznak & Brune, 1994; Buchner, 1965; Dillon & Dillon, 2004; Moran, 2001; Tanada & Kaya, 1993; Kane & Pierce, 1994), less than 1% of described insect species have been examined for microorganisms (Kane & Mueller, 2002). Insects that consume lignocellulose and host a gut microbial consortia have become of special interest recently for the application of microbial conversion of lignocellulose to biofuels, including ethanol, butanol, and hydrogen. One of the major challenges in lignocellulose conversion is the need for robust and inexpensive enzymes to deconstruct lignocellulose into fermentable sugars (Octave & Thomas, 2009); the gut microbial community of lignocellulose degrading insects may be mined for these enzymes as well as novel microorganisms themselves for the application of improved biofuels production technology.

As plants have evolved recalcitrant structures to resist predation, so have their consumers evolved mechanisms to overcome that resistance. For microorganisms, these mechanisms include lignocellulolytic enzymes that deconstruct plant polymers to sugar moieties. Some herbivorous insects also produce lignocellulolytic enzymes that enable digestion of plant polymers (Watanabe *et al.*, 1997; Zhang *et al.*, 2009). Through another mechanism, insects may host a gut microbial community that facilitates digestion of a recalcitrant lignocellulolytic diet, which the focus in the following studies. In nature, lignocellulose degradation is often a cooperative activity, one which has been reported to be most effective with a mixed culture of cellulolytic and non-cellulolytic bacteria (Haruta *et al.*, 2002; Odom & Wall, 1983). Individual members of microbial communities are often most metabolically active only when in association

with other members of the community. Additionally, the microbial activity, water chemistry, and other biogeochemical processes in the ecosystem external to the insect itself can greatly influence the functions and productivity of gut microbial communities (Röling, 2007).

Therefore, the study of lignocellulose-ecosystem-insect-microbiota interactions should be viewed as a whole process, a natural biorefinery.

### **Lignocellulose Composition and Degradation**

Plant cell walls are composed of lignocellulose, a structural polysaccharide which provides a highly resistant defensive barrier. The percent composition of lignocellulose is highly variable between plants, but is approximately 30-50% cellulose, 20-40% hemicellulose, and 10-30% lignin (Pauly & Keegstra, 2008). Cellulose is the predominant component and is in the form of crystalline microfibril bundles. These fibrils are embedded in a matrix of hemicellulose, a mixture of linear and branched polymers of diverse hexose and pentose sugars. Crossed-linked to these polysaccharides is lignin, a heterogeneous polymer of hydroxyphenylpropanoid units (Chang, 2007; Pauly & Keegstra, 2008) (Figure 1.1).

Cellulose, a homopolymer of  $\beta$ -1,4 –linked glucose units, is the most abundant biopolymer on earth and is almost exclusively found in plant cell walls; however, it is produced by a few bacteria (e.g. *Acetobacter xylinum*) and some animals (e.g. tunicates) (Lynd *et al.*, 2002). An important feature of cellulose, and relatively unusual for a polysaccharide, is its crystalline structure (Lynd *et al.*, 2002). Successive inverted glucose residues form polymer chains in which cellobiose is the repeating unit. Extensive hydrogen bonding between parallel cellulose chains results in crystallization of multiple cellulose chains into microfibrils (Taylor, 2008). Depolymerization of cellulose requires a number of multiple isomer enzymes from three

classes of cellulases: endoglucanases, exoglucanases (or cellobiohydrolases), and  $\beta$ -glucosidases (Chang, 2007).

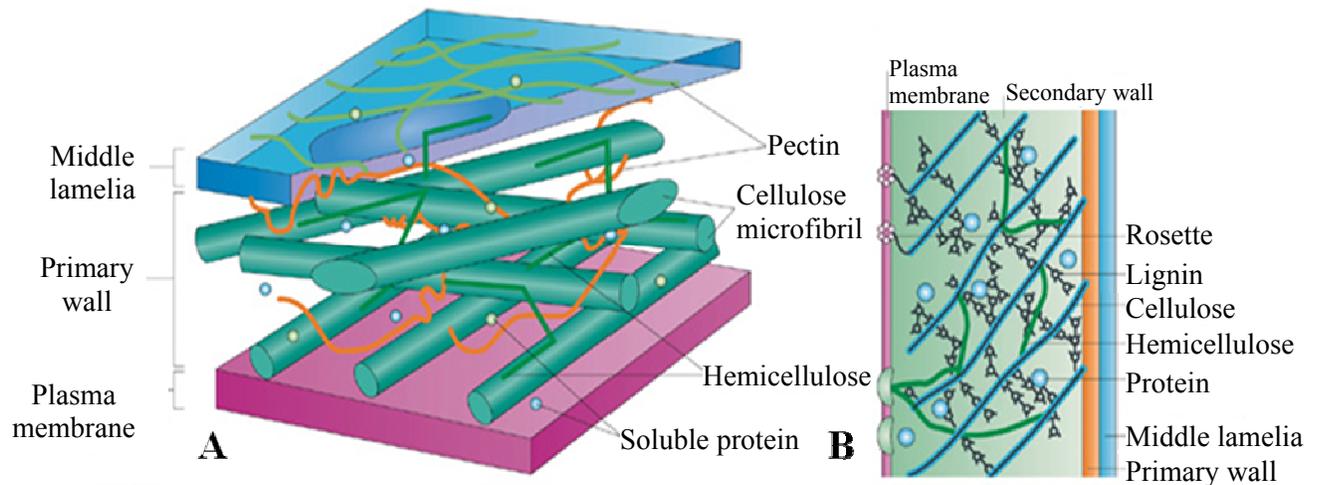


Figure 1.1. Plant cell wall structure. A, 3-D diagram of cell wall structures of cellulose microfibrils, hemicellulose, pectin, lignin and soluble proteins. B, Cross-section diagram of cell wall structures. From (Stricklen, 2008). Reprinted here with permission from publisher.

The second-most abundant sugar polymer in plants is hemicellulose, a heterogeneous mix of linear and branched polysaccharides that are cross-linked to cellulose microfibrils via hydrogen bonds (Chang, 2007; Shallom & Shoham, 2003). Hemicellulose can be denatured by mild thermochemical treatment, releasing a variety of 5- and 6-carbon sugars, including xylan, arabinose, mannose, and glucuronate (Chang, 2007; Shallom & Shoham, 2003). A hemicellulase is either a glycoside hydrolase (GH), which hydrolyzes glycosidic bonds, or carbohydrate esterase (CE), which hydrolyzes ester linkages of acetate or ferulic acids (Shallom & Shoham, 2003). Due to hemicellulose heterogeneity, many various enzymes may be required to release fermentable sugars, and the most effective enzyme cocktail will depend on the composition of the substrate.

Lignin is covalently attached to hemicellulose. This complex polymer is synthesized combinatorially from three types of phenolic monomers to generate a wide variety of different bonding motifs. The energy content of lignin is high; however, it is also highly recalcitrant due to structural qualities such as aromaticity, heterogeneity, and extensive carbon-carbon crosslinking (Chang, 2007).

### **Lignocellulose in Riparian Stream Ecosystems**

Detritus (dead organic matter) in the form of lignocellulolytic leaf litter provides the major contribution of organic matter in small streams in forested watersheds (Fisher & Likens, 1973). The decomposition of detritus reduces organic matter size and releases nutrients for re-entry into food webs (Cummins, 1974). Many streams depend on this allochthonous input of organic material for carbon and energy (Wallace, 1997). However, the structural polysaccharides of plant cell walls (cellulose and hemicellulose) are physically and chemically bound to lignin, forming a lignocellulose complex that is resistant to digestion (Maltby, 1992; Webster & Benfield, 1986). Decomposition of leaf litter in riparian ecosystems is primarily the result of complex chemical-structural modifications due to microbial lignocellulolytic enzyme activity (Webster & Benfield, 1986; Maltby, 1992; Gessner *et al.*, 1999; Cummins, 1974). Few animals synthesize the lignocellulolytic enzymes required to digest plant litter, thus animal nutrition requires the activity of these microbes, either in free-living inter-relationships or associated in the animal gut.

Traditionally, decomposition has been understood to occur in distinct and temporally separated phases (Cummins, 1974; Webster & Benfield, 1986). However, Gessner *et al.* (1999) suggested that the stages of breakdown are not so distinct and successive, but rather that they occur simultaneously, allowing potential for interaction and the syntrophic effects of multiple

species. The three phases/stages of the decay of leaf litter in streams occur are leaching, conditioning, and fragmentation (Cummins, 1974; Maltby, 1992; Webster & Benfield, 1986) (Figure 1.2).

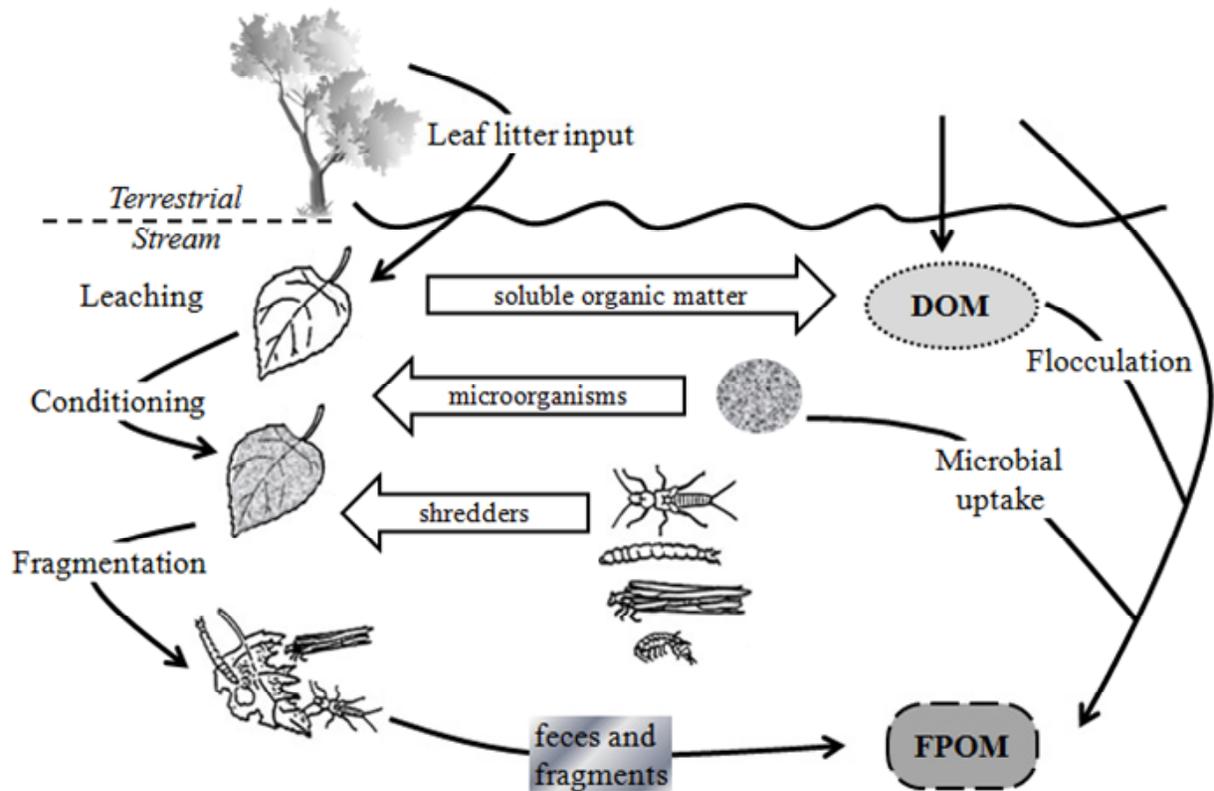


Figure 1.2. Leaf and detritus processing in riparian streams. Adapted from Cummins, 1974.

Leaching is the rapid (within 24h) loss of soluble organic matter (or dissolved organic matter, DOM) from coarse particulate organic matter (CPOM) shortly after immersion (Cummins, 1974). In aquatic environments, leaching is considered to be the initiation of leaf breakdown, and studies have estimated substantial loss of mass (up to 30%) within 24h after

immersion (Petersen & Cummins, 1974). To facilitate measurement of changes, leaf litter is pre-treated by artificial drying, killing the leaf tissue and decreasing structural integrity, resulting in the observed rapid dissociation of soluble constituents (Gessner *et al.*, 1999). Fresh leaves tend to have decreased rates of both leaching and fungal colonization (Gessner & Schwoerbel, 1989).

Conditioning refers to microbial colonization and chemical-structural modification of leaf material, which increases palatability for detritivorous macroinvertebrates, or shredders (Cummins, 1974). The detritivore diet is also enhanced by the accumulation of microbial biomass, which has high nutritional value (Cummins, 1974). The concept of conditioning has traditionally focused on the preparation and enhancement of leaf litter for shredders; however, microorganisms themselves have an established role as effective detritus decomposers (Suberkropp, 1998). Microbes directly cause loss of leaf mass via excretion of lignocellulolytic enzymes that cleave plant polymers, followed by assimilation and conversion of resultant organic matter to CO<sub>2</sub> and biomass (Cummins & Klug, 1979; Suberkropp, 1998). The specific relationships between fungi and bacteria on decaying leaves in streams are poorly understood, and both synergistic (Bengtsson, 1992) and antagonistic (Gulis, 2003) interactions have been demonstrated.

The microbial colonization of leaf litter is believed to be dominated by fungi, specifically aquatic hyphomycetes (Baldy *et al.*, 1995; Gulis, 2003; Bengtsson, 1992). While aquatic hyphomycetes do excrete polysaccharide hydrolyzing enzymes, including pectinases, cellulases, and hemicellulases (Suberkropp & Klug, 1980), there is little evidence that aquatic hyphomycetes have the necessary enzymes to degrade the lignin and lignocellulosic complexes that render the leaf matrix resistant to breakdown (Chamier, 1985). The relative contribution of bacteria to leaf decomposition is thought to increase only after particle size has been reduced in

the latter stages of decay (Suberkropp & Klug, 1976; Baldy *et al.*, 1995). Bacteria can account for approximately 10% of the total biomass and 30% of the total microbial production (Baldy *et al.*, 2002), but the contribution of bacteria to leaf decomposition has most likely been underestimated due to bias in methodology (Findlay & Arsuffi, 1989; Kirchman *et al.*, 1985; Newell & Fallon, 1991; Suberkropp & Weyers, 1996).

Fragmentation results in the reduction of detritus leaf litter from coarse to fine particulate organic matter (CPOM and FPOM, respectively). The importance of fragmentation to the overall reduction of detritus (conversion to biomass and CO<sub>2</sub> or other mineralization products) is not well understood and is likely quite variable (Gessner *et al.*, 1999). Fragmentation does increase the surface area available for microbial colonization, which would accelerate decomposition (Cummins, 1974). Along with stress and abrasion from stream turbulence, the physical fragmentation of decomposing leaf litter is mediated by feeding activity of macroinvertebrate shredders. (Cummins, 1974).

### **Macroinvertebrate Shredders**

Shredders are a functional feeding group of insects that consume coarse particulate organic matter, primarily leaf litter. In small riparian stream ecosystems, leaf litter comprises the majority of carbon and energy input (Vannote *et al.*, 1980). Thus, shredders are an important segment of the small stream ecosystem and usually comprise about 20% of the total biomass (or 10% numerical abundance) of stream macroinvertebrates (Petersen *et al.*, 1989). Although leaf litter is the primary source of both carbon and energy input into small stream systems, many organisms are unable to degrade this lignocellulosic material, which has low nutritional value due to a high C:N ratio. Furthermore, proteins complexed with tannins, lignins, and highly structured plant polysaccharide polymers (cellulose, hemicellulose, and lignin) make digestion of

leaf litter difficult (Martin *et al.*, 1980). By converting lignocellulose into a form that other organisms can use, shredders influence the bioavailability of carbon and energy within the ecosystem. Shredders can consume up to 130% of their body weight daily (Cummins *et al.*, 1973). Shredder feces consists of high surface area fine particulate organic matter (FPOM) that can be consumed by smaller macroinvertebrates and microorganisms (Cummins *et al.*, 1973; Fisher & Likens, 1973). Approximately 60% of the material ingested by shredders is excreted as feces (Cummins *et al.*, 1973; Cummins *et al.*, 1989).

