

A Survey of Bacterial Diversity From Successive Life Stages of Black Soldier Fly (Diptera: Stratiomyidae) by using 16S rDNA Pyrosequencing

Author(s): Longyu Zheng , Tawni L. Crippen , Baneshwar Singh , Aaron M. Tarone , Scot Dowd , Ziniu Yu , Thomas K. Wood , and Jeffery K. Tomberlin

Source: Journal of Medical Entomology, 50(3):647-658. 2013.

Published By: Entomological Society of America

URL: <http://www.bioone.org/doi/full/10.1603/ME12199>

BioOne (www.bioone.org) is a nonprofit, online aggregation of core research in the biological, ecological, and environmental sciences. BioOne provides a sustainable online platform for over 170 journals and books published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Web site, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/page/terms_of_use.

Usage of BioOne content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

A Survey of Bacterial Diversity From Successive Life Stages of Black Soldier Fly (Diptera: Stratiomyidae) by Using 16S rDNA Pyrosequencing

LONGYU ZHENG,^{1,2} TAWNI L. CRIPPEN,³ BANESHWAR SINGH,² AARON M. TARONE,² SCOT DOWD,⁴ ZINIU YU,¹ THOMAS K. WOOD,⁵ AND JEFFERY K. TOMBERLIN²

J. Med. Entomol. 50(3): 647–658 (2013); DOI: <http://dx.doi.org/10.1603/ME12199>

ABSTRACT Sustainable methods for managing waste associated with people and animals have been proposed in the past. Black soldier fly, *Hermetia illucens* (L.), larvae represent one of the more promising methods. Larvae reduce dry matter, bacteria, offensive odor, and house fly populations. Prepupae can be used as feedstuff for livestock. However, it is not known if such a method results in the proliferation of potential pathogens. Although some bacterial species have been cultured and identified from black soldier fly, a true appreciation of fly associated bacterial diversity is not known. Such information is needed to understand pathogen colonization on decomposing animal and plant waste in the presence of black soldier fly larvae as well as develop research strategies for maximizing the use of this fly to reduce waste without risking environmental harm. Using 454 sequencing, we surveyed bacterial diversity associated with successive life stages of the black soldier fly reared on plant material. Bacteria diversity classified (99.8%) across all life stages spanned six bacterial phyla with $\geq 80\%$ bootstrap support. Bacteroidetes and Proteobacteria were the most dominant phyla associated with the black soldier fly accounting for two-thirds of the fauna identified. Many of these bacteria would go undetected because of their inability to be cultured.

KEY WORDS decomposition ecology, waste management, black soldier fly, *Hermetia illucens*, bacteria

Bacteria are an expected component of decomposing animals (Burkepille et al. 2006, Barnes et al. 2010) and plant wastes (Miki et al. 2010), and they are associated with the insects that use these resources. These insects can be beneficial, contributing to such functions as digestion and nutrient processing (Janzen 1977, Burkepille et al. 2006) or detrimental causing health issues and spreading pathogens to other organisms (Greenberg 1973). Although many bacteria are be-

nign, some species, such as *Escherichia coli* O157:H7 and *Salmonella enterica*, are important foodborne pathogens (Erickson et al. 2004). Appropriate methods for handling animal and plant wastes, although reducing the horizontal contamination of the surrounding ecosystem, are being developed (Erickson et al. 2004).

Black soldier fly larvae, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) can be used to recycle animal tissue (Tomberlin et al. 2005) and other waste products (Sheppard et al. 1994). Because of this ability, the black soldier fly is a recognized method for sustainable recycling of animal wastes (Sheppard et al. 1994), feces (Myers et al. 2008), and plant materials (Tomberlin et al. 2009). Larvae of the black soldier fly can reduce dry matter in such waste by 50% (Sheppard et al. 1994) and house fly, *Musca domestica* L. (Diptera: Muscidae) populations by 94–100% (Bradley and Sheppard 1984), although producing viable feedstuff for livestock, poultry (Sheppard et al. 1994), and aquaculture (St. Hilaire et al. 2007). Unlike house flies, soldier flies also reduce *E. coli* populations in feces (Liu et al. 2008) and larvae can be used for the production of biodiesel (Li et al. 2011).

Recent efforts have determined that black soldier fly larvae reduce pathogenic bacteria within animal wastes (Erickson et al. 2004, Liu et al. 2008). Further-

Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice. Mention of trade names, companies, or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement of the products by the U.S. Department of Agriculture. The authors acknowledge the Texas A&M University Brazos HPC cluster that contributed to the research reported here. (brazos.tamu.edu).

¹ State Key Laboratory of Agricultural Microbiology, National Engineering Research Center of Microbial Pesticide, Huazhong Agricultural University, China.

² Department of Entomology, Texas A&M University, College Station TX 77843.

³ Southern Plains Agricultural Research Center, Agricultural Research Service, USDA, College Station, TX.

⁴ Research and Testing Laboratory, Lubbock, TX.

⁵ Department of Chemical Engineering, Texas A&M University, College Station, TX.

⁶ Corresponding author: Jeffery K. Tomberlin, 2475 TAMU, Department of Entomology, Texas A&M University, College Station, TX 77843 (e-mail: jktomberlin@tamu.edu).

more, bacteria isolated from black soldier fly larvae can be used as a probiotic to enhance manure reduction and subsequent larval development (Yu et al. 2011). Many of these beneficial bacteria could be natural constituents of the larval environment or potentially vertically transmitted. However, although the black soldier fly might suppress potential pathogens, it is not clear if other opportunistic pathogens might proliferate in their presence and present potential health and environmental risks.

Bacteria isolated previously and identified from the black soldier fly relied on classic culturing methods (Yu et al. 2011). With the development of high throughput sequencing techniques, we can now gain a more comprehensive picture of the bacterial diversity associated with the black soldier fly, including those that are nonculturable. Recent microbial ecological studies from different environmental habitats by using the analysis of 16S rRNA gene sequences have revealed an immense diversity of bacterial species (Huber et al. 2007, Roesch et al. 2007, Costello et al. 2009, Bowman et al. 2012) and individual bacterial species have been identified in quantities unattainable by classic culture techniques (Hail et al. 2011).

The increased depth in sensitivity obtainable by this technology demands the reevaluation of previously measured ecological systems. With this concept in mind, we conducted a survey of the microbial community present during successive life stages of black soldier fly maintained in colony and raised on a standardized diet consisting of plant residue (Sheppard et al. 2002). The purpose of this study was to provide some insight into the overall, and potentially the vertically transmitted, bacterial diversity associated with successive life stages of the black soldier fly. Data produced from this study will allow a greater understanding of bacterial shifts occurring during fly development as related to all bacteria and to pathogen dispersal specifically when using the black soldier fly to reduce waste and produce a feedstuff for livestock (Sheppard et al. 1994), swine (Newton et al. 1977), and aquaculture (St. Hilaire et al. 2007).

Materials and Methods

Source of Black Soldier Fly. The black soldier fly colony maintained in a greenhouse at the Forensic Laboratory of Investigative Entomological Sciences Facility, TX A&M University, College Station, TX was established in 2009 from eggs of a laboratory colony at the Coastal Plain Experiment Station, University of Georgia, Tifton, GA, USA, which originated from material collected at a poultry facility in Bacon Co., GA, USA, in 1998. Approximately 36 generations have passed since the initiation of the colony with supplemental stock added approximately every 2–4 yr.