Shredders preferentially consume conditioned leaves as they are more palatable, likely due to softening of the leaf tissue from partial degradation by conditioning microorganisms (Arsuffi & Suberkropp, 1984; Lawson *et al.*, 1984; Canhoto & Graça, 1999). Enhanced nutrition contributes to increased consumption, as conditioning increases nitrogen concentration and available protein (Kaushik & Hynes, 1971). Preferential feeding of conditioned leaves by shredders suggest that the microbial biomass is nutritionally important, either by direct assimilation of the biomass or by utilization of the physically altered leaf substrate (Barlocher & Kendrick, 1974; Buchner, 1965). The “peanut butter and cracker” theory suggests that the nutritionally valuable microbial biomass (“peanut butter”) is found on the nutritionally unsuitable leaf (“cracker”), thus leaf ingestion is necessary only for the purpose of obtaining microbial biomass (Cummins, 1974). However, evidence suggested that microbial biomass was not as quantitatively important as other components of the detritus diet of shredders (Baker & Bradnam, 1976). For one shredder, *Tipula abdominalis* larva, a portion of the ingested microbial biomass is digested and assimilated; however, ingested microbial biomass only accounts for 11-27% of larval growth (Lawson *et al.*, 1984). Although microbial biomass may contribute to shredder nutrition, leaf detritus contributes significantly to shredder growth.

Microbial biomass did not sustain the nutritional requirements for shredders, but it was unlikely that shredders were able to digest the leaf diet unassisted. Shredders, and detritivores in general, do not seem to produce themselves the necessary lignocellulolytic enzymes to digest the abundant plant polymers of their diets (Barlocher & Kendrick, 1974; Barlocher & Porter, 1986; Walters & Smock, 1991). Some shredders host gut microbial communities that are hypothesized to facilitate digestion of lignocellulose (Klug & Kotarski, 1980; Sinsabaugh *et al.*, 1985). In this cellulolytic symbiont theory, it was suggested that shredders benefit from microbial biochemical modification of leaves and/or from products of microbial fermentation (Lawson *et al.*, 1984). The necessity of microbially-mediated hydrolysis and fermentation for the digestion and assimilation of plant polymers has been demonstrated with <sup>14</sup>C-labelled cellulose in three different genera of shredders (*Pteronarcys proteus*, *Tipula abdominalis*, and *Pycnopsyche luculenta*) (Sinsabaugh *et al.*, 1985). Acetate from microbial fermentation was produced in the guts of shredders *T. abdominalis* and *Pycnopsyche guttifer* and was transported across the gut wall into the hemolymph (Lawson & Klug, 1989).

### ***Tipula abdominalis* Larva**

*Tipula abdominalis* is an aquatic crane fly, which is found riparian stream ecosystems. The larvae progress through four larval instar stages, and are shredders of leaf detritus. First instar larvae hatch from eggs late in summer and then progress relatively quickly (weeks) through second and third instar stages. They molt into the fourth instar stage in late fall and persist longest (months) in this final instar (Byers, 1996). Fourth instar larvae consume conditioned leaf litter throughout the fall, winter, and spring.

The gut morphology of *T. abdominalis* larvae consists of two main compartments: midgut and hindgut. In contrast to the linear gut morphology of other insects (e.g., *Pteronarcys*

spp., *Pycnopsyche* spp.), the anterior portion of the hindgut of *T. abdominalis* protrudes from the hindgut where material may be detained for extended digestion; this structure has been termed a “fermentation paunch” (Figure 1.3). The midgut is highly alkaline (pH 11), while the hindgut is neutral (pH 7) (Martin, 1987). Studies suggest that proteolysis occurs in the alkaline conditions in the midgut, dissociating protein complexes from plant polymers, which are then more accessible for saccharification and microbial fermentation in the pH neutral hindgut (Canhoto & Garca, 2006; Clark, 1999; Garca & Barlocher, 1998; Lawson & Klug, 1989; Sinsabaugh *et al.*, 1985).

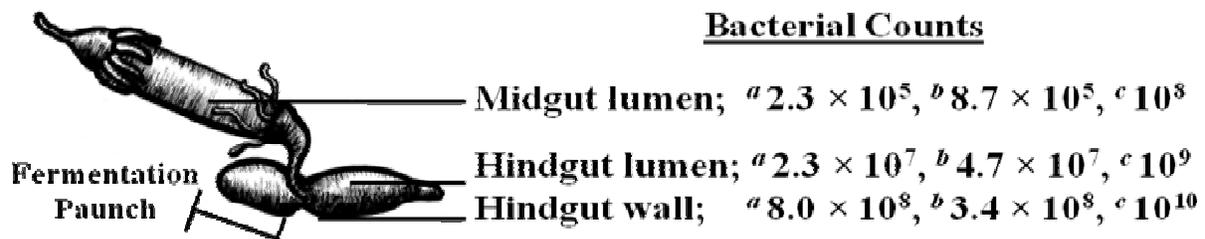


Figure 1.3. Drawing of *T. abdominalis* gut tract with viable and direct bacterial cell counts. <sup>a</sup> aerobic and <sup>b</sup> anaerobic CFU mg<sup>-1</sup> dry weight, <sup>c</sup> direct microscope counts mg<sup>-1</sup> dry weight (Klug & Kotarski, 1980). Drawing modified from (Rogers, 2005).

Scanning electron microscopy studies revealed that the *T. abdominalis* larval gut hosts a dense and morphologically diverse microbial community (Klug & Kotarski, 1980). The lumen contents of the midgut contain a microbial diversity similar in morphology to that of ingested leaf detritus. No microorganisms are associated with the wall (larval epithelial tissues) of the midgut. In contrast, the lumen and wall of the hindgut hosts a microbial community of greater density and morphological diversity, which differs from that of the ingested leaf detritus.

Aerobic and anaerobic cultivation of bacteria revealed that colony-forming units also increased from midgut lumen to hindgut lumen to hindgut wall (Figure 1.3). The density and diversity of the microbial community increases with each larval instar stage. Because the greatest number of bacteria are associated with the hindgut wall, and attachment to epithelial cells is likely a mechanism for prolonged symbioses (Dillon & Dillon, 2004), it is hypothesized that much of the resident bacteria are found in the wall-associated sub-population.

### **Molecular Methods for Microbial Community Analysis**

While much was discovered about the morphological diversity of the *T. abdominalis* hindgut microbiota, nothing was known about individual members or the phylogenetic structure of the bacterial community. Studies in the following chapters employed both culture dependent and independent techniques to describe the bacterial population associated with the hindgut. Because only a small percentage (estimated 1% or less) of total estimated bacterial species can be cultivated, culture independent molecular techniques are vital for thorough bacterial community phylogenetic characterization (Amann *et al.*, 1995; Head *et al.*, 1998).

Initial culture-independent molecular techniques to assess microbial diversity included fatty acid profiling (White & Findlay, 1988; Findlay, 1996). More recently, DNA has become the signature molecule; DNA-based techniques have allowed phylogenetic dissection of microbial communities which has provided great insight to microbial diversity (Nocker *et al.*, 2007). In polymerase chain reaction (PCR)-based methods, universal or specific primers are employed to amplify genes from a wide variety or select group, respectively, of organisms. The 16S rRNA gene has become the prevalent gene for assessing bacterial diversity (Hugenholtz *et al.*, 1998; Pace *et al.*, 1986). The 16S rRNA gene has been an advantageous genetic marker because of its ubiquitous distribution and relatively slow rate of evolution, which allows

comparison of divergent bacteria. PCR primers can be designed from highly conserved regions of the gene to capture sequence from divergent species and uncultivable bacteria (Baker *et al.*, 2003; Rosselló-Mora & Amann, 2001). However, the low evolutionary rate of the 16S rRNA gene can restrict the resolution of closely related species (Jaspers & Overmann, 2004).

Treatment of insertions and deletions, PCR-bias, potential horizontal gene transfer, and multiple copies within a single genome can also complicate the analysis of 16S rRNA genes (Pontes *et al.*, 2007; Santos & Ochman, 2004 and references therein). But despite its limitations, consensus use as well as an extensive and growing database, the 16S rRNA gene is a powerful molecular marker (Nocker *et al.*, 2007; Pontes *et al.*, 2007).

Several molecular methods have been developed for the analysis of microbial diversity, including cloning and sequencing, whole-genome shotgun cloning, amplified ribosomal DNA restriction analysis (ARDRA), restriction fragment length polymorphism (RFLP), terminal restriction length polymorphism (T-RFLP), automated ribosomal intergenic spacer analysis (ARISA), single strand conformation polymorphism (SSCP), and denaturing gradient gel electrophoresis (DGGE). In cloning and sequencing, PCR amplified sequences are cloned into vectors; the inserts are then sequenced providing the greatest level of phylogenetic resolution and putative identification of taxa (Nocker *et al.*, 2007 and references therein). In contrast to sequencing specific genes, whole-genome shotgun cloning obtains sequences of fragments of genomic DNA produced by physical shearing and size fractionation (Fleischmann *et al.*, 1995; Venter *et al.*, 2004).

ARDRA, T-RFLP, SSCP, and DGGE methods employ separation of DNA fragments based on size or sequence via gel electrophoresis. These profiling techniques may provide less phylogenetic resolution than cloning and sequencing, but they are still very useful tools for

assessing microbial diversity and to discover structural changes in microbial community structure (Nocker *et al.*, 2007 and references therein). In ARDRA, ribosomal community DNA is PCR-amplified. PCR products are digested with one or more restriction enzymes, and then size separated on acrylamide or high percent agarose gels (Massol-Deya *et al.*, 1995). In a similar method, T-RFLP, marker genes are also PCR-amplified, except the products are labeled by attaching fluorescent dye to the 5'-end of one of the primers. Labeled product is digested with one or more restriction enzymes, and then size separated on an acrylamide sequencing gel. A laser is used to produce a profile electropherogram, in which only the labeled terminal fragments are detected (Clement *et al.*, 1998; Liu *et al.*, 1997). In both SSCP and DGGE, single and double strand, respectively, PCR products of similar size are separated on an acrylamide gel. During SSCP, strand separation under denaturing conditions is achieved prior to gel loading. Electrophoresis occurs under non-denaturing conditions, in which the single-stranded DNA folds into secondary structure depending on nucleotide sequence. Different secondary structures have different migration and mobility properties in gel electrophoresis, and thus complex mixtures of community DNA can be separated (Lee *et al.*, 1996; Widjoatmodjo *et al.*, 1995). In DGGE, PCR products are separated on an acrylamide gel with an increasing denaturant gradient. Strand separation occurs as the DNA is exposed to stronger denaturing conditions, resulting in partially melted DNA that has retarded to nearly halted migration on the gel. Melting behavior is dependent on GC content and nucleotide sequence, allowing for separation and profiling of community DNA (Muyzer *et al.*, 1993; Muyzer *et al.*, 2004; Muyzer & Smalla, 1998).

In the following chapters, cloning and sequencing and DGGE were the methods chosen for analysis of the *T. abdominalis* larval hindgut bacterial community. Cloning and sequencing

provided high phylogenetic resolution, while DGGE allowed for increased sample processing so that a greater number of individuals could be analyzed.

## **Objectives**

The aim of this study was to characterize the bacterial community associated with the hindgut of the *T. abdominalis* larvae. In chapter 2, the phylogeny of the bacterial community was investigated using molecular techniques to include uncultivable species. Also, bacteria were isolated and assayed for activity towards model plant polymers. In chapters 3 and 4, bacteria associated with the leaf diet, hindgut lumen, hindgut wall, and casts from multiple individual larvae were compared to putatively identify the source of variation in community structure, as well as to distinguish a resident and stable sub-population. In chapter 5, a cellulolytic sub-population was cultivated in enrichment cultures and changes in community structure were monitored through successive generations of sub-cultivation of enrichment cultures. Bacteria from enrichment cultures were isolated under anaerobic and aerobic conditions. Chapter 6 describes *Klugiella xanthotipulae*, a novel species and genus isolated from the hindgut of *T. abdominalis*.

## CHAPTER 2

# ISOLATION OF POLYMER-DEGRADING BACTERIA AND CHARACTERIZATION OF THE HINDGUT BACTERIAL COMMUNITY FROM THE DETRITUS-FEEDING LARVAE OF *TIPULA ABDOMINALIS* (DIPTERA: TIPULIDAE)<sup>1</sup>

<sup>1</sup>Cook, Dana M., Emily DeCrescenzo Henriksen, Rima Upchurch, Joy B. Doran Peterson. 2007. Isolation of polymer-degrading bacteria and characterization of the hindgut bacterial community from the detritus-feeding larvae of *Tipula abdominalis* (Diptera: Tipulidae). Appl. Environ. Microbiol. 73: 5683-5686. Reprinted here with permission from the publisher.

## **Abstract**

*Tipula abdominalis* larval hindgut microbial community presumably facilitates digestion of the lignocellulosic diet. The microbial community was investigated through characterization of bacterial isolates and analysis of 16S rRNA gene clone libraries. This initial study revealed novel bacteria and provides a framework for future studies of this symbiosis.

## **Introduction**

Insects are the largest taxonomic group of animals on earth. Although a few thorough studies have shown that insects host an environment with high microbial diversity (Brune, 2005; Breznak & Brune, 1994; Buchner, 1965; Dillon & Dillon, 2004; Moran, 2001; Tanada & Kaya, 1993; Kane & Pierce, 1994), less than 1% of described insect species have been examined for microorganisms (Kane & Mueller, 2002). *Tipula abdominalis* is an aquatic crane fly ubiquitous in aquatic riparian environments. *T. abdominalis* larvae are shredders, a functional feeding group of insects that consume coarse particulate organic matter, primarily leaf litter. In small riparian stream ecosystems, leaf litter comprises the majority of carbon and energy inputs (Vannote *et al.*, 1980); however many organisms are unable to degrade this lignocellulosic material, which is difficult to digest due to highly structured plant polysaccharide polymers (cellulose, hemicellulose, and lignin), and which has low nutritional value due to a high C:N ratio (Martin *et al.*, 1980). By converting lignocellulose into a form that other organisms can use, *T. abdominalis* larvae influence the bioavailability of carbon and energy within the ecosystem.

The larva itself is not capable of tissue-level synthesis of cellulolytic enzymes (Sinsabaugh *et al.*, 1985), and it was proposed that the larvae benefit nutritionally from

microbially-mediated digestion of leaf lignocellulose, providing simple fermentation products which can be used by the larvae (Lawson & Klug, 1989). Scanning electron microscopy studies demonstrated a dense and morphologically diverse microbial community in the hindgut of *T. abdominalis* larvae (Klug & Kotarski, 1980). This microbial community was investigated for phylogenetic diversity and enzymatic activity towards model plant polymer substrates.

## **Material and Methods, Results, and Discussion**

Larvae collection and dissection. Larvae were collected from 2<sup>nd</sup> order streams (Vannote *et al.*, 1980) in Michigan. Hindguts were extracted and transferred to a reduced buffered salt solution (BSS) (Leadbetter & Breznak, 1996). Whole-hindguts were homogenized in 1 mL BSS. Alternatively, loosely associated microorganisms were removed by vigorous vortex washing (X3) of the hindgut wall in BSS. Washed hindgut walls were homogenized in 1 mL BSS.

Bacterial isolation and characterization. Gut homogenates were serially diluted and plated onto tryptic soy agar (Difco) and R2 agar (Reasoner & Geldreich, 1985) and incubated for up to 3 weeks at 22°C. Colonies were subcultured until pure cultures were obtained. Sequencing of the 16S rRNA genes from the cultured isolates was performed at MIDI Labs (Newark, Delaware, USA). Fifty nine isolates represented 4 classes (Table 2.1). Fifteen and nineteen isolates (25%) had  $\leq 97\%$  and 100% 16S rRNA gene sequence similarity, respectively, to known organisms in databases.

Isolates were screened for different enzymatic activities (hydrolysis of substrate) on model substrates as described previously: carboxymethylcellulose (CMC) (Wood & Kellogg, 1988); starch (Difco 272100); xylan (Mondou *et al.*, 1986); polygalacturonate (PGA) (Starr *et al.*, 1977); and methylumbelliferyl conjugates of cellobiopyranoside (MUC), arabinofuranoside

(MUA), glucoside (MUG), mannopyranoside (MUM), and xyloside (MUX) (Sharrock, 1988). Five (8.5%) isolates could hydrolyze all, and 35 (60%) isolates could hydrolyze one or more model substrates used in the current study. Isolates capable of degrading these methylumbelliferyl-conjugates demonstrate enzymatic activity for degrading plant carbohydrate polymer ends produced by partial digestion of lignocellulose, and may assist the digestion of leaf litter in the *T. abdominalis* larval hindgut.

Five groups of isolates had identical partial 16S rRNA gene sequences, but different enzymatic activities towards the study model substrates (Table 2.1). All 5 groups had high (>97%) sequence similarity to previously described bacteria. This difference in physiological characteristics would not have been observed if solely molecular techniques were employed. These results exemplify the importance of culture-dependent research in conjunction with molecular techniques. General evaluations of microbial diversity estimate that only 1-10% of known prokaryotic phylotypes have been cultured. Symbionts of the termite gut have been studied for over a century, yet only a small portion have been cultured (Breznak, 2000).

Table 2.1. Putative identity of isolates and their enzymatic activity on model plant polymers.

	Isolate	% Sim.	Cultured Strain Match	CMC	Starch	Xylan	PGA	MUA	MUX	MUC	MUG	MUM
Proteobacteria	1C49L			-	-	-	-	-	+	+	+	-
	2C74	100	<i>Caulobacter leidyia</i>	-	-	+	-	-	+	+	+	+w
	3C72			-	-	-	-	-	+	+	+	+w
	4C16	100	<i>Methylobacterium aminovorans</i>	-	-	-	-	-	-	-	-	-
	8C22P	99.1	<i>Methylobacterium zatmanii</i>	-	-	-	-	-	-	-	-	-
	5C5 <sup>*3</sup>	100	<i>Methylobacterium hispanicum</i>	+	-	-	-	-	-	-	-	-
9C110	98.4	<i>Serratia fonticola</i>	+	-	-	+	+	+	+	+	-	
Firmicutes	10C22	100	<i>Bacillus circulans</i>	+	-	-	-	-	-	-	-	-
	11C46			-	+	-	-	+	+	+	+	-
	12C70	100	<i>Bacillus cohnii</i>	-	-	-	-	-	-	+	+	-
	13C100			+w	+	-	-	-	-	-	-	-
	14 C111	99.8	<i>Bacillus firmus</i>	+	-	-	-	-	-	+w	-	-
	15C59 <sup>*3</sup>	99.8	<i>Bacillus fusiformis</i>	-	-	-	-	-	-	-	-	-
	20C75	96.6	<i>Bacillus sphaericus</i>	-	-	-	-	-	-	-	-	-
	17 C67			-	-	-	-	-	-	-	-	-
	18C68	100	<i>Bacillus silvestris</i>	-	-	+	-	-	-	-	-	-
	21C27	100	<i>Bacillus megaterium</i>	-	-	-	-	-	+	+	+	-
	22C25	100	<i>Bacillus weihenstephanensis</i>	-	-	-	-	-	-	-	-	-
	23C26	99.8	<i>Bacillus pumilus</i>	-	-	-	-	+	+w	+	+	-
	24C57	99.2	<i>Bacillus thuringiensis</i>	+	+	-	-	-	+	+	-	-
	27C64 <sup>*5</sup>	99.1	<i>Paenibacillus amylolyticus</i>	+	+	+	+	+	+	+	+	+
	29 C58s	97.6		-	-	-	-	-	-	-	-	-
	31C28	96.2	<i>Paenibacillus agaridevorans</i>	-	+	+	-	+	+	+	+	+
	32C23	95		+	+w	+	-	+	+	+	+	+
	33C53L <sup>*2</sup>	98	<i>Paenibacillus glycanilyticus</i>	-	-	-	-	+	+	+	+	+
	35C82	100	<i>Staphylococcus aureus</i>	-	-	-	-	-	-	-	-	-
	36C7 <sup>*3</sup>	99	<i>Staphylococcus saprophyticus</i>	-	-	-	-	-	-	-	-	-
39C61W <sup>*2</sup>	100	<i>Staphylococcus warneri</i>	-	-	-	-	-	-	-	-	-	
41C21	100	<i>Bacillus muralis</i>	-	-	-	-	+	+	+	-	-	
Actinobacteria	42C60W	99.2	<i>Georgenia muralis</i>	+w	-	-	-	+	+	+	-	+w
	43C17	93.5	<i>Arthrobacter nicotianae</i>	-	-	-	+	+	+	+	+	+
	44C3 <sup>*8</sup>	95	<i>Leifsonia poae</i>	-	-	-	-	-	-	-	-	-
	19C65S1	99.4	<i>Microbacterium foliorum</i>	-	+	-	-	+	+	+	-	-
	52C2 <sup>*2</sup>			+	-	-	-	-	-	-	-	-
	54C108	97	<i>Microbacterium lacticum</i>	+	-	+	+	-	-	-	-	-
	55C94 <sup>*2</sup>	99.8	<i>Micrococcus luteus</i>	-	-	-	-	-	-	-	-	-
	57C60O	100	<i>Rhodococcus luteus</i>	-	-	-	-	-	-	-	-	-
	58C40	99.4		+	+	-	-	+	+	+	+	-
	59C99	98.6	<i>Sanguibacter suarezii</i>	-	-	-	-	+	-	+	+	-

\*n: isolate represents n number of isolates with identical partial 16S rRNA gene sequences and identical enzymatic profiles for the substrates tested in this study.

(+): Hydrolysis of substrate; (-): No observable hydrolysis of substrate; (+w): weak hydrolysis of substrate.

Clone libraries. Bacterial DNA was extracted from hindgut wall homogenates (Shinzato *et al.*, 1999), and purified using Sephadex G-200 spin columns (Tsai & Olson, 1992). 16S rRNA genes were amplified from purified community genomic DNA using bacterial domain forward primer, 27F (5'- AGA GTT TGA TCM TGG CTC AG), and universal reverse primer, 1492R (5' – GGT TAC CTT GTT ACG ACT T) using puReTaq<sup>TM</sup> Ready-To-Go<sup>TM</sup> PCR Beads (Amersham Biosciences). Each PCR began at 94°C for 3.5 min., followed by 15 cycles of 94°C for 1 min., 62°C for 1 min., and 72°C for 1 min. and finished with 4 min at 72°. 15-cycle PCR products were cloned directly into the vector for library construction. Clone libraries were generated using a TOPO TA<sup>®</sup> Cloning Kit (Invitrogen, Carlsbad, CA). The anterior hindguts of two larvae were pooled to construct one library (1), and one library was constructed per hindgut for 2, 3, and 4. Clones were sequenced at Iowa State University's DNA Sequencing Facility (Ames, Iowa).

Clone sequences were analyzed for chimeras using Greengenes software tools (DeSantis *et al.*, 2006b; DeSantis *et al.*, 2006a). DOTUR was used to determine the similarity of the clones to one another at varying sequence similarity, as well as to calculate diversity indices (Schloss & Handelsman, 2005). Diversity indices approached the maximum for all libraries. Coverage values for all libraries were greater than 0.5, indicating that the most prevalent phylogenetic groups had been sampled (Table 2.2).

Table 2.2. Diversity indices calculated from the hindgut-derived 16S rRNA gene clone libraries at  $\geq 97\%$  sequence similarity ( $OTU_{0.97}$ ).

Libraries	Indices					
	N <sup>1</sup>	S <sup>2</sup>	Shannon (H)	H/Hmax <sup>3</sup>	Coverage <sup>4</sup>	Chao1
1	46	32	3.365	0.97	0.54	53
2	59	32	3.033	0.88	0.61	83
3	124	73	4.084	0.95	0.63	134
4	76	36	3.237	0.90	0.68	105
total clones	305	122	4.348	0.91	0.8	175.36

1. N, total number of clones.
2. S, number of operational taxonomic units (OTUs).
3. Hmax,  $\ln(S)$ .
4. Calculated from Good's equation, Coverage =  $1-(n1/N)$ .

Percentage of sequence similarity to previously reported sequences was determined by comparing the 16S rRNA gene sequences to the RDP database (Cole *et al.*, 2003) using the program RDPquery (Dyszynski & Sheldon). From 305 clones, 122 operational taxonomic units sharing  $\geq 97\%$  sequence similarity ( $OTU_{0.97}$ ) were identified, representing 9 classes (Figure 2.1). The majority of clones had highest sequence similarity to *Clostridia* and *Bacteroidetes*, representing 65% and 19% of the total clones, respectively. Clones were compared to one another, as well as to previously described uncultured and cultured bacteria, at varying percent sequence similarities. Clones were more similar to one another than to previously described sequences, and more similar to uncultured than cultured bacteria (Figure 2.2). At  $\geq 97\%$  sequence similarity, 80% of clones were similar to another clone from this collection, while only 8% and 2% were similar to previously described uncultured and cultured bacteria, respectively.

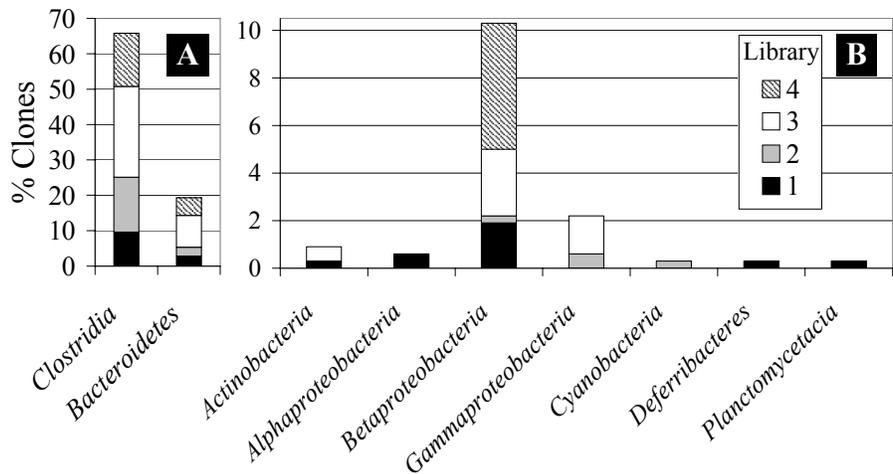


Figure 2.1. Phylogenetic distribution of clones by class. Note change in scale: (A) v axis scale 0-70. (B) v axis scale 0-10.

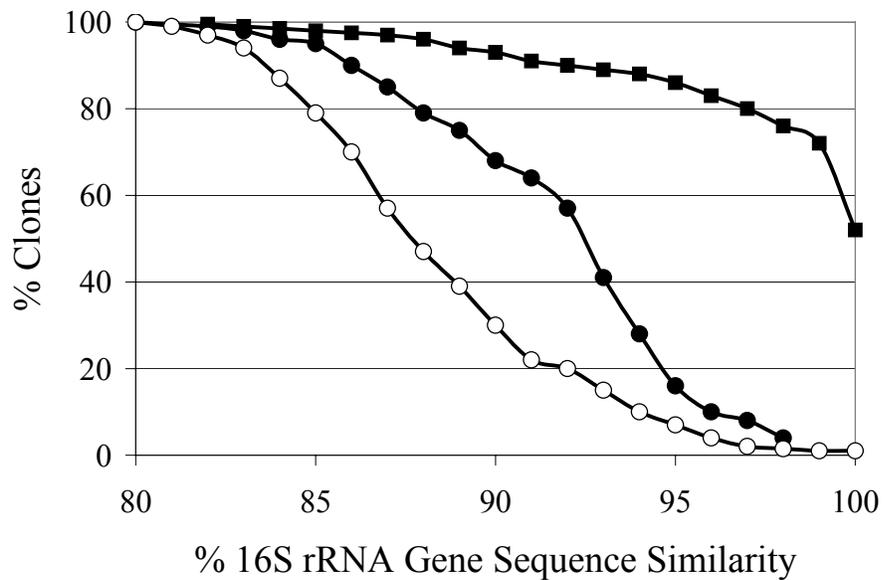


Figure 2.2. Percentage of clones similar to another clone from this study (squares), previously described uncultured (closed circles), or cultured (open circles) bacteria at x% sequence similarity.

In summary, our data indicate that the hindgut bacterial community is phylogenetically diverse. Many of the members are more related to one-another than to previously described bacteria. Isolates demonstrated enzymatic activities that are physiologically relevant to the hindgut environment and digestion of a lignocellulosic diet. While these isolates demonstrate the ability to degrade model plant polymers, it will be important in future studies to link the isolate activity *in vitro* with function *in vivo* in the larval gut. Leaf litter degradation by shredders is an important component of the stream ecosystem and those insects consuming lignocellulose rich diets have developed numerous mechanisms for surviving on a nutrient poor resource. One mechanism employed is the establishment of a gut microbial consortium capable of lignocellulose degradation, and identifying microorganisms with such capabilities is essential to understanding carbon and energy cycling in stream environments.

Accession numbers. Isolate 16S rRNA gene sequences were submitted to GenBank (Benson *et al.*, 2005) with accession numbers AY504427 to AY504477 and AY497196 to AY497203. For clones, one sequence per operational taxonomic unit of  $\geq 99\%$  sequence similarity (OTU<sub>0.99</sub>) was submitted with accession numbers EF176774 to EF176920.

## Addendum

LIBSHUFF analysis data removed from manuscript by publisher is presented here. Statistical comparisons of clone libraries made using LIBSHUFF method (Singleton *et al.*, 2001) (Table 2.3). Analysis of four libraries gave six pair-wise comparisons, half of which were significantly different. This suggests some variability exists in the bacterial community associated with the *T. abdominalis* larval hindgut wall between individual larvae.

Table 2.3. LIBSHUFF results. Highlighted values indicate  $p$ -values above the critical value of 0.0043 (experimentwise  $p$ -value 0.05, critical  $p$ -value calculated using Bonferroni Correction  $p = 1 - (1 - p_{\text{critical}})^{k(k-1)}$ ,  $k$  = number of libraries). In the pair-wise comparisons, when one  $p$ -value in either the  $x \rightarrow y$  or  $y \rightarrow x$  comparison, the libraries are statistically significant different. The comparisons of libraries that were not statistically different are highlighted.

Comparison			Comparison			Comparison		
	$p$ -Value			$p$ -Value			$p$ -Value	
$x$	$x \rightarrow y$	Statistically Different	$x$	$x \rightarrow y$	Statistically Different	$x$	$x \rightarrow y$	Statistically Different
$y$	$y \rightarrow x$		$y$	$y \rightarrow x$		$y$	$y \rightarrow x$	
Library 1	<0.0001	yes	Library 1	0.0196	no	Library 2	<0.0001	yes
Library 2	0.0003		Library 4	0.0044		Library 4	<0.0001	
Library 1	0.676	no	Library 2	0.7955	no	Library 3	0.0005	yes
Library 3	0.0656		Library 3	0.0215		Library 4	0.1552	

## Acknowledgements

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**CHAPTER 3**  
**COMPOSITION OF MICROBIAL COMMUNITIES ASSOCIATED WITH HINDGUT**  
**WALL, HINDGUT LUMEN, CASTS, AND LEAF LITTER DIET OF *TIPULA***  
***ABDOMINALIS* LARVAE<sup>1</sup>**

<sup>1</sup>Cook, Dana M. and Joy Doran Peterson. 2010. Composition of microbial communities associated with hindgut wall, hindgut lumen, casts, and leaf litter diet of *Tipula abdominalis* larvae. To be submitted to Appl. Environ. Microbiol.

## **Abstract**

The hindguts of *Tipula abdominalis* larvae host a dense and diverse microbiota that assists in digestion of recalcitrant lignocellulosic leaf detritus. Variation in phylogenetic structure between subpopulations of the hindgut attached to the wall epithelium or in the lumen, the ingested leaf detritus, and the larval casts was assessed via clone library construction of community 16S rRNA genes. Some larvae were starved in an attempt to decrease the influx of transient microorganisms from the diet into the hindgut, and thus decrease individual variation from non-resident bacteria. The phylogenetic structure of communities associated with ingested leaf detritus is significantly different from that associated with hindgut communities. Clone libraries results suggest that communities associated with the starved wall and fresh wall are most similar. *Clostridia* and *Bacteroidetes* dominate in hindgut associated communities, while *Betaproteobacteria* dominate in leaf and cast associated communities.

## **Introduction**

*Tipula abdominalis* is an aquatic crane fly ubiquitous to small riparian streams. Its larvae are primary shredders of leaf litter and, thus, are an important part of the carbon and energy cycling in these ecosystems (Martin *et al.*, 1980; Petersen *et al.*, 1989). The *T. abdominalis* larval hindgut harbors a dense and diverse microbial community (Klug & Kotarski, 1980). The hindgut microbiota are presumed to enable the efficient digestion of nutrient poor, recalcitrant leaf litter (Lawson & Klug, 1989; Lawson *et al.*, 1984; Sinsabaugh *et al.*, 1985).

Defined study of this complex microbial community is compounded by the difficulties in distinguishing the resident population from transient bacteria passing through the gut tract on ingested material. Resident species are distinguished by their ability to colonize and divide at a

rate equal to or greater than the elimination rate (Dillon & Dillon, 2004). In the *T. abdominalis* larval gut, bacterial counts increase through the tract, from midgut lumen to hindgut lumen to hindgut wall (Klug & Kotarski, 1980). Because epithelial attachment is a likely mechanism for gut colonization, bacterial inventories have been conducted from larval hindgut wall tissues (Cook *et al.*, 2007). *T. abdominalis* larva hatch sterile, and shredders generally do not consume feces, thus the leaf diet is likely the inoculum source for the hindgut microbiota (Klug & Kotarski, 1980).