Maintenance of the Colony. Flies were reared according to the methods of Sheppard et al. (2002). Briefly, eggs were held in 12-cm³ plastic tubs at 27°C with ambient humidity until hatch. Approximately 200 g of a Gainesville diet (20% corn meal, 30% alfalfa meal, 50% wheat bran) saturated with water was

added to the tub (Hogsette 1985). After 48 h at 27°C the larvae were transferred into a new 40- by 15- by 12-cm plastic tub and covered by the diet to a depth of ≈2 cm. The top on the tub was cover once per day with 2 cm of fresh diet. After 15 d at 27°C, the larvae were split into new 65- by 50- by 12-cm plastic tubs to keep the larval density consistent. When dispersing larvae (i.e., prepupae), which are black, accounted for ≈50% of the population, feeding was suspended and remaining medium allowed to dry. Emerging adults were transferred into a 1.5-m³ mesh tent held in a greenhouse under natural light at 27°C with ambient humidity. Eggs were collected by placing 5- by 3-cm blocks of dry, triple-layered, corrugated cardboard, containing ≈60 flutes, adhered to the sides of a 30-cm square metal pan containing feed, into each tent.

Sample Collection and DNA Extraction. We designed this study to provide the broadest spectrum of bacterial communities associated with the various life stages of the black soldier fly. Based on these results, we will be able to conduct future studies focused on those stages of development and associated bacterial communities of greatest interest to waste management with this species.

For analysis of successive developmental stages, one layer each of corrugated cardboard block (≈20 flutes or 8,000 eggs) was allowed to hatched. Resulting larvae were separated into three pans to allow pupation and emergence of adults. Feeding and dispersing larvae, as well as pupae were taken as needed from these pans for analysis and adults were sampled immediately after eclosion. A second generation of eggs (egg2) was collected from corrugated cardboard block (as described above) placed in with the newly eclosed adults. Samples either were processed immediately or stored at –80°C until DNA extraction could be performed.

DNA extractions were performed from ≈0.25-g eggs (<1 h old), two 7-d-old larvae, two dispersing larvae (prepupae), two pupae, or two newly emerged adults. These samples were selected randomly and entire specimens were homogenized in 1.5-ml PBS. Briefly, samples were placed in 1.5-ml microcentrifuge tubes with Tris-EDTA buffer with 10% SDS, proteinase K (100 μg/ml), and lysozyme (2 mg/ml). Samples were homogenized and incubated at 56°C for 1 h while shaken at 900 rpm. Sodium chloride buffer (NaCl, 5M) was added to bring the total concentration above 0.5M. Cetyl trimethylammonium bromide (CTAB) /NaCl buffer was added and the sample was thoroughly mixed and incubated at 65°C for 10 min. Sequential extraction in a 1X volume was performed using chloroform and isoamyl alcohol (24:1), phenol, chloroform, and isoamyl alcohol (25:24:1), and isopropanol by centrifugation at 4,000 × g for 2 min. The DNA was precipitated by two washes in 70% ethanol, dissolved in nuclease free water, quantified by spectrophotometry, the DNA products from three replicate samples were mixed in equal concentrations and aliquoted for analysis by 454 FLX pyrosequencing.

Bacterial Tag-Encoded FLX-Titanium Pyrosequencing. The bTEFAP method was performed by the Research and Testing Laboratory (Lubbock, TX) (Dowd et al. 2008). The 16S rDNA universal eubacterial primers 28 F (5'- GAGTTTGATCNTGGCT-CAG) and 519R (5'- GTNTTACNGCGGCKGCTG) were used for amplifying the \approx 500-bp region of 16S rDNA genes (Handl et al. 2011). HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used for polymerase chain reaction (PCR) under the following conditions: 94°C for 3 min followed by 32 cycles of 94°C for 30 s, 60°C for 40 s and 72°C for 1 min, and a final elongation step at 72°C for 5 min by using designed special fusion primers with -unique tag sequences for each sample as follows: 5' LinkerA-Tags-28 F and 5' LinkerB-519R. After PCR, amplicon products from different samples were mixed in equal concentrations, and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, Beverly, MA). In preparation for FLX sequencing (Roche, Nutley, NJ), the DNA fragments size and concentration were measured using DNA chips under a Bio-Rad Experion Automated Electrophoresis Station (Bio-Rad Laboratories, Hercules, CA) and a TBS-380 Fluorometer (Turner Biosystems, Sunnyvale, CA). A 9.6×10^6 sample of double-stranded DNA molecules/ μ l with an average size of 550 bp were combined with 9.6 million DNA capture beads, and then amplified by emulsion PCR. After bead recovery and bead enrichment, the bead-attached DNAs were denatured with NaOH, and sequencing primers were annealed. A two-region 454 sequencing run was performed on a 70 by 75 GS PicoTiterPlate by using a Genome Sequencer FLX System (Roche). All FLX related procedures were performed following Genome Sequencer FLX System manufacturers instructions (Roche).

Pyrosequencing Data Analysis. All sequences (4,820) were checked for chimera formation by using web based chimera check program Decipher (Wright et al. 2012). Suspected chimera (494) and five other sequences that did not align were deleted from the dataset and only 4,321 sequences were used for further analyses. Hierarchical classification of the 4,321 16S rDNA sequences were carried out according to the Bergey's bacterial taxonomy (Garrity et al. 2004) using Naïve Bayesian rRNA classifier version 2.2 (Wang et al. 2007) as implemented in Ribosomal Database Project (RDP) Multiclassifier version 1.0. Only sequences having \geq 80% bootstrap support were considered classified at a particular hierarchical level. Sequences with bootstrap support $<$ 80% were considered as unclassified. Sequences that were not assigned at a particular taxonomic level were also considered as unclassified. Bar graphs were created for phylum and class level sequence abundances by using Excel 2007 (Microsoft Corporation, Redmond, WA). Heat maps graphics were generated for all classified genera by using natural log transformed percent relative abundance profiles using default distance (column means and row means for X and Y axis respectively) based dendrogram in gplots package of R version 2.13.0 (R Development Core Team 2006). The 0% values were con-

verted into 0.01% for log transformation. The heat map of the bacterial genera associated with different life stages were also clustered based on NJ tree of classified genera (Y-axis) (see below for detail) and based on FastUniFrac based clustering of life stages (X-axis) (Fig. 1a), which helps in better comparison of bacteria by phenotypic and taxonomic characteristics important to bacterial community functional analysis.

Neighbor-joining (NJ) trees were constructed from all sequences (4,321) and from sequences that were classified at the genus level (1473). NJ trees were rooted based on 16S rRNA gene sequence of *Thermatoga maritima* (M21774) and *Aquifex pyrophilus* (M83548). NJ tree of all sequences was constructed to determine the closest relative of unclassified sequences and NJ tree of all classified genera was constructed to group genera based on evolutionary relationships. Both data sets were aligned based on 16S rRNA secondary structure in Infernal aligner (Nawrocki and Eddy 2007, Nawrocki et al. 2009), as implemented in the Ribosomal Database Project (RDP) under tool Aligner (<http://rdp.cme.msu.edu/>) (accessed on 01 June 2012). Hypervariable ambiguous regions were manually deleted from the multiple sequence alignment in MEGA5 (Tamura et al. 2011). Evolutionary distances of aligned sequences were calculated by neighbor joining (NJ) method with the Kimura two-parameter correction (Saitou and Nei 1987) for 100 bootstrap replications (for all sequences) and for 1,000 bootstrap replications (for the data sets with all genera) in PAUP* v.4.0b10 (Swofford 2003). Calculated evolutionary distances were used for construction of rooted NJ trees in PAUP* v.4.0b10 (Swofford 2003). NJ trees of all classified genera was used as an input file for construction of unweighted FastUniFrac based clustering of bacterial communities (Hamady et al. 2009) associated with different life stages of black soldier fly. Jackknifing with 1000 permutations was performed for node support of the FastUniFrac tree. *P*-tests were performed using 1,000 permutations for each pair of samples (life stages) and for all samples together in FastUniFrac (Hamady et al. 2009). All trees were edited using Archaeoptryx version 0.957 beta (Han and Zmasek 2009) and FigTree v1.3.1 (<http://tree.bio.ed.ac.uk/>).