16S rRNA gene clone libraries were constructed to determine the variation between the microbiota associated with the hindgut wall and lumen in larvae sampled directly from the environment as well as from larvae starved for five days. Starvation was intended to decrease the influx of transient microorganisms from the diet into the hindgut and thus decrease individual variation from non-resident bacteria. Hindgut bacterial communities associated with the leaf litter diet as well as casts from starved larvae were also evaluated. Hindgut wall associated subpopulations were presumed to contain a greater proportion of resident bacteria than that the hindgut lumen microbiota because attachment to gut epithelium is a mechanism for colonization and potential retention in the hindgut (Dillon & Dillon, 2004). Differences between the wall and lumen subpopulations were expected to decrease in starved larvae as the input of transient bacteria was reduced. It was also hypothesized that declining substrate available to the hindgut community from lack of nutrient input could disrupt the resident bacterial population during the starvation process. Therefore, starvation was only maintained until the larvae stopped producing casts.

## Materials and Methods

Sample collection. Larvae, leaf packs, and stream water were collected from 2<sup>nd</sup> order streams (Vannote *et al.*, 1980) in Athens, Georgia. Half of the larvae were sacrificed, and hindguts were extracted immediately following collection. The other larvae were maintained individually in filter-sterilized stream water and starved for five days. These larvae were then sacrificed, and hindguts were extracted. Water was exchanged daily and casts were collected from starved larvae for each of the five days. To extract the hindguts, extreme posterior and anterior ends of the larval body were removed, and the gut tracts were gently pulled from the body and processed essentially as previously described (Cook *et al.*, 2007). Midguts were separated and removed. A longitudinal incision was made on the hindgut wall, allowing the hindgut to open flat and exposing the lumen material. Lumen material was washed from the hindgut wall by vigorous vortexing in saline for three washes. The sample labeled “Lumen” was collected from combined wash liquids from each individual larva. Casts from each individual larva over the five day starvation period were combined. Casts and lumen were homogenized separately for each larva in a tissue grinder with saline and then centrifuged at 10,000 x g for 10 min. Each individual hindgut was homogenized in approximately 1 mL of saline in a tissue grinder and centrifuged as described above. Approximately 30 grams (wet weight) of the leaf pack from which the larvae were collected was used for DNA extraction. The leaf pack was homogenized in an autoclaved blender with saline then centrifuged at 10000 x g for 10 min. For all samples, supernatants were discarded and bacterial DNA was extracted from resuspended pellets as previously described (Cook *et al.*, 2007; Shinzato *et al.*, 1999) and purified using Sepharose 4B spin columns (Jackson *et al.*, 1997).

Construction of 16S rRNA gene clone libraries. DNA from sixteen individuals within groups fresh wall, fresh lumen, starved wall, starved lumen, and cast were pooled (leaf group was originally a pooled group). From the pooled DNA groups, 16S rRNA genes were amplified using the bacterial domain forward primer, 27F (5'- AGA GTT TGA TCM TGG CTC AG), and the universal reverse primer, 1492R (5' – GGT TAC CTT GTT ACG ACT T) using Invitrogen (Carlsbad, CA) Platinum Taq Polymerase, following the manufacturer's instructions. Each PCR began at 94°C for 3.5 min., followed by 20 cycles of 94°C for 1 min., 62°C for 1 min., and 72°C for 1 min. and finished with 4 min at 72°. Twenty-cycle PCR products were purified with Clean & Concentrator™ (Zymo Research; Orange, CA) and cloned into the vector for library construction. Clone libraries were generated using a TOPO TA® Cloning Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Clones were sequenced at the University of Georgia Sequencing Facility (Athens, GA).

Analysis of 16S rRNA gene clone libraries. Raw sequence data was edited with Chromas Lite (Technelysium Pty Ltd; Tewantin, Queensland, Australia). Sequence alignments were constructed with the Greengenes software workbench (DeSantis *et al.*, 2006; DeSantis *et al.*, 2006). Sequences were assigned bacterial taxonomy with the Classifier tool on the Ribosomal Database Project (Cole *et al.*, 2009). Libraries were checked for chimeras using the Greengenes Chimera Check with the Bellerophon tool. In the program mothur, a phylogenetic distance matrix, average neighbor operational taxonomic unit (OTU) clustering, and community richness and diversity indices were calculated (Schloss *et al.*, 2009). The significance of differences between groups were determined using the LIBSHUFF method (Singleton *et al.*, 2001). UniFrac distances between libraries were calculated with the Environment Distance Matrix analysis using

normalized weighted abundance (Lozupone *et al.*, 2006). The UniFrac method measures the phylogenetic distance (0.0 – 1.0) between sets of taxa.

Accession numbers. 16S rRNA gene clone sequences were submitted to NCBI (Benson *et al.*, 2005) with accession numbers GU450332 - GU451043.

## **Results and Discussion**

Clone sequences were associated with three bacterial phyla: *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Figure 3.1). *Betaproteobacteria* clone sequences dominated the leaf and cast libraries, while *Clostridia* clone sequences comprised a majority in the larval hindgut libraries. The second largest group of clones associated with the hindgut libraries were *Bacteroidetes* sequences. Only one *Bacteroidetes* sequence was found in the leaf library and none were detected in the cast library.

The shift from *Proteobacteria* associated with the leaf diet, to *Clostridia* and *Bacteroidetes* in the hindgut, then back to *Proteobacteria* in the cast, coincides with physiological changes within the hindgut. The leaf diet and larval casts are primarily aerobic environments with anaerobic microenvironments, which would allow growth of aerobic and facultatively anaerobic *Proteobacteria* while restricting the growth of *Clostridia*, which are exclusively anaerobic, and *Bacteroidetes*, many of which are anaerobic. Anaerobic conditions within the hindgut would allow outgrowth of *Clostridia* and *Bacteroidetes*. Aerobic microenvironments also exist in the hindgut and strict aerobes have been isolated from the *T. abdominalis* larval hindgut (Cook *et al.*, 2007).

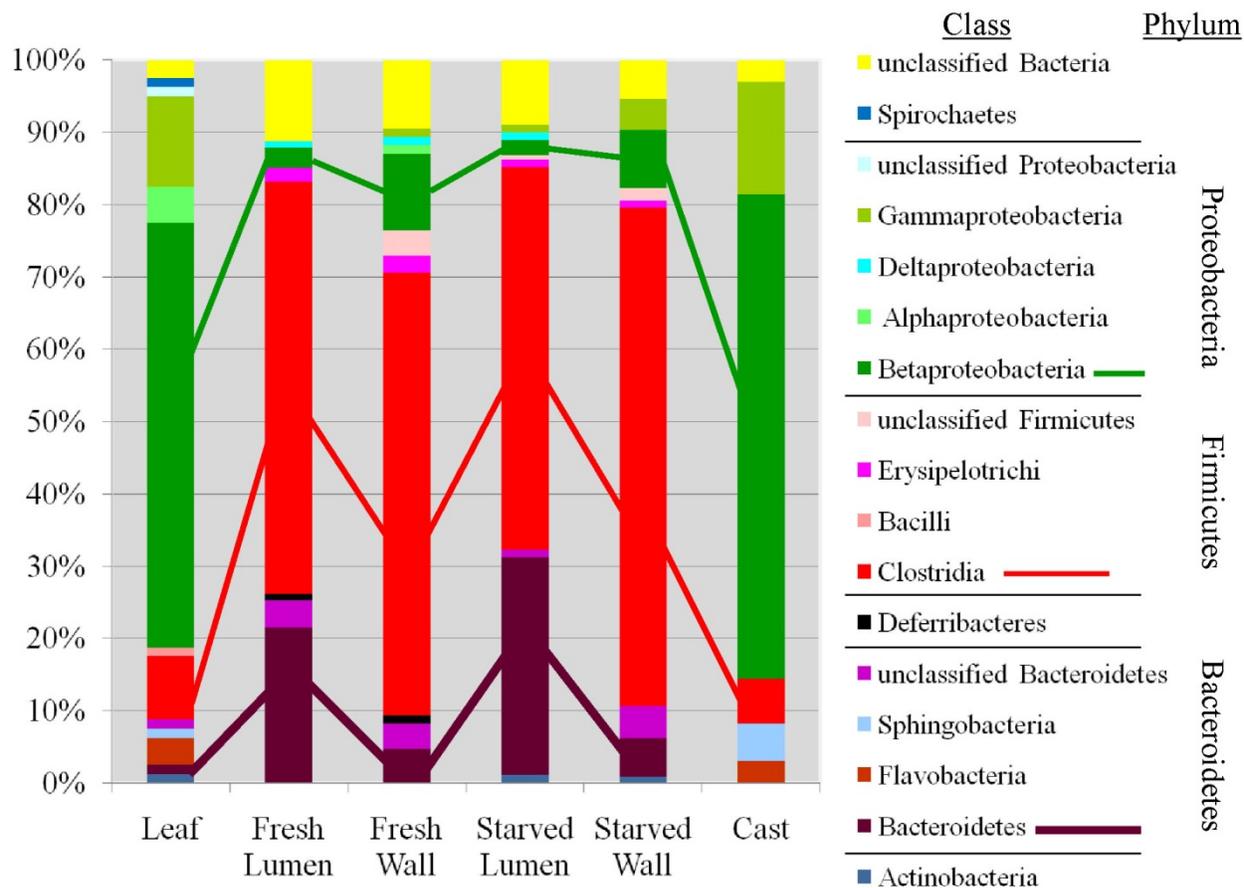


Figure 3.1. Phylogenetic distribution of clones by class, confidence threshold 95% (Classification where sequences could not be assigned with a bootstrap confidence estimate above the 95% threshold are displayed under an artificial 'unclassified' taxon).

A total of 73 clones were assigned to an unclassified *Bacteria*, *Firmicutes*, *Bacteroidetes*, or *Proteobacteria* taxon. Forty two of those clones formed fifteen OTUs at 97% sequence similarity with two or more representatives (Table 3.1). Nine unclassified OTU<sub>0.97</sub> contained representatives from two or more libraries, including previously described cloned libraries from *T. abdominalis* larval hindgut wall – associated bacterial community (Cook *et al.*, 2007). Although these clones were assigned to an unclassified taxon based on their similarity to database (NCBI, RDP) archives, their detection in multiple libraries suggest a conserved association with the *T. abdominalis* larval hindgut.

Table 3.1. Distribution of unclassified OTU<sub>0.97</sub> with two or more representatives within clone libraries. OTUs above dashed line represented in two or libraries.

unclassified OTU <sub>0.97</sub>	number clones in OTU	Distribution of Unclassified Clones in Libraries					Cook <i>et al.</i> 2007
		Leaf	Fresh Lumen	Fresh Wall	Starved Lumen	Starved Wall	
1	3			2			1
2	3			1	2		
3	3		2	1			
4	2		1			1	
5	3			1		2	
6	5		1		4		
7	3		1		1	1	
8	5		4		1		
9	2				1	1	
10	2					2	
11	2	2					
12	2		2				
13	2		2				
14	2				2		
15	3				3		

Bacterial communities from the fresh and starved lumen had the greatest diversity (Table 3.2). Chao1 predicts higher estimations of OTUs at 97% sequence similarity for larval hindgut communities than for leaf diet and cast communities. Diversity indices were approximately 90% of the maximum for all libraries.

Table 3.2. Community richness and diversity calculations for 16S rRNA gene clone libraries at OTU<sub>0.97</sub>.

Library	$N^a$	$S^b$	Chao1 <sup>c</sup>	$H^d$	$H/H_{\max}^e$	Coverage <sup>f</sup>
Leaf	80	39	64.3	3.258	0.89	0.71
Fresh Lumen	148	75	126.75	3.924	0.91	0.69
Fresh Wall	85	48	106.67	3.602	0.93	0.61
Starved Lumen	189	72	95.25	3.908	0.91	0.84
Starved Wall	112	53	109.1	3.584	0.90	0.70
Cast	97	34	61.2	3.110	0.88	0.82

<sup>a</sup>  $N$ , total number of sequences.

<sup>b</sup>  $S$ , number of OTUs at 97% sequence similarity.

<sup>c</sup> Chao1, estimate number of OTUs at 97% sequence similarity;  $= S + (n_1)^2/(2n_2)$ , where  $n_1$  is the number of singletons and  $n_2$  is the number of doubletons.

<sup>d</sup>  $H$ , Shannon diversity index.

<sup>e</sup>  $H_{\max}$ ,  $\ln(S)$ .

<sup>f</sup> Calculated from Good's equation: coverage =  $1 - (n_1/N)$ .

LIBSHUFF results indicate that libraries from the starved and fresh wall were not statistically significantly different (Table 3.3). Those libraries had the lowest UniFrac distance, indicating that they were more similar to each other. Although all other libraries were significantly different from one another, cast and leaf, fresh and starved lumen, fresh lumen and starved wall, and fresh lumen and fresh wall libraries had relatively low UniFrac distances (0.208 – 0.370 relative to 0.604 – 0.771). Lower UniFrac distances indicate that the libraries share more similarities. If starvation had decreased the influx of transient microorganisms so that the

starved lumen subpopulation would be more similar to the wall-associated communities, then the starved lumen would have had lower Unifrac distance values between the starved and fresh wall-associated communities. However, UniFrac results indicate that the starved lumen community were more distant (less similar) to both the starved and fresh wall-associated communities (UniFrac distances 0.604 and 0.618, respectively), suggesting that declining substrate available to the hindgut community from lack of nutrient input disrupted the resident bacterial population during the starvation process (Table 3.3).

In conclusion, the microbial communities associated with the hindgut of *T. abdominalis* were significantly different from the microbiota of the leaf diet and casts. As resident species are distinguished by their ability to colonize and divide at a rate equal to or greater than the elimination rate (Dillon & Dillon, 2004), this change in community structure within the hindgut provides further evidence for the presence of a resident population selectively colonizing the hindgut. Furthermore, clones assigned to unclassified taxa (low similarity to previously described sequences in database archives) detected in multiple libraries suggest novel bacteria in a conserved association with the *T. abdominalis* larval hindgut.

Table 3.3. LIBSHUFF comparisons and UniFrac distances between 16S rRNA gene clone libraries. Highlighted values indicate  $p$ -values above the critical value of 0.0017 (experimentwise  $p$ -value 0.05, critical  $p$ -value calculated using Bonferroni Correction  $p = 1 - (1 - p_{\text{critical}})^{k(k-1)}$ ,  $k$  = number of libraries). In the pair-wise comparisons, libraries are statistically significant different when one  $p$ -value in either the  $x \rightarrow y$  or  $y \rightarrow x$  comparison. The comparison of libraries that were not statistically different is highlighted.

		Comparisons				Comparisons								
		$p$ -Value		$p$ -Value		$p$ -Value		$p$ -Value						
X	Y	$x \rightarrow y$	Statistically Different	UniFrac Distances	X	Y	$x \rightarrow y$	Statistically Different	UniFrac Distances	X	Y	$x \rightarrow y$	Statistically Different	UniFrac Distances
Cast	Fresh Lumen	<0.0001	yes	0.759	Fresh Lumen Starved Lumen	Starved Lumen Starved Wall	<0.0001	yes	0.354	Starved Lumen Starved Wall	Starved Lumen Starved Wall	<0.0001	yes	0.604
Cast	Fresh Wall	<0.0001	yes	0.695	Fresh Lumen Starved Wall	Fresh Lumen Starved Wall	<0.0001	yes	0.357	Fresh Wall Starved Wall	Fresh Wall Starved Wall	0.9956	no	0.126
Cast	Leaf	<0.0001	yes	0.208	Fresh Wall Leaf	Fresh Wall Leaf	<0.0001	yes	0.696	Leaf Starved Lumen	Leaf Starved Lumen	<0.0001	yes	0.771
Cast	Starved Lumen	<0.0001	yes	0.764	Fresh Wall Starved Lumen	Fresh Wall Starved Lumen	0.57	yes	0.618	Leaf Starved Wall	Leaf Starved Wall	<0.0001	yes	0.695
Cast	Starved Wall	<0.0001	yes	0.686	Fresh Lumen Fresh Wall	Fresh Lumen Fresh Wall	<0.0001	yes	0.370	Fresh Lumen Leaf	Fresh Lumen Leaf	<0.0001	yes	0.768
							0.3001							

**CHAPTER 4**

**DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) ANALYSIS OF  
MICROBIAL COMMUNITIES ASSOCIATED WITH HINDGUT WALL, HINDGUT  
LUMEN, AND LEAF LITTER DIET OF *TIPULA ABDOMINALIS* LARVAE<sup>1</sup>**

<sup>1</sup>Cook, Dana M., Christopher E. Bagwell, and Joy Doran Peterson. 2010. Denaturing gradient gel electrophoresis (DGGE) analysis of microbial communities associated with hindgut wall, hindgut lumen, and leaf litter diet of *Tipula abdominalis* larvae. To be submitted to Appl. Environ. Microbiol.

## **Abstract**

The hindguts of *Tipula abdominalis* larvae host a dense and diverse microbiota that assists in digestion of recalcitrant lignocellulosic leaf detritus. Variation in phylogenetic structure between subpopulations of the hindgut attached to the wall epithelium or in the lumen, and casts from individual larvae was assessed via denaturing gradient gel electrophoresis (DGGE).

## **Introduction**

*Tipula abdominalis* is an aquatic crane fly ubiquitous to small riparian streams. Its larvae are primary shredders of leaf litter and, thus, are an important part of the carbon and energy cycling in these ecosystems (Martin *et al.*, 1980; Petersen *et al.*, 1989). The *T. abdominalis* larval hindgut harbors a dense and diverse microbial community (Klug & Kotarski, 1980). The hindgut microbial consortium is presumed to enable the efficient digestion of nutrient poor, recalcitrant leaf litter (Lawson & Klug, 1989; Lawson *et al.*, 1984; Sinsabaugh *et al.*, 1985). The microbial community associated with leaf litter diet is dominated by *Betaproteobacteria*, while the microbiota associated with the hindgut is dominated by *Clostridia* and *Bacteroidetes*; the communities associated with the hindgut wall and lumen are significantly different (Cook & Doran Peterson, 2010; Cook *et al.*, 2007). In the *T. abdominalis* larval gut, bacterial counts increase through the tract, from midgut lumen to hindgut lumen to hindgut wall (Klug & Kotarski, 1980). Epithelial attachment is a likely mechanism for gut colonization, and evidence suggests that a phylogenetically stable resident subpopulation is associated with hindgut wall (Cook & Doran Peterson, 2010).

The objective of the current study was to determine the variation between the microbiota associated with the hindgut wall and lumen from individual larvae using denaturing gradient gel electrophoresis (DGGE) of the variable V3 region of the 16S rRNA gene. The microbial community associated with the leaf litter diet was also evaluated and used as standard to assist in alignment of DGGE bands and gels. It was hypothesized that a greater individual variation would be detected in the lumen microbiota because these subpopulations harbor a greater proportion of transient bacteria. Larvae were sampled from the environment and dissected immediately following collection, or starved for five days. Starvation was intended to decrease the influx of transient microorganisms from the diet into the hindgut and thus decrease individual variation from non-resident bacteria.

## **Materials and Methods**

Sample collection. Larvae, leaf packs (diet on which they were feeding), and stream water were collected from 2<sup>nd</sup> order streams (Vannote, *et al.*, 1980) in Athens, Georgia. Half of the larvae were sacrificed, and hindguts were extracted immediately following collection. The other larvae were maintained individually in filter-sterilized stream water (exchanged daily) and starved for five days. These larvae were then sacrificed, and hindguts were extracted. To extract the hindguts, extreme posterior and anterior ends of the larval body were removed, and the gut tracts were gently pulled from the body. Midguts were separated and removed. A longitudinal incision was made on the hindgut wall, allowing the hindgut to open flat and exposing the lumen material. Lumen material was washed from the hindgut wall by vigorous vortexing in saline for three washes. Lumen was collected from combined wash liquids from each individual larva. Each individual hindgut was homogenized in approximately 1 mL of saline in a tissue grinder

and centrifuged as described above. Approximately 30 grams (wet weight) of the leaf pack from which the larvae were collected was used for DNA extraction. The leaf pack was homogenized in an autoclaved blender with saline then centrifuged at 10,000 x g for 10 min. For all samples, supernatants were discarded and bacterial DNA was extracted from resuspended pellets as described previously (Cook *et al.*, 2007; Shinzato, *et al.*, 1999) and purified using Sepharose 4B spin columns (Jackson, *et al.*, 1997).

Denaturing gradient gel electrophoresis (DGGE). The variable V3 region of the 16S rRNA gene was amplified from purified community genomic DNA using forward primer 341F (5' GC-clamp) and reverse primer 519R (Muyzer *et al.*, 1993). PCR amplification was performed with Invitrogen (Carlsbad, CA) Platinum Taq Polymerase following the manufacturer's instructions with temperature cycling as described previously (Muyzer, *et al.*, 1993). Two hundred ul of PCR product was purified and concentrated with Clean & Concentrator™ (Zymo Research; Orange, CA). DGGE was performed on an 8% (wt/vol) polyacrylamide gel with a 35-55% denaturing gradient. Gel casting and electrophoresis (200V, 60C, 4 hrs) was performed with the Bio-Rad (Hercules, CA) D-Code System following the manufacturer's instructions. After electrophoresis, gels were stained in 1X TAE containing 0.5 mg/L ethidium bromide for 30 min and then destained in 1X TAE for 5 min. Gels were visualized with a Fotodyne (Hartland, WI) UV transillumination system.

DGGE analysis. For each gel, a binary matrix was created from the banding patterns: each band position was scored as 0 for absence and 1 for presence of a band. To provide a reference for band position across samples, horizontal lines were drawn with Gel-Pro Analyzer software (Media Cybernetics; Silver Spring, MD). Leaf lanes that were loaded near the left and right side of each gel were sub-samples of one pool of PCR products from a single community

DNA source. Therefore, leaf lanes produced nearly identical banding patterns. Bands from the leaf lanes, as well as prominent bands in all other lanes, served as references to align bands across gels. One binary matrix was constructed from fresh lumen, fresh wall, starved lumen, and starved wall gels, each containing 2 leaf lanes. From the binary matrix, a distance matrix was calculated by pair-wise comparisons of all samples with the following equation: distance =  $1 - (\# \text{ bands similar} / \text{average } \# \text{ bands})$ . Unweighted pair group method with arithmetic mean (UPGMA) analysis was performed with the distance matrices using MEGA3 (Kumar *et al.*, 2004).

## Results and Discussion

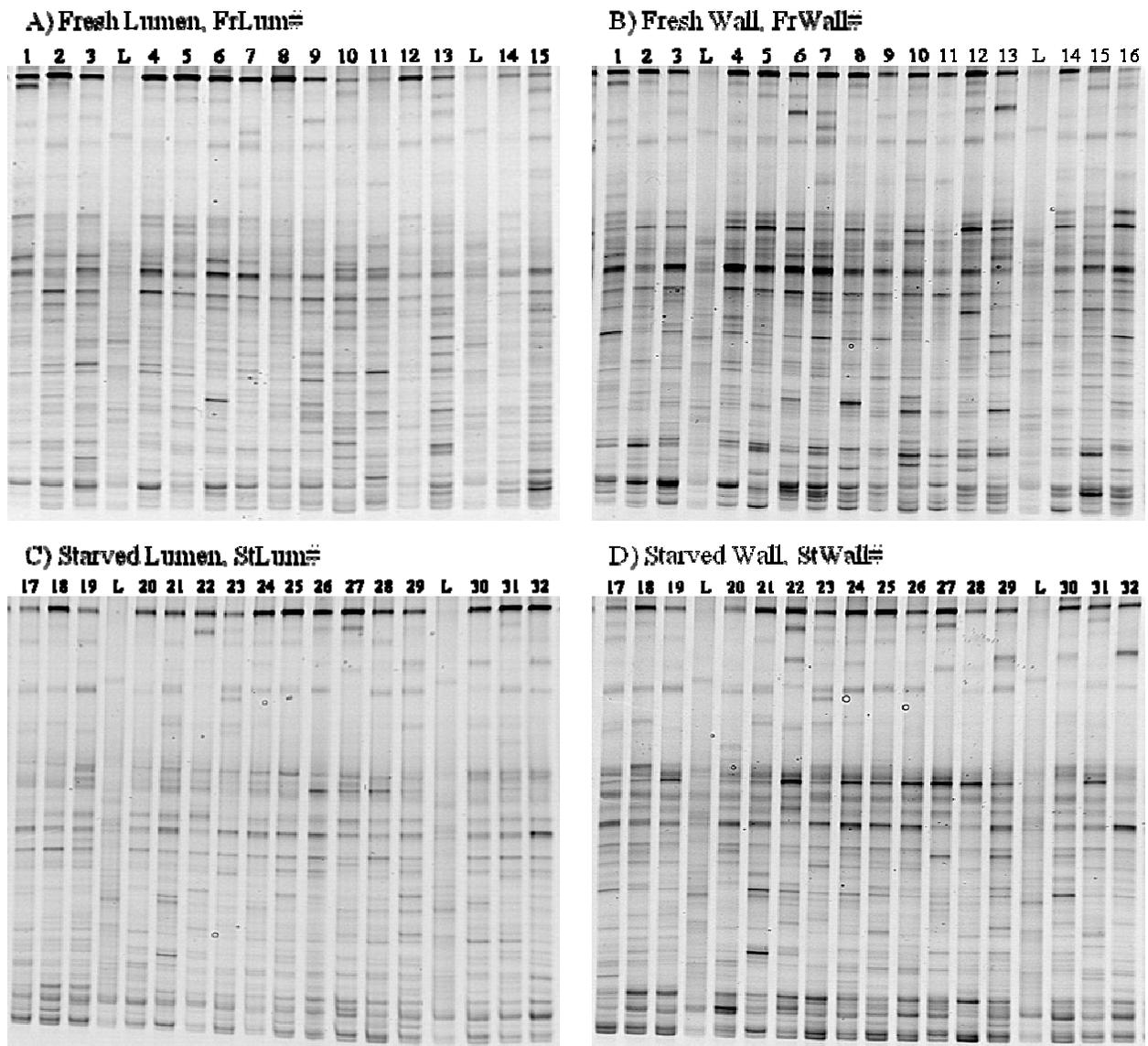
Images of DGGE results are shown in Figure 4.1. PCR amplification from sample fresh lumen16 was unsuccessful. Results from UPGMA clustering of DGGE banding patterns are presented in Figure 4.2. Since they were replicates from one pool of PCR products, the banding pattern of leaf lanes were identical, or very similar, and those samples formed an out-group cluster divergent from all other samples (cluster II). Hindgut community samples branched to form two clusters: cluster A consisting of almost entirely fresh lumen samples, and cluster B consisting of mostly fresh wall, starved wall, and starved lumen samples. Cluster B branched to form two main clusters (and a 3<sup>rd</sup> branch containing two outliers): cluster 1 consisting mostly of fresh wall samples, and cluster 2 consisting entirely of starved wall and starved lumen samples.

UPGMA clustering trend suggests that the starved wall and starved lumen bacterial communities are most similar to each other. This is in contrast to previous studies with clone libraries, which indicated that communities associated with the fresh and starved wall were most similar, while the starved wall and starved lumen were relatively more distant (Cook & Doran

Peterson, 2010). However, those clone libraries were constructed from bacterial DNA pooled from multiple larvae; individual variation detected by DGGE in the current study could compound when combined in the larger collective community from several individuals. Furthermore, this methodological discrepancy could be due to high microdiversity within multiple phylotypes (Sánchez *et al.*, 2009). Although discrepancies between DGGE and other molecular techniques are reported in the literature (e.g. Alonso-Sáez & Gasol, 2007; Niemi *et al.*, 2001; Sánchez, *et al.*, 2009), DGGE has been shown to detect numerically important organisms (Calábria de Araújo & Schneider, 2008) and to provide reasonably comprehensive descriptions of microbial communities compared to clone libraries (Lovell *et al.*, 2008).

The microbial community associated with the fresh hindgut lumen had higher average distances between clusters (longer branch length, Figure 4.2), suggesting that variability between bacterial communities from individual larval hindgut lumen, presumably from a higher abundance of transient bacteria, causes the subpopulation associated with the lumen to diverge from the subpopulation associated with the hindgut wall. This divergence is diminished in bacterial communities associated with the hindgut wall and lumen from starved larvae, presumably because starvation reduces the influx of transient bacteria, resulting in decreased variability. In conclusion, evidence supports the hypothesis that the *T. abdominalis* larval hindgut lumen harbors a subpopulation of transient, non-colonizing bacteria, while bacteria associated with the larval hindgut wall represents a resident consortium.

Figure 4.1 Gel images of DGGE results. Number indicates source larva. Lanes label "L" contain PCR product from community DNA associated with the leaf litter diet.



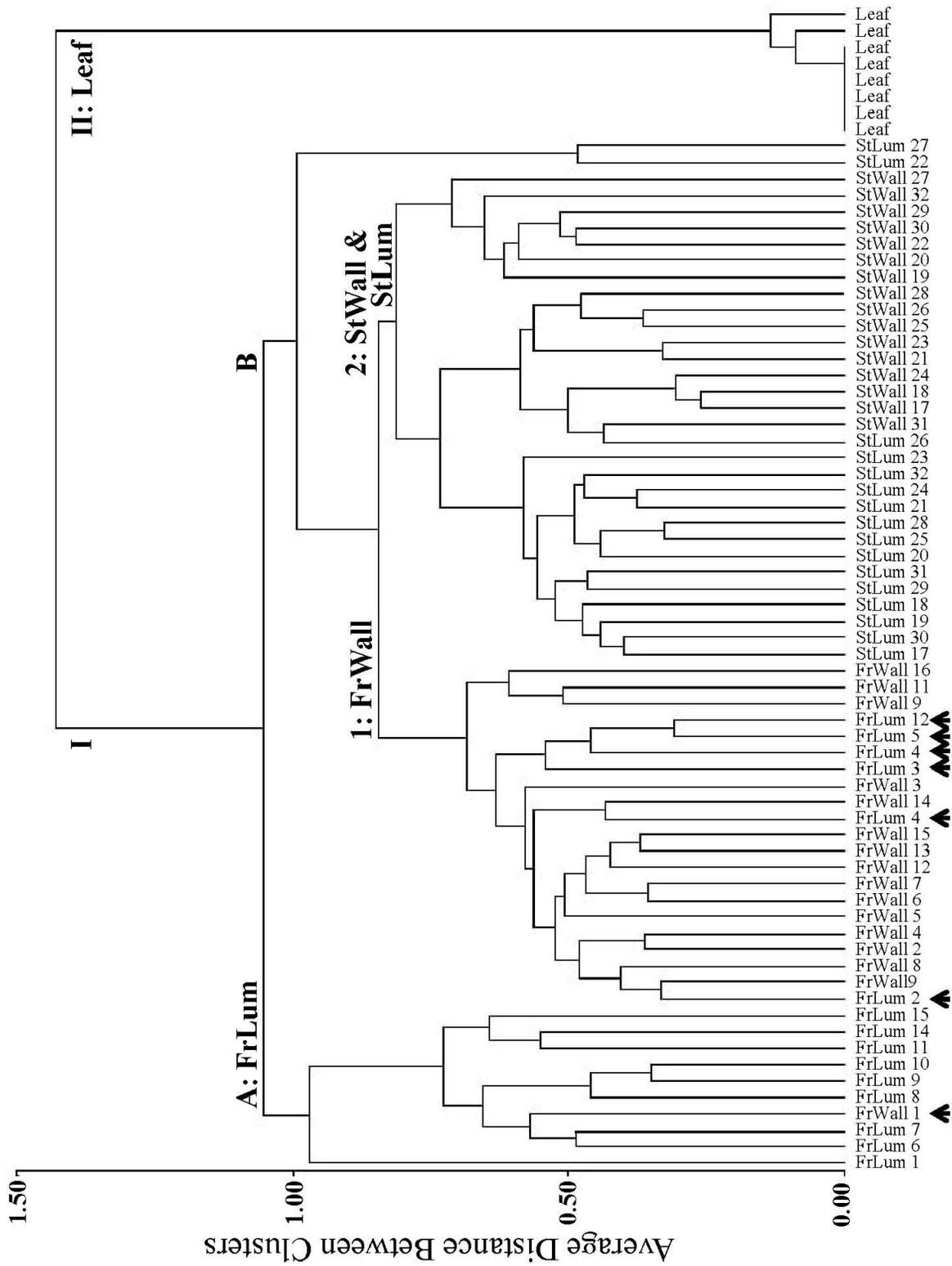


Figure 4.2. Dendrogram of UPGMA clustering of DGGGE banding patterns. Arrows indicate outliers from the general clustering trend.

**CHAPTER 5**

**ENRICHMENT FOR CELLULOLYTIC BACTERIAL COMMUNITIES FROM  
THE HINDGUT OF THE DETRITUS-FEEDING LARVAE OF *TIPULA ABDOMINALIS*  
(DIPTERA: TIPULIDAE)**

Cook, D. M. and Doran Peterson, J. 2010. Enrichment for cellulolytic bacterial communities from the hindgut of the detritus-feeding larvae of *Tipula abdominalis* (Diptera: Tipulidae). To be submitted to Appl. Environ. Microbiol.