Diversity indices were calculated using tools available in RDP pyrosequencing pipeline. Rarefaction curves were generated in Excel 2007 (Microsoft Corporation, Redmond, WA) using result obtained from tools aligner, complete linkage clustering, and rarefaction of RDP pyrosequencing pipeline (Cole et al. 2009). Shannon (1948) and Chao1 and Bunge (2002) indices were calculated using tool Shannon & Chao1 index of RDP pyrosequencing pipeline (Cole et al. 2009). Percentage coverage of species richness was calculated from rarefaction and Chao1 indices using method as described in Will et al. (2010). All raw sequence files were submitted to Sequence Read Archive (SRA). Study accession number ERP001565 can be used for the retrieval of raw sequences used in this study.

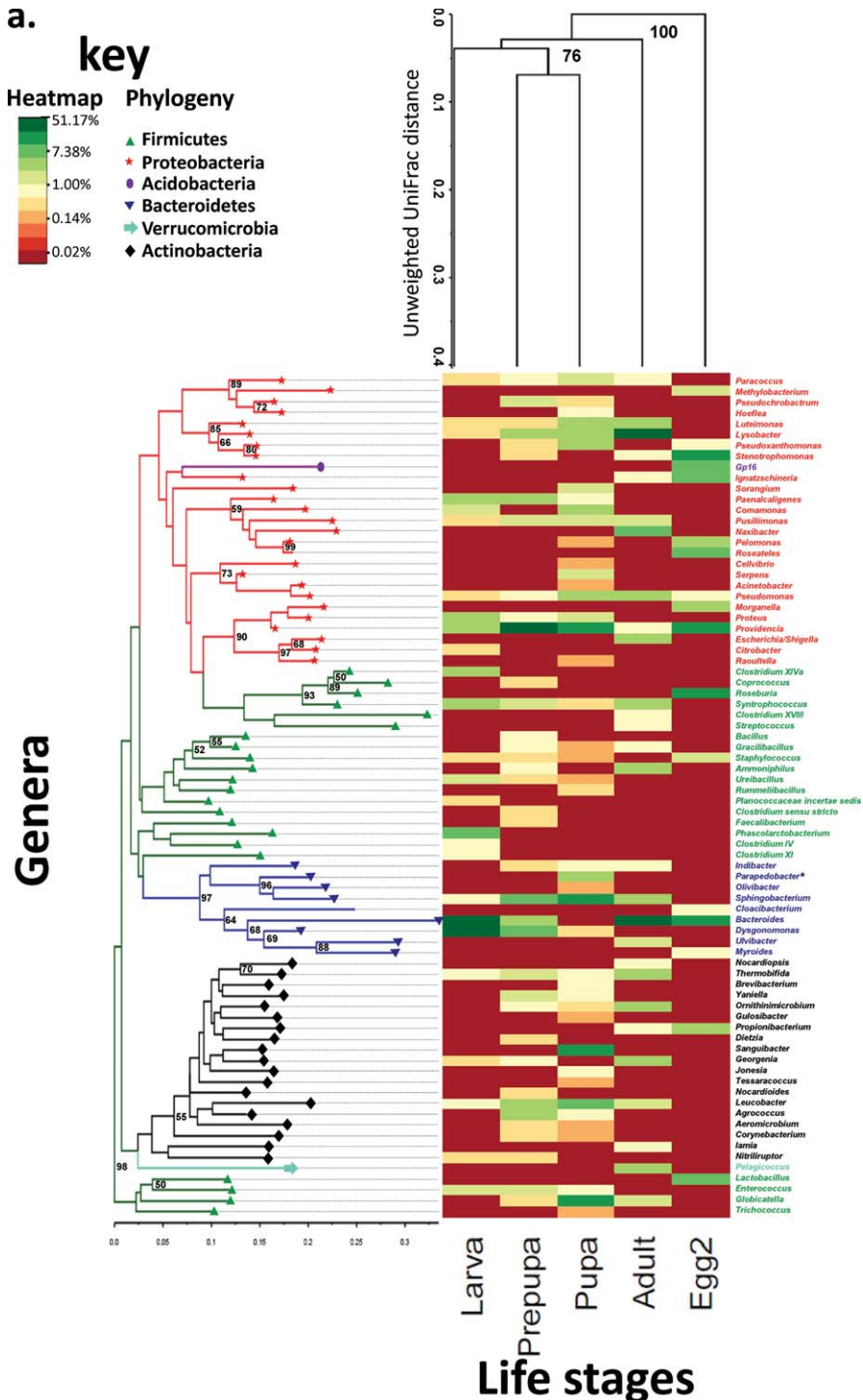


Fig. 1. Heat map of the bacterial genera associated with different life stages of black soldier fly: a) Y-axis shows neighbor-joining phylogram of 16S rDNA sequence from all classified genera and X-axis shows unweighted UniFrac distance based clustering of the life stages (numbers on nodes indicates jackknife support values), b) Y-axis shows clustering of classified genera based on percentage relative abundance using default distance (row mean) in gplots package of R, and X-axis shows clustering of life stages based on percentage relative abundance using default distance (column mean) in gplots package of R. Heat maps displayed in natural log transformed percentage relative abundance profiles, where the 0% values were converted into 0.01% for log transformation. *indicates polyphyly. (Online figure in color).