## **Abstract**

*Tipula abdominalis* larvae are primary shredders of leaf litter detritus in low-order riparian streams. The dense and diverse hindgut bacterial consortium presumably enables the larvae to survive on a cellulose-rich diet. Cellulose degradation can be enhanced by co-culturing cellulolytic anaerobes and non-cellulolytic aerobes; in nature, cellulose degradation occurs through the cooperation of many microorganisms suggesting a synergistic relationship. In such functionally specific systems, bacterial community composition can be influenced by both history (inoculating population) and environment (medium). To examine bacterial community composition and cooperative cellulose degradation from sub-populations of the hindgut, enrichment cultures were established in glass-stoppered bottles, which allowed aeration and cooperative cellulose degradation by aerobes and anaerobes. Cultures became anaerobic, with visible degradation of filter paper. Successive generations of the enrichment culture communities were cultivated, and aerobes and anaerobes were isolated from the third generation on nutrient limited media containing cellulose or cellobiose. Denaturing gradient gel electrophoresis (DGGE) analysis of the variable V3 region of the 16S rRNA genes was used to compare the communities and isolates obtained from the third generation enrichment cultures.

## **Introduction**

The hindgut of *Tipula abdominalis* larvae hosts a dense and diverse bacterial community that facilitates digestion of the lignocellulosic leaf diet. Many species of microorganisms coexist in communities by interacting with one another. Individual members of microbial communities are often most metabolically active only when in association with other members of the community. An example of such cooperative activity is lignocellulose degradation, which has

been reported to be most effective with a mixed culture of cellulolytic and non-cellulolytic bacteria (Haruta *et al.*, 2002; Odom & Wall, 1983). In a specific example, a cellulolytic anaerobe, *Clostridium straminisolvens* CSK1, isolated from a constructed cellulose-degrading microbial community, had greater cellulose-degrading efficiency when it was mixed with a non-cellulolytic aerobe isolated from the same community than in pure culture (Kato *et al.*, 2004). These studies indicate that non-cellulolytic aerobes can enhance cellulose degradation, presumably by establishing and maintaining anaerobic conditions, neutralizing pH, and consuming metabolites that might interfere with cellulose degradation.

The hindgut of *T. abdominalis* larvae is an environment of cellulose degradation from which non-cellulolytic aerobes and cellulolytic anaerobes have been identified (Cook *et al.*, 2007). To investigate cellulose degradation by cellulolytic anaerobes with other members of the *T. abdominalis* hindgut bacterial community, enrichment cultures for cellulose degradation were established. Because different sub-populations seem to be associated with either the lumen or wall epithelium of the hindgut (Klug & Kotarski, 1980; Cook *et al.*, 2007), three separate enrichment cultures were inoculated with bacterial communities from 1) the lumen, 2) the hindgut wall, and 3) whole hindguts. Changes in community structure were monitored over three generations (successive transfers) of enrichment cultures.

## **Materials and Methods**

Sample collection and preparation. Thirty larvae were collected from a 2<sup>nd</sup> order stream in Athens, GA. To extract the hindguts, extreme posterior and anterior ends of the larval body were removed, and the gut tracts were gently pulled from the body. Midguts were separated and removed. Hindguts were then transferred to reduced buffered salt solution (BSS)

(Leadbetter & Breznak, 1996). A longitudinal incision was made on the hindgut wall, allowing the hindgut to open flat to expose the lumen material. The lumen material was separated from the hindgut gut wall; loosely associated microorganisms were removed by 30 s vigorous vortex washing (X3) of the hindgut wall in BSS. Lumen material from the washes were pooled, centrifuged at 13,000 x g for 10 min, and homogenized in 6 mL BSS. Washed hindgut walls were pooled and homogenized in 6 mL of BSS.

Enrichment cultures. Cellulolytic enrichment media (g/L: 1.0 K<sub>2</sub>HPO<sub>4</sub>, 1.0 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 MgSO<sub>4</sub> • 7H<sub>2</sub>O, 2 CaCO<sub>3</sub>, 0.5 NaCl, 0.001 resazurin, 20 shredded Whatman #1 filter paper) was prepared in 150mL glass-stoppered bottles (Gottschalk *et al.*, 1981). Three replicates of enrichment cultures were established from the hindgut wall, lumen material, and whole hindgut. Generation 1 hindgut wall and lumen enrichment cultures were inoculated with 0.5 mL wall and lumen homogenate, respectively. Generation 1 whole hindgut enrichment cultures were inoculated with 250ul each of hindgut wall and lumen homogenate. After inoculation all cultures were incubated at 22°C x 12 d. Thirty mL Generation 1 enrichment cultures was centrifuged 10, 000 x g for 10 min. The supernatants were discarded and the pellets were used to inoculate Generation 2 enrichment cultures (150 mL). After 12 d incubation, 30 mL Generation 2 enrichment cultures was centrifuged 10, 000 x g for 10 min. The supernatants were discarded and the pellets were used to inoculate Generation 3 enrichment cultures (150 mL) (Figure 5.1).

Bacterial isolation and screening. Generation 3 enrichment cultures were serially diluted on media (g/L: 7.5 K<sub>2</sub>HPO<sub>4</sub>, 3.5 KH<sub>2</sub>PO<sub>4</sub>, 0.5 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0 NaCl, 0.05 MgSO<sub>4</sub> • 7H<sub>2</sub>O, 0.05 CaCl<sub>2</sub>, 1.0 yeast extract, 15 agar) containing either 10 g/L crystalline cellulose (Sigma), or 5 g/L cellobiose both aerobically and anaerobically (media also contained 1 mg resazurin and 0.5 g L-cysteine HCl per L) (Gottschalk *et al.*, 1981). Three hundred eighty four isolates (192 each

aerobes and anaerobes) were tested for enzymatic activity towards model substrates as described previously: carboxymethylcellulose (CMC, Sigma) (Wood & Kellogg, 1988); xylan from oat spelts (Sigma) (Mondou *et al.*, 1986). The cultivable sub-population was compared to the greater population: dense (poor separation of colonies for isolation) plates were washed with TSB to collect culture, from which DNA was extracted.

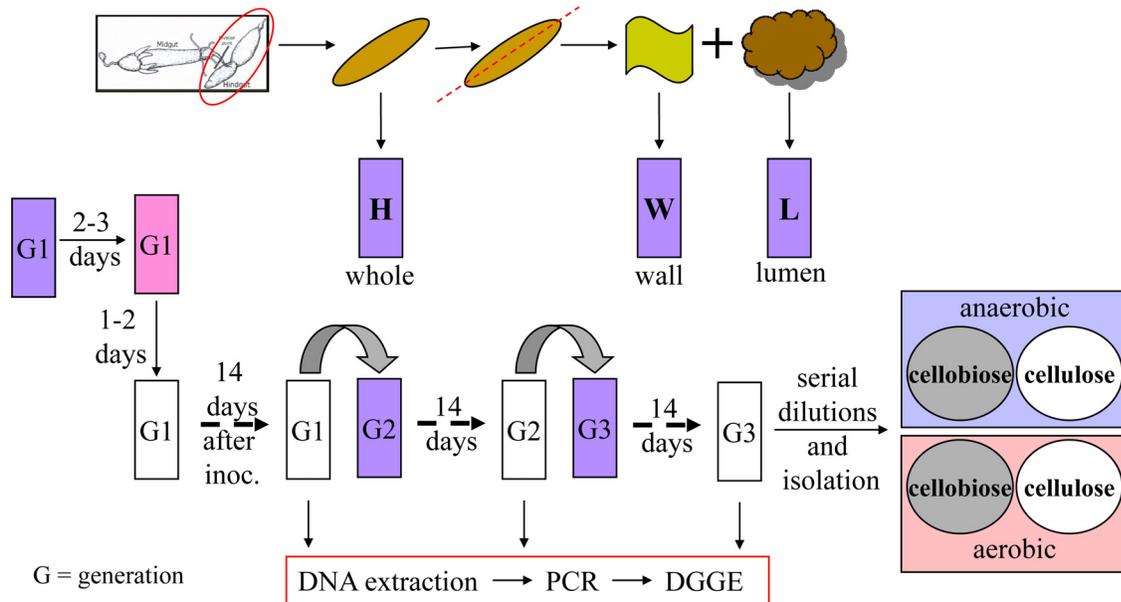


Figure 5.1. Methods diagram for construction of enrichment cultures, succession of generations, and cultivation of isolates.

DNA extraction, PCR, and DGGE. Bacterial DNA was extracted from original homogenates, enrichment cultures, and culture washed from serial dilutions (Shinzato *et al.*, 1999) and purified using Sepharose 4B spin columns (Jackson *et al.*, 1997). The variable V3 region of the 16S rRNA genes were amplified from purified community genomic DNA using reverse primer 519R and forward primer 341F with an additional GC-rich sequence at the 5' end (Muyzer *et al.*, 1993). PCR amplification was performed with Invitrogen (Carlsbad, CA) Platinum Taq Polymerase following the manufacturer's instructions and temperature cycling as

described previously (Muyzer *et al.*, 1993). Product was purified and concentrated with QIAquick PCR Purification Kit (Qiagen; Valencia, CA). DGGE was performed with an 8% (wt/vol) polyacrylamide gel with a denaturing gradient of 35-55%. Gel casting and electrophoresis (200V, 60C, 4 hrs) was performed with the Bio-Rad (Hercules, CA) D-Code System following the manufacturer's instructions. After electrophoresis, gels were stained in 1X TAE containing 0.5 mg/L ethidium bromide for 30 min, then destained in 1X TAE for 5 min. Gels were visualized with a Fotodyne (Hartland, WI ) UV transillumination system.

DGGE analysis. For each gel, a binary matrix was created from the banding patterns: each band position was scored as 0 for absence and 1 for presence of a band. To provide reference for band position across samples, horizontal lines were drawn with Gel-Pro Analyzer software (Media Cybernetics; Sliver Spring, MD). From the binary matrix, a distance matrix was calculated by pair-wise comparisons of all samples with the following equation: distance = 1 – (# bands similar / average # bands). UPGMA analysis was performed with the distance matrices using MEGA3 (Kumar *et al.*, 2004).

## **Results and Discussion**

Enrichment cultures. All enrichment cultures became anaerobic by five days after inoculation (Figure 5.2), as indicated by a transition from blue to pink to colorless (reduction of resazurin in culture). In most cases, enrichment cultures from the whole gut became anaerobic the quickest, while those from the hindgut wall were the slowest. Because the cultures were contained in glass-stoppered bottles which allowed for some oxygen diffusion, it was presumed that aerobic and facultative anaerobic bacteria in the cultures were maintaining anaerobic conditions by consuming oxygen. Shredded filter paper was the only carbon source present in

the medium, and by ten days following inoculation, visible degradation of the filter paper substrate was evident.

Bacterial isolation and screening on model substrates. Serial dilutions were performed from third generation enrichment cultures on media containing either cellobiose or cellulose both aerobically and anaerobically. Cellobiose was chosen as a substrate because it is readily fermented by cellulolytic *Clostridium* spp. (Gottschalk *et al.*, 1981). Also, cellobiose is a product of cellulose degradation and may be utilized by non-cellulolytic bacteria in a cooperative cellulose-degrading community. Cellobiose can inhibit cellulose degradation; therefore, bacteria that consume cellobiose would facilitate cellulose degradation.

One hundred ninety two each of aerobically and anaerobically cultivated isolates were selected from the serial dilutions. Because facultative metabolism has not yet been determined, isolates will be referred to as aerobic or anaerobic. Isolates were tested for enzymatic activity towards model substrates carboxymethylcellulose (CMC) and xylan (Table 5.1). CMC was chosen as model substrate for cellulose, which was the only supplemented carbon source present in the enrichment culture medium. Although xylan was not present in the enrichment culture medium, previously described *T. abdominalis* larval hindgut isolates have demonstrated activity towards these substrates (Cook *et al.*, 2007), and organisms capable of degrading such plant polymer substrates are of interest for biotechnology applications.

Whether isolates were obtained on cellulose or cellobiose containing medium did not correlate to activity on xylan or CMC (Table 5.1). In contrast, significantly more anaerobic isolates were able to degrade CMC and xylan than aerobic isolates. Of the anaerobic isolates, 57% were positive for degradation of CMC, while only 11% of aerobic isolates were positive.

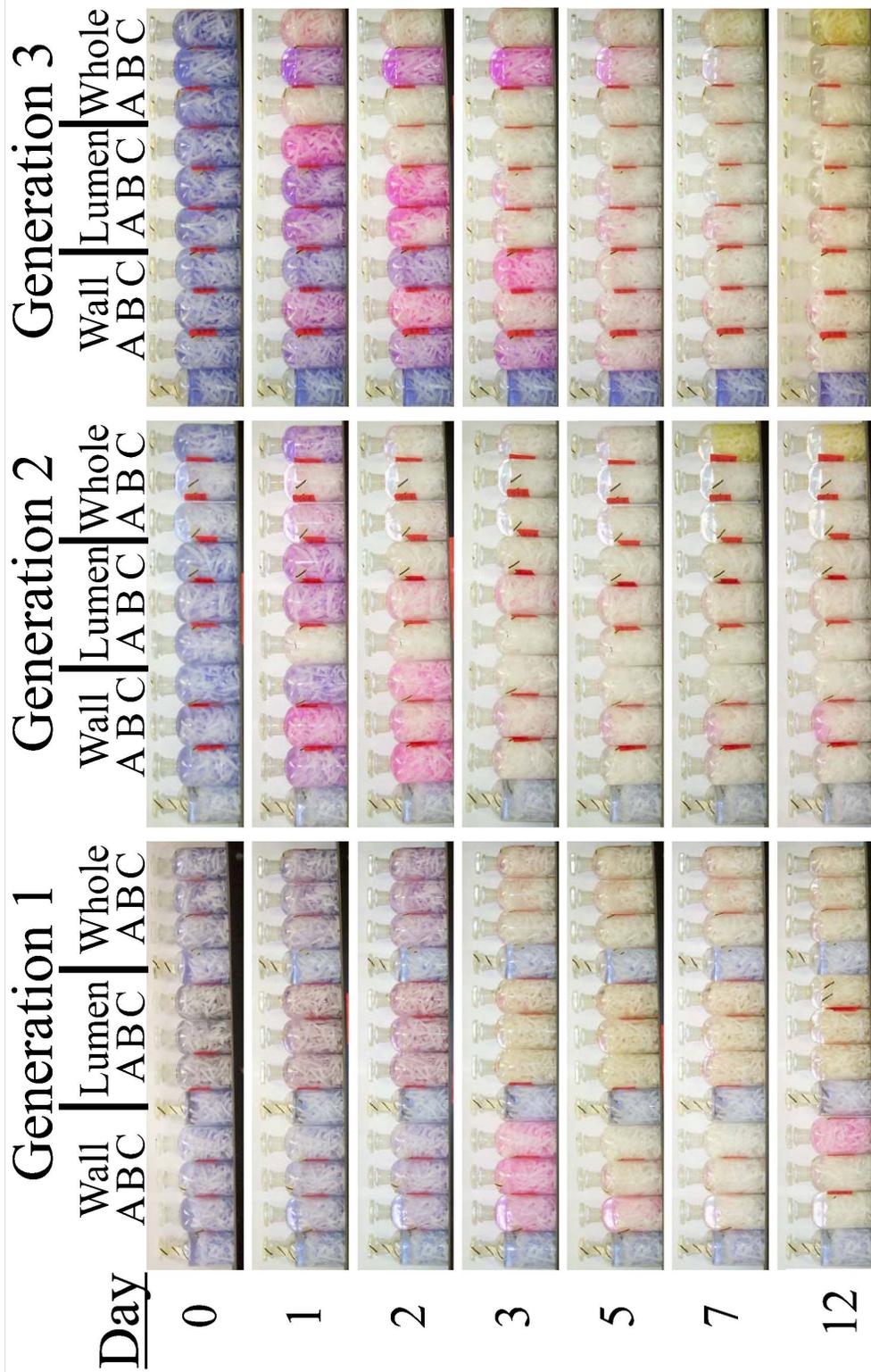


Figure 5.2. Progression of cellulolytic enrichment culture over 12 days and through three generations. Bottle on left is uninoculated control.

Likewise, 52% and 7% of the anaerobic and aerobic isolates, respectively, were positive for xylan degradation (Figure 5.3). Isolates were usually positive on both xylan and CMC, 80% of the positive isolates had activity towards both substrates.

Table 5.1. Results of isolate screening on xylan and CMC, and number of isolates that were positive on both substrates. 192 each of aerobically and anaerobically cultivated isolates.