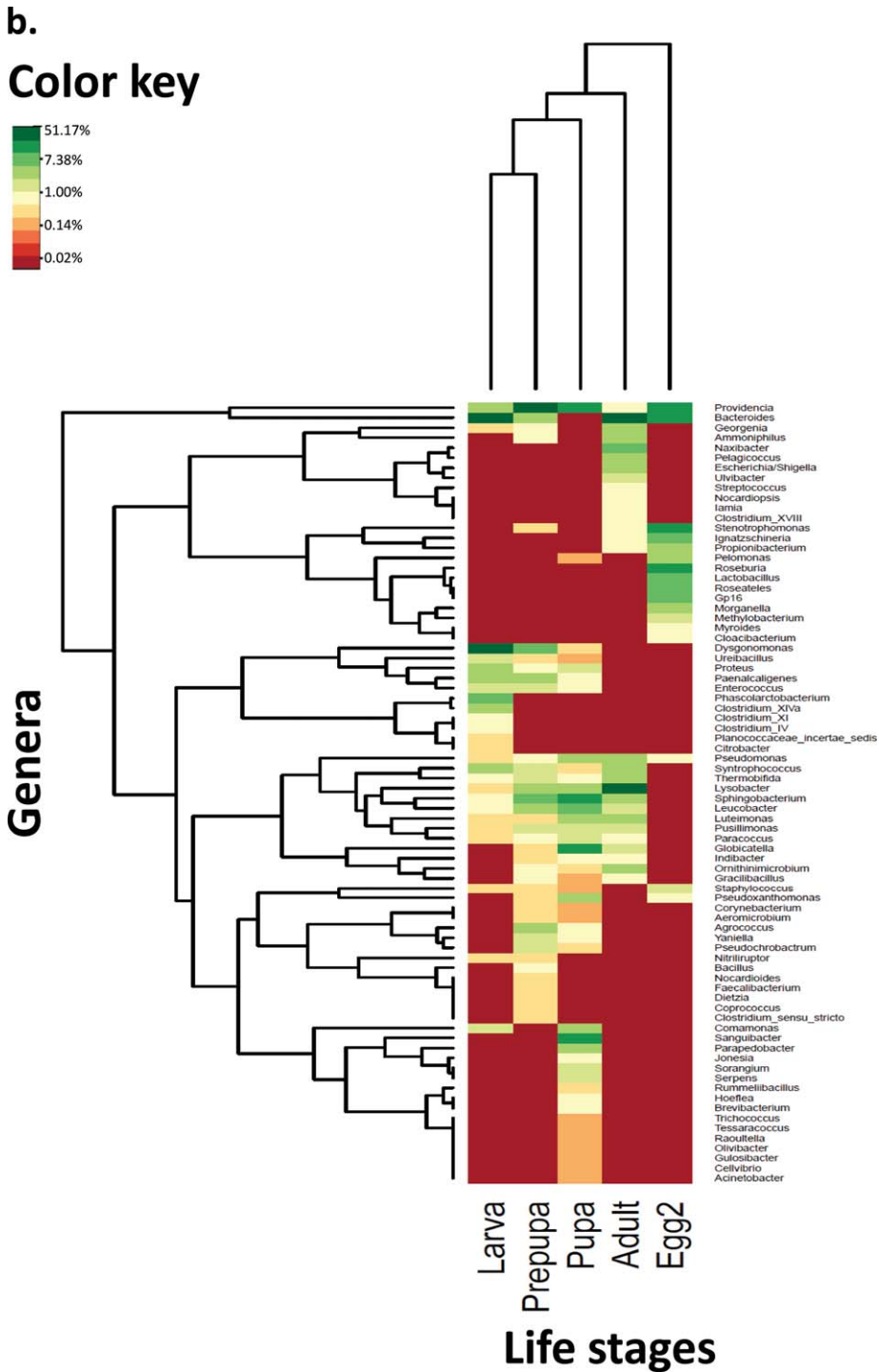


Fig. 1. (Continued).

Results

General Characteristics of 454-Sequences. In this study we obtained 4,321 sequences ranging in length from 200 to 541 bp (average length 327 bp) from the

different life stages of the black soldier fly. The number of sequences obtained from larval, prepupal, pupal, adult, and egg2 samples were 476; 1,080; 1,847; 794; and 124, respectively. Approximately 99.8, 98.2, 89.0,

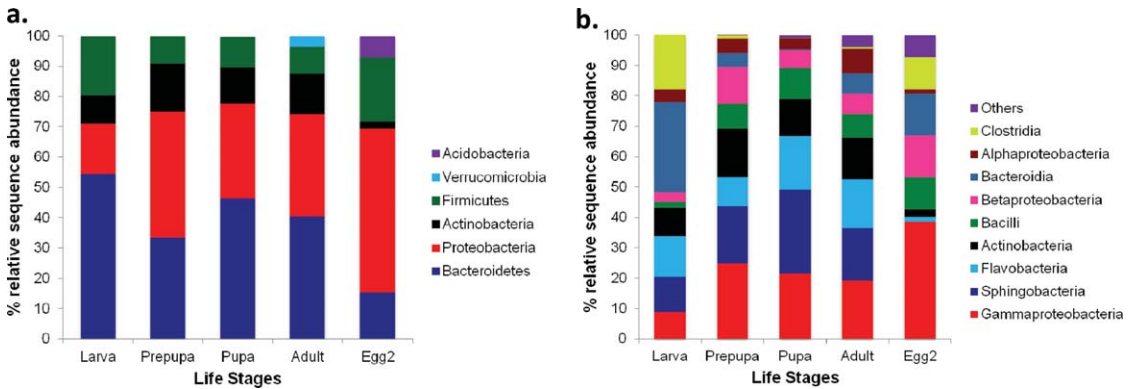


Fig. 2. Abundance profiles of bacteria associated with different life stages of the black soldier fly at a) phylum level, b) class level. Y-axis shows percentage relative abundance of the classified bacteria. X-axis shows the life stages. Others include all classes that were present at <1% relative abundance (Opitutae, Deltaproteobacteria, Acidobacteria GP16, Erysipelotrichia). Each column represents the DNA products from three replicate samples were mixed in equal concentrations and aliquotted for analysis by 454 FLX pyrosequencing. (Online figure in color).

75.0, and 34.0%, of all sequences were classified with $\geq 80.0\%$ bootstrap support into 6 phyla, 13 classes, 21 orders, 53 families, and 78 genera, respectively (data not shown).

Taxonomic Distribution of 454-Sequences. Of the bacterial phyla identified (Fig. 2a; Supp Table 1), Bacteroidetes (42.0%) and Proteobacteria (33.4%) were the most dominant phyla associated with the black soldier fly across all life stages. Proteobacteria was the most dominant phylum associated with the prepupa (41.7%) and egg (54.0%) stages whereas Bacteroidetes was the most dominant phylum associated with larva (54.4%), pupa (46.3%), and adult (40.5%) (Fig. 2a; Supp Table 1). Of the bacterial classes identified (Fig. 2b; Supp Table 2), Gammaproteobacteria, Sphingobacteria, Flavobacteria, Actinobacteria, and Bacilli comprised almost 78.0% of bacteria across all life stages, in decreasing order. Gammaproteobacteria (19.0% to 39.0%) was the most dominant on each stage except the larval stage, where Bacteroidia (29.0%) dominated. Of the bacterial orders identified (Supp Table 3), Sphingobacteriales, Flavobacteriales, Actinomycetales, Enterobacteriales, Burkholderiales, and Bacteroidales comprised almost 78% of the bacteria across all life stages, in decreasing order. Approximately 49% of all bacteria associated with larval stage belong to Bacteroidales and Clostridiales whereas >50% of all bacteria associated with pupal and adult stages belong to Sphingobacteriales, Flavobacteriales, and Actinomycetales.

Of the bacterial families identified (Supp Table 4), $\approx 55\%$ were gram-positive families. Families Sphingobacteriaceae, Flavobacteriaceae, and Enterobacteriaceae comprised >50% of bacteria across all life stages, in decreasing order. Porphyromonadaceae (19.0%), Enterobacteriaceae (26.6%), Sphingobacteriaceae (27.7%), Flavobacteriaceae (23.2%), and Xanthomonadaceae (23.7%) were the most dominant families associated with the larval, prepupal, pupal, adult, and egg samples, respectively. Sphingobacteriaceae and Flavobacteriaceae were found heavily associated with

larvae (27.3%), prepupae (29.8%), pupae (50.0%), and adults (41.6%), but not with egg stage (1.8%).

FastUniFrac based *P*-test suggests that bacterial communities are significantly different in pairwise comparisons between life stages ($P \leq 0.001$) and bacterial communities associated with each of the life stages significantly clustered ($P \leq 0.001$). FastUniFrac clustering demonstrates that prepupal and pupal stages shared the most bacterial genera although this group is not well-supported (Jackknife value <50) and the egg stage shared the least number of bacterial genera with other life stages. The heat map clustered by percentage relative abundance of classified genera (Fig. 1b) reveals a difference in community abundance between the egg stage and nonegg stages. Of the bacterial genera identified (Fig. 1a and b; Supp Table 5), *Providencia*, *Bacteroides*, *Sphingobacterium*, *Dysgonomonas*, and *Sanguibacter* comprised >50% of the bacteria across all life stages, in decreasing order *Dysgonomonas* and *Bacteroides* constituted 60.1% of bacteria associated with larval stage. *Providencia* was the most dominant associated with prepupal (51.2%) and pupal (19.4.0%) stages, *Bacteroides* (25.6%) and *Lyso-bacter* (23.7%) the most dominant on adults, and *Stenotrophomonas* (15.4%) and *Bacteroides* (14.5%) were the most dominant on the egg stage.

Bacterial Richness and Diversity Indices. The Shannon diversity index (H') at the species and genus level (3 and 5% sequence divergence, respectively) was much higher (3.6–4.7) in larvae, prepupae, pupae, and adults than in egg (2.7) (Table 1). However, H' at the phylum level (20% sequence divergence) was similar (2.3–2.8) in all life stages (Table 1). Similar trends were observed with rarefaction and Chao1 estimators (Table 1; Fig. 3).

Neighbor-Joining Clusters. A majority of deeper nodes are unresolved both at the order level (Fig. 4), as well as at the genus level (Fig. 1a–Y-axis), but all ingroup taxa formed well-supported monophyletic group. Order level monophyly of group Sphingobacteriales + Flavobacteriales + Bacteroidales is strongly

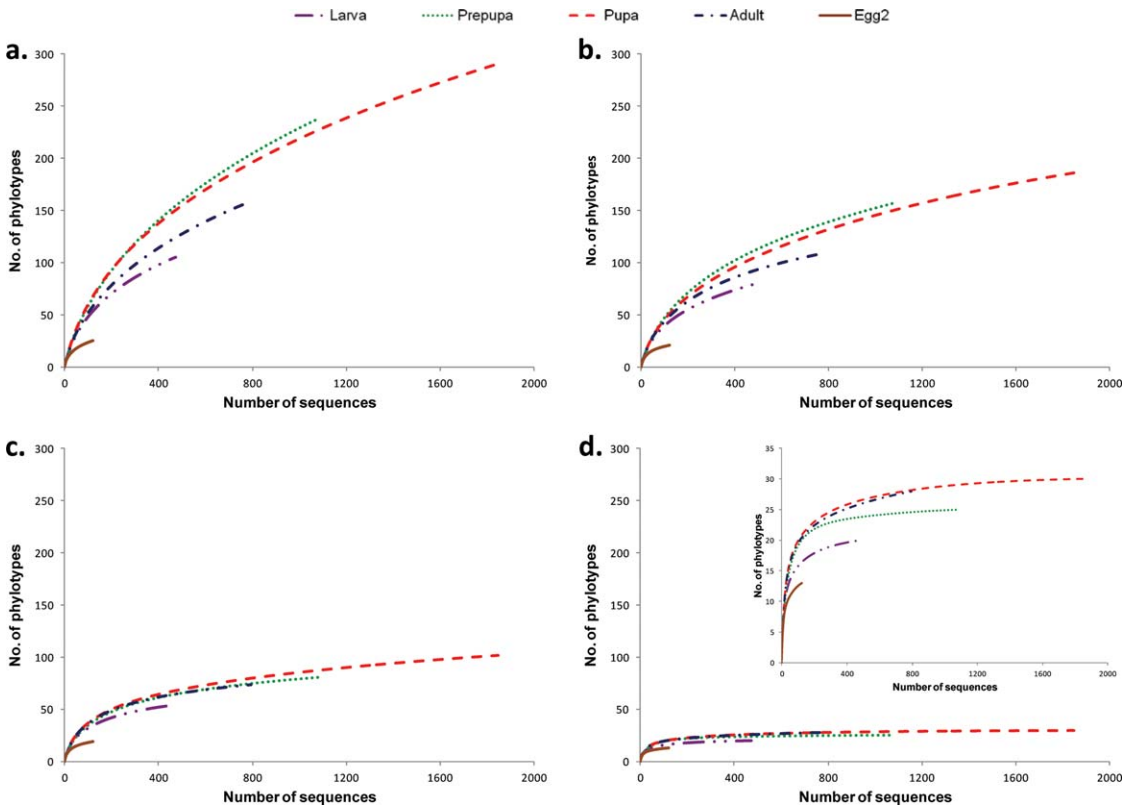


Fig. 3. Rarefaction curves from different life stages of the black soldier fly showing percentage dissimilarity and number of phylotypes identified using identical scale ranges: a) at 3% sequence divergence, b) at 5% sequence divergence, c) at 10% sequence divergence, and d) at 20% sequence divergence; inset displays an expanded scale for clarity. (Online figure in color).

supported. Sequences classified, based on RDP classifier, to orders Enterobacteriales, Burkholderiales, Myxococcales, Puniceococcales, Pseudomonadales, Sphingomonadales, Rhodospirillales, Acidimicrobiales, and Nitriliruptorales also formed well-supported monophyletic groups (Fig. 4). Many sequences considered unclassified based on RDP classifier, clustered with classified orders in the NJ tree with $\geq 50\%$ bootstrap supports. The majority of bacteria in the environment are yet to be sequenced and would be represented in the unclassified group in RDP classifier, therefore construction of the NJ tree allows determination of closest relative of these bacterial groups. The majority of unclassified sequences (476 sequences) were clustered with Pseudomonadales (225 sequences) and Rhodospirillales (100 sequences) with strong bootstrap support. At the genus level, *Parapedobacter* is polyphyletic (Fig. 1a).

Discussion

The purpose of this study was to provide a survey of bacteria associated with the various stages of black soldier fly development. The characterization of bacteria associated with distinct life stages of black soldier fly will also help identify bacteria that are possible players in interkingdom signaling attracting insects

that use decomposing resources for adult and larval nutrition (Ma et al. 2012) and allow analysis of the transfer of possible bacterial pathogens between life stages. This information will enhance commercial utilization of black soldier fly for the reduction of animal wastes and pathogen reservoirs (Sheppard et al. 1994, Tomberlin et al. 2005, Myers et al. 2008).

Our results indicate the bacterial species associated with black soldier fly are quite diverse. These results correspond with those of Jeon et al. (2011), which also used pyrosequencing to examine the bacteria fauna present. However, Jeon et al. (2011) examined the bacterial diversity in the larval stage only, in flies fed on different resources, and determined food substrate influenced the microbial fauna present. Black soldier fly larvae fed food waste possessed greater bacterial diversity at the phylum level than those fed cooked rice or calf forage. In contrast, our study reports on the bacterial diversity in each stage of development of flies fed an identical diet and demonstrates that bacterial species composition shifts within the life cycle. Black soldier fly bacterial diversity at the phylum level was similar between different life stages; however diversity at the genus level was higher during larval, prepupal, pupal, and adult stages than egg stage. This is not surprising considering black soldier fly eggs are sessile and have less exposure to environmental microbes.