	Aerobic					
	Cellulose			Cellobiose		
	Xylan	CMC	both	Xylan	CMC	both
Wall	0	4	0	1	2	1
Lumen	1	0	0	0	2	0
Whole	5	6	5	6	8	6
	Anaerobic					
	Cellulose			Cellobiose		
	Xylan	CMC	both	Xylan	CMC	both
Wall	8	14	8	8	10	5
Lumen	21	22	21	20	20	20
Whole	20	20	20	23	23	23

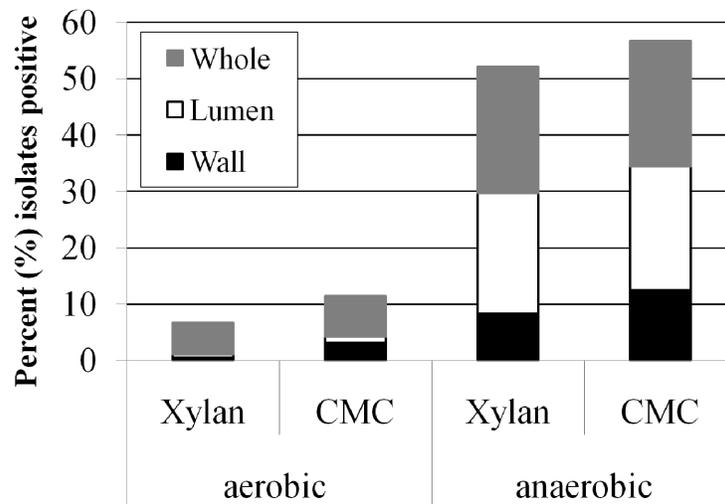


Figure 5.3. Percent aerobically and anaerobically cultivated isolates positive on xylan and CMC. Wall, lumen, and whole indicate the inoculum source for the enrichment culture from which the isolates were cultivated (Figure 5.1).

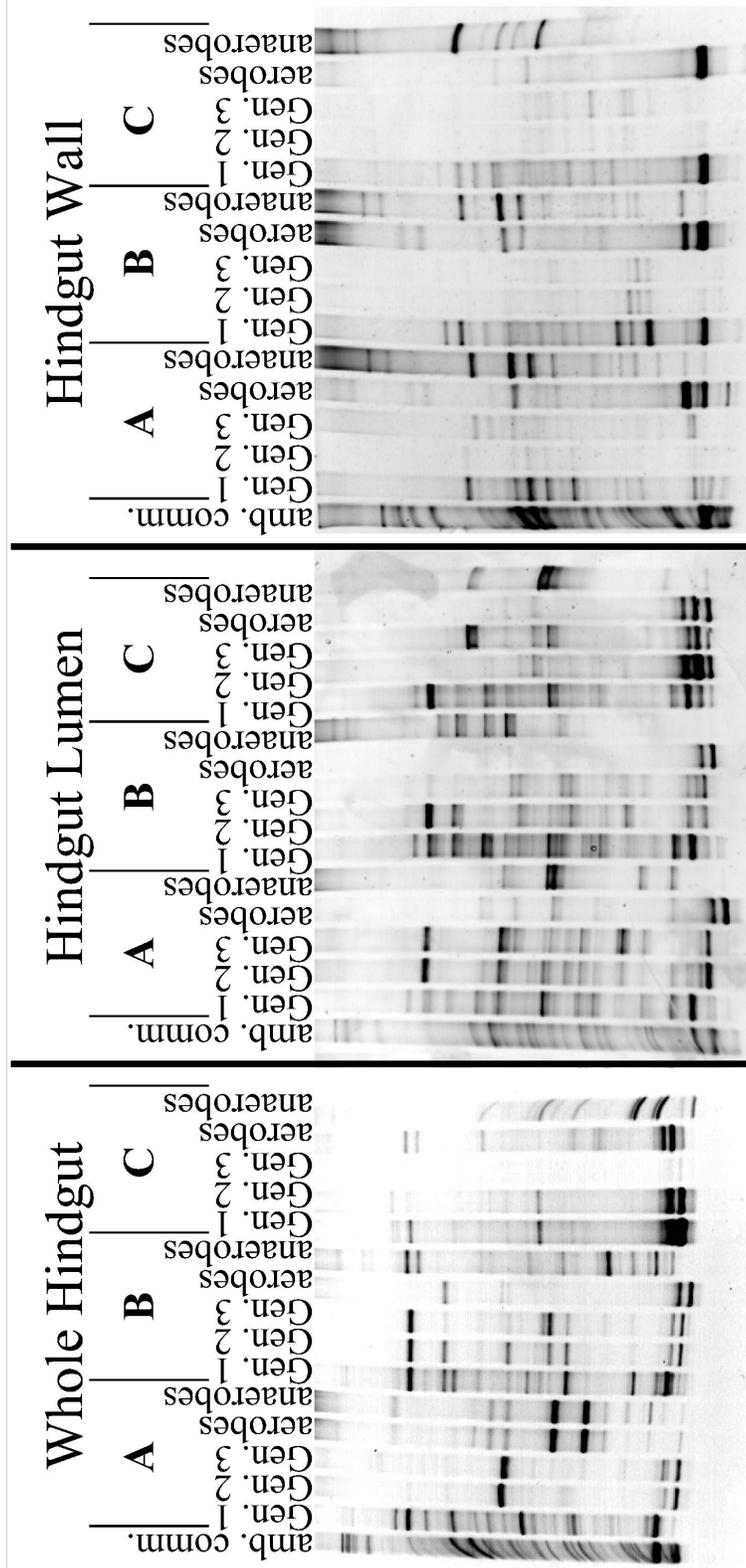


Figure 5.4. Images of DGGE analysis of enrichment cultures. Abbreviations: amb. comm., ambient community that was used as inoculum; Gen. 1-3 in replicates A, B, and C; aerobes and anaerobes, plate washes from aerobic and anaerobic culture from serial dilutions for isolation of colonies.

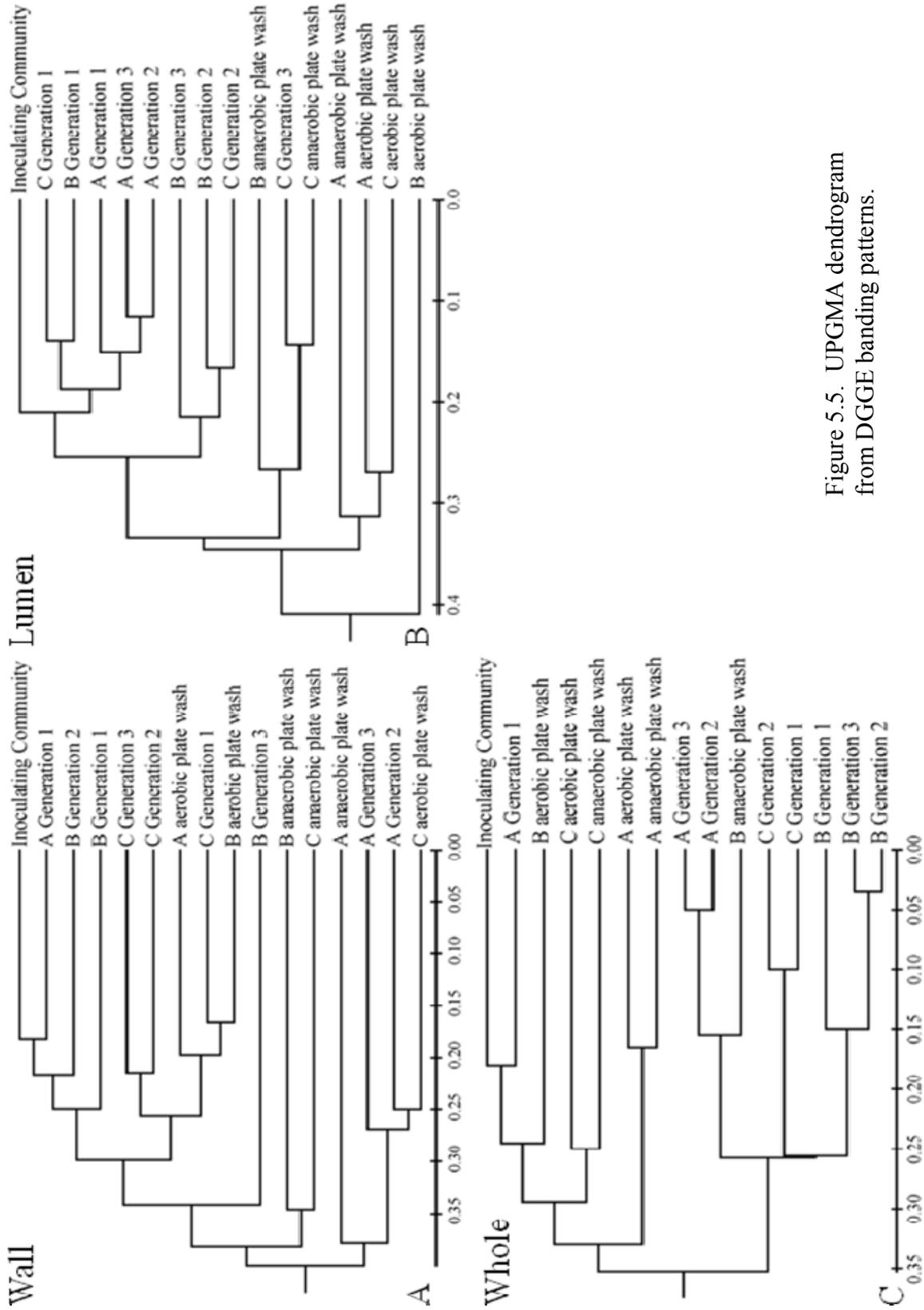


Figure 5.5. UPGMA dendrogram from DGGE banding patterns.

DGGE analysis. Gel images are shown in Figure 5.4. Enrichment cultures and plate washes had fewer bands than the inoculating community, indicating a decrease in diversity. Many of the bands from enrichment cultures also appeared in either the aerobic or anaerobic plate washes. It was hypothesized that the inoculating community would cluster with first generation enrichment cultures, and second and third generations would form clusters each respectively a greater distance from inoculating community and first generations, indicating a reduction to a constant cellulolytic sub-population. The inoculating communities did cluster with one or more replicates of first and second generation enrichment cultures; however, third generations and plate washes also clustered with the inoculating community. In two incidences (replicates A and B in lumen and whole, respectively), first, second, and third generations of the same replicate clustered together (Figure 5.5).

In contrast to expectations, a phylogenetically consistent cellulolytic bacterial community did not emerge from three generations of enrichment culture; however, a functionally stable community was obtained. Despite differences in community structure, anaerobic conditions were stably maintained and degradation of filter paper continued in enrichment cultures. Other studies which have shown that extremely dynamic bacterial communities can maintain stable ecosystem function (Langenheder *et al.*, 2006; Fernández *et al.*, 1999). Variation in phylogenetic composition of the hindgut bacterial community between individual larvae has been demonstrated previously (Cook *et al.*, 2007), yet all function in facilitation of leaf diet digestion similarly.

## **Acknowledgements**

REU student Matthew Farron is acknowledged for work in characterizing isolates.

## CHAPTER 6

### ***KLUGIELLA XANTHOTIPULAE* GEN. NOV., SP. NOV., A NOVEL MEMBER OF THE FAMILY *MICROBACTERIACEAE*<sup>1</sup>**

<sup>1</sup>Cook, Dana M., Emily DeCrescenzo Henriksen, Theresa E. Rogers, and Joy Doran Peterson. 2008. *Klugiella xanthotipulae* gen. nov., sp. nov., a novel member of the family *Microbacteriaceae*. *Int. J. Syst. Evol. Microbiol.* 58: 2779 – 2782. Reprinted here with permission from publisher.

## Abstract

An actinobacterium, designated strain 44C3<sup>T</sup>, was isolated in Michigan, USA, from the hindgut of the larvae *Tipula abdominalis*, an aquatic crane fly, and was subjected to polyphasic taxonomic investigation. Phylogenetic analysis of the 16S rRNA gene sequence revealed the strain represented a separate clade within the family *Microbacteriaceae*. It had highest 16S rRNA gene sequence similarity with the type strain of *Cryobacterium psychrotolerans* JCM 13925<sup>T</sup> (DQ515963) (96.5%). Strain 44C3<sup>T</sup> represented a novel B-type peptidoglycan. The peptidoglycan contained the diamino acid lysine, the peptide Gly – D-Glu was detected in the partial hydrolysate, and alanine was the N-terminus of the interpeptide bridge. No other amino acids found in some B-type peptidoglycans (including diaminobutyric acid, ornithine, homoserine, and hydroxyglutamic acid) could be detected. The major menaquinones were MK-12 and MK-11, the major fatty acids were ai-C<sub>15:0</sub>, ai-C<sub>17:0</sub>, i-C<sub>16:0</sub>, and the DNA G + C content was 60.9 mol%. Analysis of chemotaxonomic and phylogenetic data supports the designation of strain 44C3<sup>T</sup> as a novel genus within the family *Microbacteriaceae*. The name *Klugiella xanthotipulae* gen. nov., sp. nov. is proposed. The type strain of *Klugiella xanthotipulae* is 44C3<sup>T</sup> (=DSM 18031 = ATCC BAA-1524).

## Introduction

Strain 44C3<sup>T</sup> was isolated from the hindgut of *Tipula abdominalis* larvae as described previously (Cook *et al.*, 2007). *T. abdominalis* is an aquatic crane fly; the larvae primary shredders of leaf litter in small, riparian streams. The hindgut of *T. abdominalis* larvae hosts a dense and diverse bacterial community (Klug & Kotarski, 1980), which is suggested to facilitate digestion of the lignocellulosic diet (Lawson & Klug, 1989).

## Materials and Methods, Results, and Discussion

Strain 44C3<sup>T</sup> was maintained as 40 % (wt/v) glycerol suspensions at -20°C. Culture for biochemical and molecular studies was obtained by cultivation on tryptic soy agar (TSA; Difco) or in tryptic soy broth (TSB; Difco) at 28°C for 48 h. Cultures were incubated at 4, 10, 22, 28, 30, 37, and 45 °C to determine range and optimum temperature for growth. At 28 °C, growth was tested at pH 6-12, and with NaCl concentrations of 0.5-9% to determine pH and NaCl optima and range. Colony morphology was observed on TSA after 48 h growth at 28°C. Gram stain was performed, and standard physiological tests were performed with API NE, API Staph, API Strep, and API Coryne test kits (bioMérieux; Marcy l'Etoile, France). For phase contrast microscopy observation, cells were viewed at 100X magnification using the Leica SP2 upright microscope (Leica Microsystems Inc., Exton, PA) and images were captured with the Zeiss AxioCam (Carl Zeiss Micro-Imaging, Inc., Thornwood, NY) at the Center for Advanced Ultrastructure Research at the University of Georgia. A pixel to  $\mu\text{m}$  ratio was calculated using imaging software and was used to determine cell size.

Strain 44C3<sup>T</sup> stained Gram-variable, but was Gram-type positive, was aerobic, grew optimally at 28°C, and was able to grow at 4-30°C. While limited growth did occur at 4°C, it was not observed until 672 h (4 weeks) incubation. Irregular rod-shaped cells (0.6-3.4  $\mu\text{m}$  x 0.4-0.8  $\mu\text{m}$ ) were observed, but spores were not found. Small, smooth, and yellow colonies formed on TSA. Detailed biochemical and physiological characteristics of the strain are given in the genus and species description.

Cell wall sugars, menaquinone, amino acid, and acyl type analyses were performed by the DSMZ under the direction of Dr. Peter Schumann using previously described methods (Groth *et al.*, 1996; Schleifer, 1985; Schleifer & Kandler, 1972; Stanek & Roberts, 1974; Uchida *et al.*,

1999). Total cellular fatty acids were analyzed using the MIDI-FAME procedure essentially as described previously (Haack *et al.*, 1994); gas chromatographs were compared to profiles generated by authentic standards and archived profiles from known cultures grown under standard conditions using the MIDI Microbial Identification software (MIDI, Newark, DE).