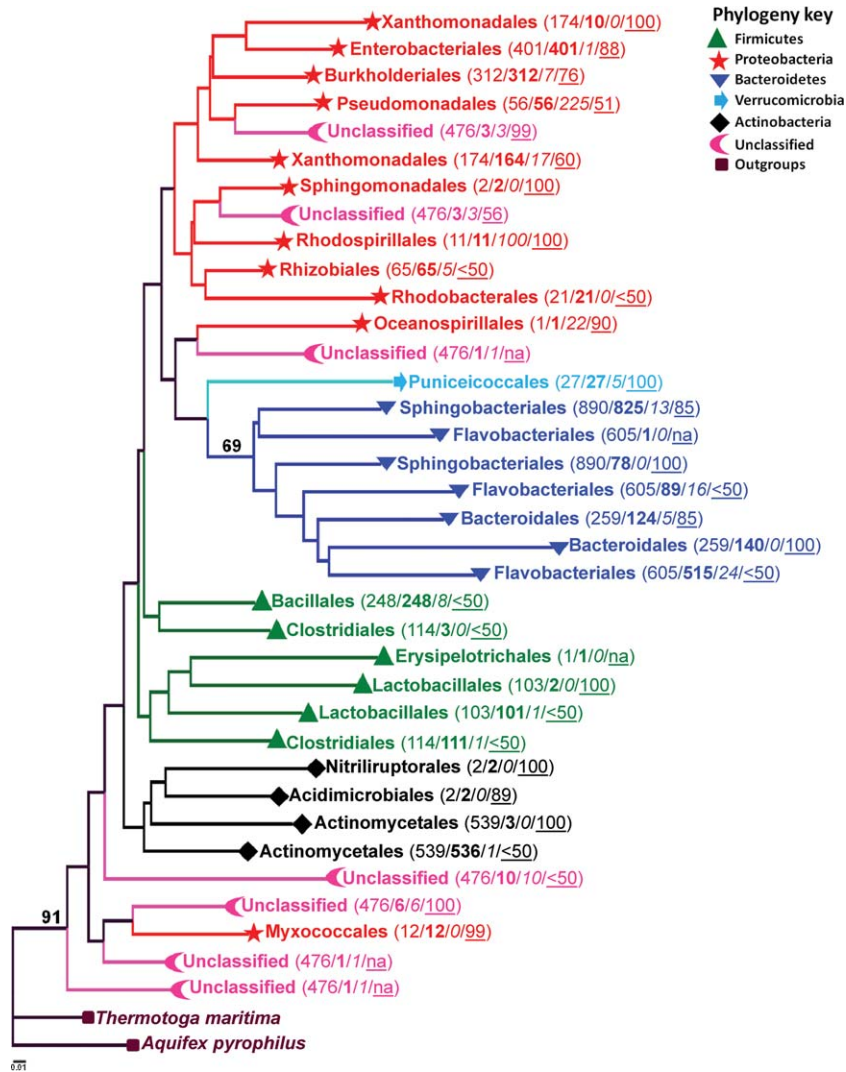


Fig. 4. Rooted neighbor-joining phylogram of 16S rRNA gene sequences associated with black soldier fly. Only nodes with bootstrap support $\geq 50\%$ are shown on the tree with associated sequence data: (standard/bold/italics/underlined); where numbers in standard font indicates total number of sequences assigned to a particular order or unclassified group based on RDP Multiclassifier; numbers in bold font indicate total number of sequences from an order or unclassified sequences that are clustered in the selected clade; numbers in italics indicate total number of unclassified sequences that are clustered in the selected clade; and numbers that are underlined indicates bootstrap support for the selected clade. (Online figure in color).

Rarefaction curve at 20% sequence dissimilarity showed saturation, indicating that the sequencing effort at phylum level covered essentially the full extent of the taxonomic diversity for all samples. A comparison of rarefaction values and Chao1 richness estimator, demonstrated percent coverage from 58% to 100% (see Table 1), which is comparable to a similar pyrosequencing-based microbial diversity study (Will et al. 2010). Actual percent coverage at 3 and 5% sequence divergence may be higher than shown here, because at lower sequence divergences (<5%), rarefaction analyses underestimate and Chao1 estimator overestimate bacterial diversity (Roesch et al. 2007, Will et al. 2010).

Only 5% of the genera found on the egg samples were also found on the emergent adult, indicating that much of the fauna is lost through molting and pupation. Overall, 78 bacterial genera were identified after removal of unknowns. Of the genera, 20.5% were shared by the larval, prepupal and pupal stages, and 11.5% by the larval, prepupal, pupal, and adult stages. Clustering analyses demonstrated that the prepupae and pupae stages had more bacterial genera in common (33.3%), which is not unexpected considering to their close sequential association during development. The bacteria found on the nonegg stages clustered closer than the egg stage; suggesting possible shared flora acquired from common environmental sources

for these predominantly mobile stages of development. While bacterial genera found on the egg stage were the most divergent from the other stages and the least abundant and could encompass species able to be vertically transmitted. The identities of these egg-associated genera will be useful for investigating the importance of vertically transmitted microbes in black soldier fly oviposition preferences and interactions of black soldier fly life history traits with bacterial species.

Many bacteria present during the more motile stages would originate from outside environmental sources by direct contact. Although bacteria on the fly from environmental sources are of interest, we hypothesize that the bacteria, which are possible players in the interkingdom signaling during oviposition, would associate more consistently with the adult and egg stages (Ma et al. 2012). Bacteria associated with the egg stage of the house fly, *Musca domestica* L. (Diptera: Muscidae) are known to be essential for adults to detect and measure oviposition sites (Lam et al. 2007). Failure to lay eggs within a given window of time results in the larvae dying because of lack of resource or cannibalism by older larvae already present (Lam et al. 2007). For house flies, it was found that these bacteria serve as an initial resource for newly emerged larvae (Lam et al. 2009a), and also suppress fungi to which the larvae are susceptible (Lam et al. 2009b). Because black soldier fly has been shown to outcompete *M. domestica* through its effects on microbial communities serving as food for its competitor, the microbes present in black soldier fly dominated resources (as compared with those on *M. domestica* dominated resources), may provide clues regarding the antimicrobial mechanisms active in black soldier fly colonization. Bacteria have also been determined to be essential for attracting blow flies (Diptera: Calliphoridae) (Chaudhury et al. 2002, 2010) and mosquitoes (Diptera: Culicidae) (Ponnusamy et al. 2010) to oviposition sites, meaning that an understanding of black soldier fly–bacterial interactions will also shed light on a broader class of bacterially mediated insect oviposition preferences.

Bacteria of the class Gammaproteobacteria were the most numerous in the bacterial community present during the second generation of the sessile egg stage. In relative proportion to the total classified population; lower numbers of Gammaproteobacteria were present on the more motile larval, prepupal, and adult stages, as well as the pupal stage. Gammaproteobacteria were 39% of the population in the egg stage, but averaged only 19% (range, of 9–25%) of the population in other stages. Five orders, Alteromonadales, Oceanospirillales, Enterobacteriales, Pseudomonadales, and Xanthomonadales, within class Gammaproteobacteria were represented across all fly stages. *Providencia* was the most prominent, representing 49% of Gammaproteobacteria across all life stages. Although the study design does not allow absolute commentary on vertical transmission, *Providencia* appears to be a candidate for vertical transmission in black soldier fly as it was found in adults and eggs.

Flies breed on dead animals and mechanically vector over 100 pathogen species into surrounding communities (Sanderson et al. 2006, Sawabe et al. 2006, Hald et al. 2008, Forster et al. 2009). In addition, many advocate the use of the black soldier fly larvae as feedstuff for livestock and poultry (Hale 1973; Newton et al. 1977, 2005); because the black soldier fly adults do not feed, they may be superior fly populations to have in association with food production facilities than other flies (such as *M. domestica*) as they will not spread microbes through feeding. However, the potential for the introduction and amplification of pathogens by these insects into waste products (fertilizer) or the feed that livestock consume is of concern. The mobile adult stage would be the most important in vectoring pathogenic bacteria by infecting subsequently visited resources or by transferring microbes vertically. In addition the structure of the overall bacterial community, and its incorporation of pathogenic species, would be influenced by its constituents' capacity to release antibiotics toxic to other species, to either help themselves colonize or to protect themselves from others trying to occupy the same space and use the same resources.

Enterobacteriales were found on all stages of black soldier fly development and many members have pathogenic potential. Their pathogenicity has an economic impact on the food safety industry (Linton and Hinton 1988). Xanthomonadales, also found on all stages of black soldier fly development, can be opportunistic pathogens and some members (e.g., *Lysobacter*) produce antibiotics (Christensen and Cook 1978). *Lysobacter* was heavily represented on the nonegg stages, particularly on the adult fly (23.6%), and present on the prepupal and pupal stages, potentially explaining antimicrobial effects of black soldier fly colonization. Some members of the order Burkholderiales have the capacity to be opportunistic pathogens. They were a minor component found associated with the larval through egg stages of development. Of the order Bacteroidales, the genus *Bacteroides* was heavily represented and associated with the egg and motile stages (ranging from 2.3 to 27.7%) during fly development, and is a common component of infections and sepsis.

The phylum Firmicutes contains two well-known classes of pathogens. *Bacilli* and *Clostridia* produce toxins that are extremely dangerous, *Bacillus anthracis* (Anthrax), *Clostridium tetani* (Tetanus), and *C. botulinum* (Botulism). Bacteria from this phylum were only a minor component of the community found in this study. The genus *Staphylococcus*, a minor component in the egg through pupa stages, is well known for developing antibiotic resistance and causing nosocomial infections. Representatives from the families Enterococcaceae were associated with nonegg stages, whereas Lactobacillaceae were identified from the egg stage. Within the class Clostridia one order, Clostridiales, was represented as a minor constituent on larvae, prepupae, pupae, and adults, but a large contributor to the bacterial community on eggs (11.1%).

Many of the species identified in this study of the bacterial fauna present on the fly at various stages of development, act as ecological consumers and decomposers (Burkepille et al. 2006). Bacteria from the order Rhodobacterales were found as a minor component on the nonegg stages. Rhodobacterales have diverse metabolic capabilities capable of carbon dioxide and nitrogen fixation, as well as chemoorganotrophic metabolizing sulfur-containing compounds (Garrity et al. 2005). Many bacteria from the order Rhizobiales are plant symbionts facilitating nitrogen fixation (Skorpil and Broughton 2006). However, one genus of Rhizobiales, *Methylobacterium*, is a methane-oxidizing bacterium (Patt et al. 1976). The methanotroph was identified primarily during the egg2 stage. In contrast, nitrogen fixing Rhizobiales were primarily found during the nonegg stages. Although present in small quantities, given their ability to produce volatiles, these bacteria may participate in interkingdom signaling attracting insects to use resources.

The class Betaproteobacteria was represented by the order Burkholderiales, some members of which have the capacity to degrade chlororganic pesticides and polychlorinated biphenyls; however, their capacity to be opportunistic pathogens limits their commercial usage for the degradation of pesticides. Interestingly, the presence of such microbes raises concerns of an enhanced development of resistance to pesticides in insect communities associated with black soldier fly.

Conclusion. The possibility of bacteria retained through successive black soldier fly life stages should result in scrutinizing the initial bacterial load and diversity on these flies before introduction into waste or feed to mitigate any inadvertent disease transmission. This also raises the prospect of using black soldier fly to purposefully introduce bacterial species beneficial to bioremediation, competitive exclusion of pathogenic organisms, and the reduction of animal wastes. They are also potentially useful in bioengineering biowaste management systems associated with black soldier fly. However, as initially pointed out, this study served as a survey of those bacteria species present throughout the life cycle of the black soldier fly. Future research should continue along the path of identifying particular species that can enhance the ability of the black soldier fly to digest wastes and waste associated pathogens, thus eliminating those that pose potential harm to humans and other animals.

Acknowledgments

Funding for L. Z., B. S., J.K.T., and A.M.T. was provided partially by Texas Agrilife Research and the College of Agriculture and Life Sciences at Texas A&M University. Additional funds for T.L.C., B. S., J.K.T., and A.M.T. were provided by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice through Grant 2010-DN-BX-K243. T.K.W. is the T. Michael O'Connor Endowed Professor at Texas A&M University.

References Cited

- Barnes, K. M., D. E. Gennard, and R. A. Dixon. 2010. An assessment of the antibacterial activity in larval excretion/secretion of four species of insects recorded in association with corpses, using *Lucilia sericata* Meigen as the marker species. *Bull. Entomol. Res.* 100: 635–640.
- Bowman, J. S., S. Rasmussen, N. Blom, J. W. Deming, S. Rysgaard, and T. Sicheritz-Ponten. 2012. Microbial community structure of Arctic multiyear sea ice and surface seawater by 454 sequencing of the 16S RNA gene. *ISME J.* 6: 11–20.
- Bradley, S. W., and D. C. Sheppard. 1984. House fly oviposition inhibition by larvae of *Hermetia illucens*, the black soldier fly. *J. Chem. Ecol.* 10: 853–859.
- Burkepille, D. E., J. D. Parker, C. B. Woodson, H. J. Mills, J. Kubanek, P. A. Sobczyk, and M. E. Hay. 2006. Chemically mediated competition between microbes and animals: microbes as consumers in food webs. *Ecology* 87: 2821–2831.
- Chao, A., and J. Bunge. 2002. Estimating the number of species in a stochastic abundance model. *Biometrics* 58: 531–539.
- Chaudhury, M. F., J. B. Welch, and L. A. Alvarez. 2002. Response of fertile and sterile screwworm (Diptera: Calliphoridae) flies to bovine blood inoculated with bacteria originating from screwworm infested animal wounds. *J. Med. Entomol.* 39: 130–134.
- Chaudhury, M. F., S. R. Skoda, A. Sagel, and J. B. Welch. 2010. Volatiles emitted from eight wound-isolated bacteria differentially attract gravid screwworms (Diptera: Calliphoridae) to oviposit. *J. Med. Entomol.* 47: 349–354.
- Christensen, P., and F. Cook. 1978. *Lysobacter*, a new genus of nonfruiting, gliding bacteria with a high base ratio. *Int. J. Syst. Bacteriol.* 28: 367–393.
- Cole, J. R., Q. Wang, and E. Cardenas. 2009. The ribosomal database project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* 37: D141–145. (<http://pyro.cme.msu.edu/>).
- Costello, E., C. Lauber, M. Hamady, N. Fierer, J. Gordon, and R. Knight. 2009. Bacterial community variation in human body habitats across space and time. *Science* 326: 1694–1697.
- Dowd, S., T. Callaway, R. Wolcott, Y. Sun, T. McKeehan, R. Hagevoort, and T. Edrington. 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). *BMC Microbiol.* 8: 125.
- Erickson, M. C., M. Islam, C. Sheppard, J. Liao, and M. P. Doyle. 2004. Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar enteritidis in chicken manure by larvae of the black soldier fly. *J. Food Prot.* 67: 685–690.
- Forster, M., K. Sievert, S. Messler, S. Klimpel, and K. Pfeffer. 2009. Comprehensive study on the occurrence and distribution of pathogenic microorganisms carried by synanthropic flies caught at different rural locations in Germany. *J. Med. Entomol.* 46: 1164–1166.
- Garrity, G., J. Bell, and T. Lilburn. 2004. *Bergey's manual of systematic bacteriology*. Springer, New York.
- Garrity, G., J. Bell, and T. Lilburn. 2005. *Bergey's manual of systematic bacteriology*, 2nd ed. Springer-Verlag, New York.
- Greenberg, B. 1973. *Biology and disease transmission*. Princeton University Press, Princeton, NJ.
- Hail, D., I. Lauzière, S. E. Dowd, and B. Bextine. 2011. Culture independent survey of the microbiota of the glassy-winged sharpshooter (*Homalodisca vitripennis*) using 454 pyrosequencing. *Environ. Entomol.* 40: 23–29.