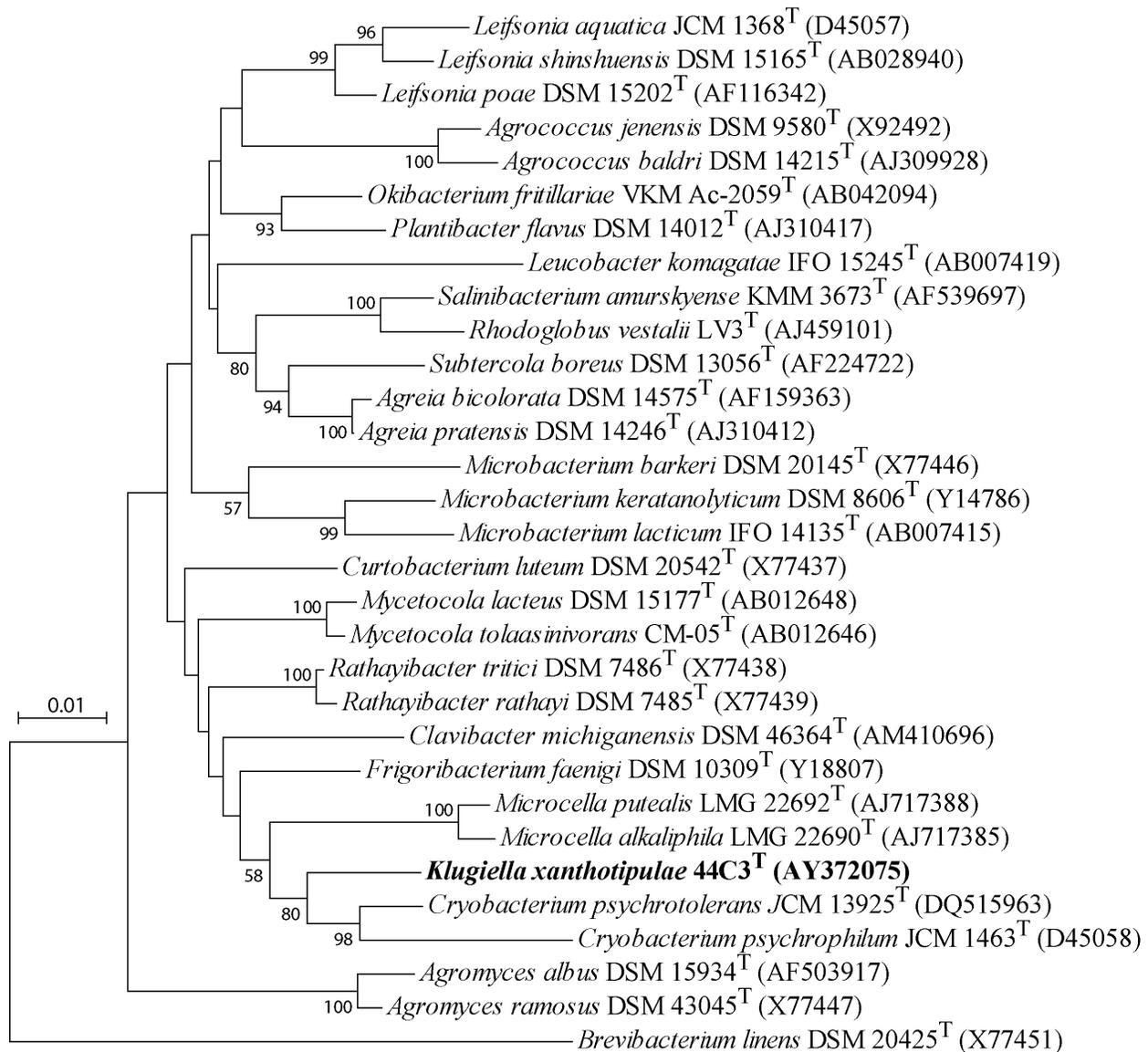
Rhamnose was the only cell wall sugar. The major menaquinones were MK-12 and MK-11. The major fatty acids were ai-C<sub>15:0</sub> (54.7 %), ai-C<sub>17:0</sub> (18.3 %), and i-C<sub>16:0</sub> (17.9 %); detailed fatty acid profile is given in the species description. No glycosyl residues were found in the peptidoglycan, thus the peptidoglycan of is of the acetyl type. Analysis of the cell wall amino acids revealed that the peptidoglycan of strain 44C3<sup>T</sup> contained alanine, glycine, glutamate, and lysine in a molar ratio of molar ratios 1.6: 0.9: 1.0: 1.0. No other amino acids found in some B-type peptidoglycans (including diaminobutyric acid, ornithine, homoserine, or hydroxyglutamic acid) could be detected. As usual for B-type peptidoglycans, the peptide Gly – D - Glu was detected in the partial hydrolysate. The peptide D-Ala – Ala was found confirming alanine as the N-terminus of the interpeptide bridge. Three additional peptides were found, which were most likely composed of lysine and alanine residues. While these data are not sufficient to propose a structure, they do not concur with published peptidoglycan structures, and support the conclusion that 44C3<sup>T</sup> represents a novel B-type peptidoglycan.

Sequencing of the 16S rRNA gene was performed at MIDI Labs (Newark, DE). Putative identity of strains was determined by searching catalogued sequences in GenBank (Benson *et al.*, 2005) using the BLAST tool (Altschul *et al.*, 1990). Sequence alignments of strains and most closely related actinobacteria were created using ClustalX (Thompson *et al.*, 1997), and edited in GeneDoc (Nicholas, 1997). Distances were calculated using the Jukes-Cantor algorithm (Jukes & Cantor, 1969), and branching order was calculated using neighbor-joining (Saitou & Nei,

1987). A phylogenetic tree was constructed using the program MEGA 3.1, which internally calculates bootstrap values (Kumar *et al.*, 2004). To confirm phylogenetic position of 44C3<sup>T</sup>, a minimum evolution algorithm analysis was also performed using the MEGA 3.1 program. Extraction of genomic DNA was performed using French pressure cell lysis (Thermo Spectronic), followed by purification by chromatography on hydroxyapatite as described previously (Cashion *et al.*, 1977). The G + C content was determined according to Mesbah *et al.* (Mesbah *et al.*, 1989), and confirmed by DSMZ methods (Cashion *et al.*, 1977; Mesbah *et al.*, 1989; Tamaoka & Komagata, 1984; Visuvanathan *et al.*, 1989) under the direction of Dr. Peter Schumann.

The nearest phylogenetic neighbors of strain 44C3<sup>T</sup>, as determined by 16S rRNA gene sequence (1501 bp), were distantly related members of the family *Microbacteriaceae* (similarities ranging from 92.5 to 96.5 %). Strain 44C3<sup>T</sup> formed a distinct subclade within the family, and had highest sequence similarity to *Cryobacterium psychrotolerans* JCM 13925<sup>T</sup> (DQ515963) (96.5%) (Figure 6.1). The G + C content of the genomic DNA was 60.9 mol%.

Although other genera of the *Microbacteriaceae* family contain rhamnose, as well as one or more other sugars in the cell wall (references in Table 6.1); rhamnose was the only sugar detected for the strain 44C3<sup>T</sup>. Strain 44C3<sup>T</sup> is similar to *Mycetocola*, *Frigoribacterium*, and *Microcella* for the presence of lysine as a diamino acid of the cell wall, but is different for major menaquinones, major fatty acids, and G + C content. For the quinone system, *Agrococcus* (Groth *et al.*, 1996) have menaquinones similar to 44C3<sup>T</sup>, but they differ in other chemotaxonomic characteristics including peptidoglycan amino acids, major fatty acid composition, and G + C content (Table 6.1).



**Figure 6.1.** Neighbor-joining, Jukes-Cantor phylogenetic dendrogram based on 16S rRNA gene sequence similarity, showing position of strain 44C3<sup>T</sup> among its phylogenetic neighbors. Numbers at branch nodes are bootstrap values (1000 resamplings; only values > 50 are given). *Brevibacterium linens* DSM 20425<sup>T</sup> (X77451) served as an outgroup. GenBank accession numbers are given in parentheses. Bar, 1 % sequence divergence.

**Table 6.1.** Differential chemotaxonomic characteristics of the genus *Klugiella* and related genera of the family *Micrococcobacteriaceae*.

Data for reference taxa: *Mycetocola* (Tsukamoto *et al.*, 2001), *Agrococcus* (Groth *et al.*, 1996), *Frigoribacterium* (Kämpfer *et al.*, 2000), *Cryobacterium* (Suzuki *et al.*, 1997), *Microcella* (Tiago *et al.*, 2005), *Microbacterium* (Takeuchi & Hatano, 1998, Yokota *et al.*, 1993a, Yokota *et al.*, 1993b).

ND, no data available.

Characteristic	<i>Klugiella</i>	<i>Mycetocola</i>	<i>Agrococcus</i>	<i>Frigoribacterium</i>	<i>Cryobacterium</i>	<i>Microcella</i>	<i>M. dextranolyticum</i> <i>M. lacticum</i> <i>M. laevaniformans</i> <i>M. hominis</i>
Diamino acid*	Lys	Lys	DAB	Lys	DAB	Lys or Orn	Lys
Major cell wall sugar(s)†	Rha‡	ND	Glc, Rha	ND	Rha, Fuc	ND	Gal, Rha, (NMan, 6dT, Xyl, Glc)§
Major fatty acids	ai-15:0, ai-17:0, i-16:0	ai-15:0, ai-17:0	ai-15:0, i-16:0, i-15:0	ai-15:0, i-16:0	ai-15:0, i-15:0, ai-17:0	i-16:0, ai-15:0, i-14:0, i-15:0	ai-15:0, ai-17:0, i-16:0
Major menaquinones	11, 12	10	11, 12	9	10	12, 13 or 13, 14	11, 12
G + C content (mol%)	60.9	63.9-65.2	74	71.7	65	68.8	68.3-71.2

\* Lys, lysine; DAB, diamminobutyric acid; Orn, ornithine.

† Rha, rhamnose; Glc, glucose; Fuc, fucose; Gal, galactose; Man, mannose; 6dT, 6-deoxtalose; Xyl, xylose.

‡ Only sugar detected.

§ Compounds in parentheses are variable between species.

Chemotaxonomic characteristics that differentiate strain 44C3<sup>T</sup> from other representatives of the nearest phylogenetic neighbors detected by 16S rRNA gene sequence analysis are reported in Table 6.1. It is evident from genotypic and phenotypic data presented that strain 44C3<sup>T</sup> represents a new genus and novel species within the family *Microbacteriaceae*, for which the name *Klugiella xanthotipulae* gen. nov., sp. nov. is proposed. *Klugiella* can be distinguished from other genera of the *Microbacteriaceae* by the major menaquinones MK-12 and MK-11, along with cell wall diamino acid lysine. Some *Microbacterium* spp. (Table 6.1) have the major menaquinones MK-12 and MK-11 as well as the cell wall diamino acid lysine; *Klugiella* can be differentiated by having rhamnose as the only detectable cell wall sugar, and lower G + C content of approximately 61 mol%.

#### **Description of *Klugiella* gen. nov.**

*Klugiella* [Klu.gi.el'la. N. L. fem. n. named after Michael J. Klug, an American entomologist/microbiologist who, along with S. Kotarski, first described the microbial community of the *Tipula abdominalis* larval gut, from which 44C3<sup>T</sup> was isolated].

Gram-type positive, Gram-reaction variable, mesophilic, and aerobic. Cells are non-motile, non-sporeforming, irregular rods (0.6-3.4 x 0.4-0.8 µm). The peptidoglycan type is B, lysine is the diamino acid of the peptidoglycan, and alanine is the N-terminus of the interpeptide bridge. The major menaquinones are MK-12 and MK-11. The major fatty acids are ai-C<sub>15:0</sub>, ai-C<sub>17:0</sub>, and i-C<sub>16:0</sub>. The G + C content of genomic DNA is about 61 mol%. 16S rRNA gene sequence similarity indicates membership of the family *Microbacteriaceae*. The type species is *Klugiella xanthotipulae*.

### **Description of *Klugiella xanthotipulae* sp. nov.**

*Klugiella xanthotipulae* [xan.tho.ti.pu.lae. Gr. adj. *xanthos* yellow; N.L. fem. n. *tipula*, of *Tipula*, isolated from *Tipula abdominalis*].

Colonies are convex, circular, and yellow. Growth occurs at 4-30 °C with an optimum at 28°C, and at a pH range of 6-11 with an optimum at pH 8. Can grow at 1% (w/v) NaCl, but not 3%. Catalase-positive, oxidase-negative. Positive for pyrazinamidase, β-glucuronidase, β-galactosidase, and α-glucosidase; negative for α-galactosidase, arginine dihydrolase, leucine arylamidase, pyrolydonyl arylamidase, alkaline phosphatase, N-acetyl-β-glucosaminidase, urease, indole production, acetoin production, hydrolysis of gelatin, and reduction of nitrates. Negative for acid production from L-arabinose, lactose, ribose, and sorbitol. Acid is produced from fructose, mannose, maltose, trehalose, mannitol, xylitol, melibiose, raffinose, xylose, sucrose, and α-methyl-D-glucoside. Rhamnose is the only sugar of the cell wall. Cell wall acyl type is acetyl. The menaquinones are MK-12, MK-11, MK-10, MK-13, MK-9 (43:38:7:6:1). The fatty acid profile contains ai-C<sub>15:0</sub> (54.71 %), ai-C<sub>17:0</sub> (18.28 %), i-C<sub>16:0</sub> (17.92 %), i-C<sub>15:0</sub> (1.37 %), and i-C<sub>14:0</sub> (1.03 %). The G + C content of the genomic DNA is 60.9 mol%.

The type strain, 44C3<sup>T</sup> (= DSM 18031 = ATCC BAA-1524), was isolated from the hindgut of *Tipula abdominalis* larvae collected in Michigan, USA.

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**CHAPTER 7**  
**CONCLUSIONS**

The model of the *T. abdominalis* larval gut as a natural biorefinery is presented in Figure 7.1. In this model, the substrate (conditioned leaf litter) is ingested by larvae. Maceration of the substrate during ingestion decreases particle size and increases surface area to volume ratios. Upon entering the alkaline midgut, proteolysis degrades complexed proteins making polysaccharide polymers more accessible for further processing. In the neutral pH hindgut, bacterial enzymes saccharify cellulose and hemicellulose. These sugars are then consumed by bacteria and converted to acetate and other fermentation products, which can be transported across the gut to the hemolymph to support larval energy and growth requirements (Lawson & Klug, 1989). In the fermentation paunch, material is retained for extended processing (Klug & Kotarski, 1980). Lastly, waste and by-products are excreted and are valuable to other organisms in the ecosystem. This model of the *T. abdominalis* larva as a natural biorefinery can be applied towards developments in technology for industrial biomass refinery processes.

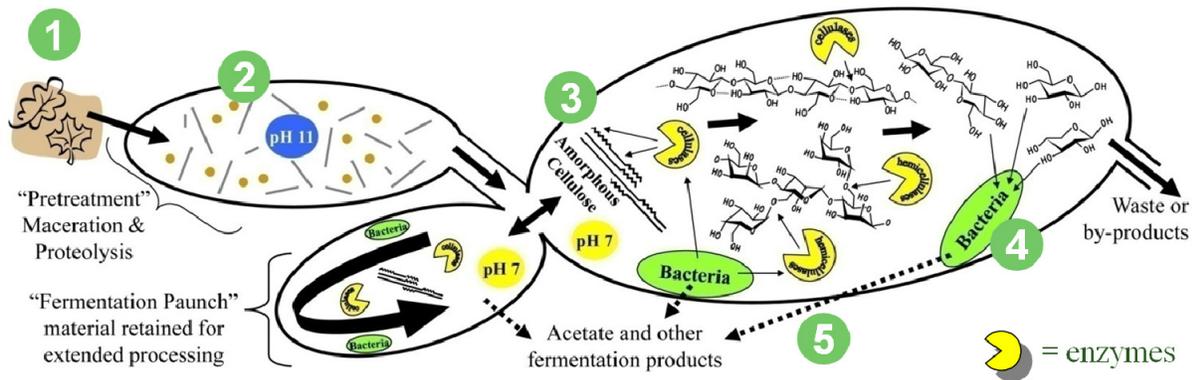


Figure 7.1. Model of the *T. abdominalis* larval gut as a natural biorefinery.

The *Tipula abdominalis* larval hindgut hosts a novel phylogenetically diverse and dynamic bacterial community. Based on culture-independent methods, *Clostridia* and *Bacteroidetes* comprise a majority in the hindgut community. In contrast, *Betaproteobacteria* comprise a majority of the microbiota associated with the ingested leaf diet and larval casts.

Although trends in phylogenetic structure of the hindgut community seem apparent at the class level, statistical analysis indicates that the sub-communities associated with the hindgut wall epithelial and lumen are significantly different. Hindgut bacterial community structure is also variable between individual larvae. Variability could be compounded by the presence of transient bacteria, which cause difficulty in identifying the resident bacteria which presumably facilitate the digestion of the larvae's lignocellulosic diet. Although phylogeny of the hindgut community is very dynamic, the function remains stable. In cellulolytic enrichment cultures cultivated from the hindgut microbiota, anaerobic cellulose degradation was stable while the phylogenetic structure varied. It is probable that specific species interactions may not be vital for this system; rather, structure of community member metabolism and enzyme activity may dictate the colonization of the hindgut.

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