- Hald, B., H. Skovgard, K. Pedersen, and H. Bunkenborg. 2008. Influxed insects as vectors for *Campylobacter jejuni* and *Campylobacter coli* in Danish broiler houses. *Poultry Sci.* 87: 1428–1434.
- Hale, O. M. 1973. Dried *Hermetia illucens* larvae (Stratiomyidae) as a feed additive for poultry. *J. Georgia Entomol. Soc.* 8: 16–20.
- Hamady, M., C. Lozupone, and R. Knight. 2009. Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. *ISME J.* 4: 17–27.
- Han, M., and C. Zmasek. 2009. PhyloXML: XML for evolutionary biology and comparative genomics. *BMC Bioinform.* 10: 356.
- Handl, S., S. E. Dowd, J. F. Garcia-Mazcorro, J. M. Steiner, and J. S. Suchodolski. 2011. Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. *FEMS Microbiol. Ecol.* 76: 301–310.
- Hogsette, J. A. 1985. New diets for production of house flies and stable flies (Diptera: Muscidae) in the laboratory. *J. Econ. Entomol.* 85: 2291–2294.
- Huber, J. A., D. B. Mark Welch, H. G. Morrison, S. M. Huse, P. R. Neal, D. A. Butterfield, and M. L. Sogin. 2007. Microbial population structures in the deep marine biosphere. *Science* 318: 97–100.
- Janzen, D. H. 1977. Why fruits rot, seeds mold, and meat spoils. *Am. Nat.* 111: 691–713.
- Jeon, H., S. Park, J. Choi, G. Jeong, S.-B. Lee, Y. Choi, and S.-J. Lee. 2011. The intestinal bacterial community in the food waste-reducing larvae of *Hermetia illucens*. *Curr. Microbiol.* 62: 1390–1399.
- Lam, K., D. Babor, B. Duthie, E. M. Babor, M. Moore, and G. Gries. 2007. Proliferating bacterial symbionts on house fly eggs affect oviposition behaviour of adult flies. *Anim. Behav.* 74: 81–92.
- Lam, K., C. Geisreiter, and G. Gries. 2009a. Ovipositing female house flies provision offspring larvae with bacterial food. *Entomol. Exp. Appl.* 133: 292–295.
- Lam, K., K. Thu, M. Tsang, M. Moore, and G. Gries. 2009b. Bacteria on housefly eggs, *Musca domestica*, suppress fungal growth in chicken manure through nutrient depletion or antifungal metabolites. *Naturwissenschaften* 9: 1127–1132.
- Li, Q., L. Zheng, N. Qiu, H. Cai, J. K. Tomberlin, and Z. Yu. 2011. Bioconversion of dairy manure by black soldier fly (Diptera: Stratiomyidae) for biodiesel and sugar production. *Waste Manag.* 31: 1316–1320.
- Linton, A. H., and M. H. Hinton. 1988. Enterobacteriaceae associated with animals in health and disease. *Society for Applied Bacteriology Symposium Series*: 17: 71S–85S.
- Liu, Q., J. K. Tomberlin, J. A. Brady, M. R. Sanford, and Z. Yu. 2008. Black soldier fly (Diptera: Stratiomyidae) larvae reduce *Escherichia coli* in dairy manure. *Environ. Entomol.* 37: 1525–1530.
- Ma, Q., A. Fonseca, W. Liu, A. T. Fields, M. L. Pimsler, A. F. Spindola, A. M. Tarone, T. L. Crippen, J. K. Tomberlin, and T. K. Wood. 2012. *Proteus mirabilis* interkingdom swarming signals attract blow flies. *ISME J.* 6: 1356–1366.
- Miki, T., M. Ushio, S. Fukui, and M. Kondoh. 2010. Functional diversity of microbial decomposers facilitates plant coexistence in a plant–microbe–soil feedback model. *Proc. Natl. Acad. Sci. U.S.A.* 107: 14251–14256.
- Myers, H. M., J. K. Tomberlin, B. D. Lambert, and D. Kattes. 2008. Development of black soldier fly (Diptera: Stratiomyidae) larvae fed dairy manure. *Environ. Entomol.* 37: 11–15.
- Nawrocki, E., and S. Eddy. 2007. Query-dependent banding (QDB) for faster RNA similarity searches. *PLOS Comp. Biol.* 3: 0540–0554.
- Nawrocki, E., D. Kolbe, and S. Eddy. 2009. Infernal 1.0: inference of RNA alignments. *Bioinformatics* 25: 1335–1337.
- Newton, G. L., C. V. Booram, R. W. Barker, and O. M. Hale. 1977. Dried *Hermetia illucens* larvae meal as a supplement for swine. *J. Anim. Sci.* 44: 395–400.
- Newton, G. L., C. Sheppard, D. W. Watson, G. Burtle, and R. Dove. 2005. Using the black soldier fly, *Hermetia illucens*, as a value-added tool for the management of swine manure. (http://www.cals.ncsu.edu/waste_mgmt/smithfield_projects/phase2report05/cd.web%20files/A2.pdf).
- Patt, T. E., G. C. Cole, and R. S. Hanson. 1976. *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. *J. Syst. Bacteriol.* 26: 226–229.
- Ponnusamy, L., D. M. Wesson, C. Arellano, C. Schal, and C. S. Apperson. 2010. Species composition of bacterial communities influences attraction of mosquitoes to experimental plant infusions. *Microb. Ecol.* 59: 158–173.
- R Development Core Team. 2006. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Roesch, R., R. R. Fulthorpe, A. Riva, G. Casella, A.K.M. Hadwin, A. D. Kent, S. M. Daroub, F.A.O. Camargo, W. G. Farmerie, and E. W. Triplett. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME J.* 1: 283–290.
- Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406–425.
- Sanderson, M. W., J. M. Sargeant, X. Shi, T. G. Nagaraja, L. Zurek, and M. J. Alam. 2006. Longitudinal emergence and distribution of *Escherichia coli* O157 genotypes in a beef feedlot. *Appl. Environ. Microbiol.* 72: 7614–7619.
- Sawabe, K., K. Hoshino, H. Isawa, T. Sasaki, T. Hayashi, Y. Tsuda, H. Kurahashi, K. Tanabayashi, A. Hotta, T. Saito, et al. 2006. Detection and isolation of highly pathogenic H5N1 avian Influenza A virus from blow flies collected in the vicinity of an infected poultry farm in Kyoto, Japan, 2004. *Am. J. Trop. Med. Hyg.* 75: 327–332.
- Shannon, C. E. 1948. A mathematical theory of communication. *Bell Syst. Tech. J.* 27: 379–423.
- Sheppard, D. C., G. L. Newton, S. A. Thompson, and S. Savage. 1994. A value added manure management system using the black soldier fly. *Biores. Technol.* 50: 275–279.
- Sheppard, D. C., J. K. Tomberlin, J. A. Joyce, B. Kiser, and S. M. Sumner. 2002. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). *J. Med. Entomol.* 39: 695–698.
- Skorpił, P., and W. Broughton. 2006. Molecular interactions between *Rhizobium* and legumes. *Prog. Mol. Subcell. Biol.* 41: 143–164.
- St. Hilaire, S., C. Sheppard, J. K. Tomberlin, S. Irving, M. A. McGuire, E. E. Mosley, R. W. Hardy, and W. Sealey. 2007. Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. *J. World Aqua. Soc.* 38: 59–67.
- Swofford, D. 2003. PAUP*: phylogenetic analysis using parsimony (*and other methods) computer program, version 4. Sinauer Associates, Inc., Sunderland, MA.
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731–2739.

- Tomberlin, J. K., D. C. Sheppard, and J. A. Joyce. 2005. Black soldier fly (Diptera: Stratiomyidae) colonization of pig carrion in south Georgia. *J. Forensic Sci.* 50: 152–53.
- Tomberlin, J. K., P. H. Adler, and H. M. Myers. 2009. Development of the black soldier fly (Diptera: Stratiomyidae) in relation to temperature. *Environ. Entomol.* 38: 930–934.
- Wang, Q., G. M. Garrity, J. M. Tiedje, and Cole, J. R. 2007. Naive Bayesian classifier of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73: 5261–5267.
- Will, C., A. Thurmer, and A. Wollherr. 2010. Horizon-specific bacterial community composition of German grassland soils, as revealed by pyrosequencing-based analysis of 16S rRNA genes. *Appl. Environ. Microbiol.* 76: 6741–6759.
- Wright, E. S., L. S. Yilmaz, and D. R. Noguera. 2012. DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. *Appl. Environ. Microbiol.* 78: 717–725. (<http://decipher.cce.wisc.edu/FindChimeras.html>).
- Yu, G., P. Cheng, Y. Chen, Y. Li, Z. Yang, Y. Chen, and J. K. Tomberlin. 2011. Inoculating poultry manure with companion bacteria influences growth and development of black soldier fly (Diptera: Stratiomyidae) larvae. *Environ. Entomol.* 40: 30–35.

Received 7 September 2012; accepted 24 January 2013.
