Microbial Ecology of Tropical Arboreal Ants

By

Alissa Susanne Hanshew

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Microbiology)

at the

University of Wisconsin – Madison

2013

Date of final oral examination: 8/23/13

The dissertation was approved by the following members of the Final Oral Committee:
    Cameron R. Currie, Professor, Bacteriology
    Margaret McFall-Ngai, Professor, Medical Microbiology and Immunology
    Heidi Goodrich-Blair, Professor, Bacteriology
    Garret Suen, Assistant Professor, Bacteriology
    Kenneth F. Raffa, Professor, Entomology

Dissertation Abstract

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Under the supervision of Professor Cameron R. Currie

At the University of Wisconsin – Madison

Ants are one of the most dominant families of arthropods on Earth, with more than 12,000 described species. Their life histories range from tiny, cryptic, and nearly eyeless ground dwellers, to almost two-inch long hunters with one of the most painful stings known to man. Despite their ubiquity, and our knowledge that most if not all animals rely upon microbial symbionts, ant bacterial communities remain understudied. The work presented in this dissertation expands our knowledge of the microbial communities present in thirteen tropical arboreal ant species. First, I explore the presence and putative function of culturable Actinobacteria in three ant-plant-fungi mutualisms. I show that these three disparate ant species are host to Actinobacteria that may serve as protective or nutritional symbionts. Next I characterize and compare the bacterial communities of different species pairings of *Cecropia*-obligate *Azteca* ants. I found that the bacterial communities of these ants were not correlated with *Azteca* species, *Cecropia* species, or geographic location. Life stage may explain some of the differences and similarities in these ants, but there are likely other variables, such as varied rates of predation, that I am unable to account for that may better explain our data. Lastly, we explore

the effect of host species, nesting strategy, and diet on microbial communities of seven arboreal ant species at La Selva Biological Station. Microbial community composition was highly correlated with host species, but not genera or subfamily. Likewise, correlations can be drawn between trophic level and the presence, or absence, of particular bacterial community members.

Acknowledgements

As with any large undertaking, a PhD is not completed in a vacuum. There are likely people whom I have forgotten to mention here. I am no less grateful for their assistance.

I cannot thank Kevin McIver, Traci Kinkel, and other members of the McIver lab at the University of Maryland-College Park enough for encouraging me to apply to graduate school. Their support and feedback was invaluable in how to pick a research focus and find a graduate program. Certainly Julie Myers deserves a heartfelt thank you for the time she put into helping me figure out how to explain to other people why I deserved a chance at graduate level education.

Likewise, my experiences at Fairmont Heights High School were, and continue to be, instrumental in helping me see the big picture. No experience will ever be as challenging as teaching high schoolers science. Not even getting my PhD. I remember on a regular basis that science is nothing without the ability to explain it in plain language to anyone, regardless of their level of education.

I appreciate the academic freedom my advisor, Cameron Currie, has given me in my research. I did not come here to work on the bacteria associated with arboreal ants, but my experiences in the Currie lab have hopefully provided me the knowledge I will need to continue working in microbial ecology, no matter what the host may be. My committee members, Heidi Goodrich-Blair, Margaret McFall-Ngai, Ken Raffa, and Garret Suen have been incredibly helpful over the past five years in asking me to push the boundaries of my knowledge and asking me questions that help me find the holes in my research and how I present it. Likewise, I am grateful

Lastly, my family, both the one I was born into and the one I have chosen for myself. My parents of course deserve special thanks for their love, support, and encouragement. As the first Ph.D. in my family, I'm sure much of my family still doesn't understand just what it is I do. My future husband, the future Dr. Charlie Mason, has also been instrumental in this process, providing countless hours of discussion about the nuances of the work we do, and likely more hours helping me forget it all. You can see the product of part of our collaboration in Appendix 1. Much of the rest should likely never be repeated in public, and certainly not committed to print!

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Chapter 1. Introduction

At more than 12,000 described species, ants are one of the most dominant families of arthropods in terrestrial ecosystems, especially in the tropics [1, 2]. As with other arthropods, and in fact all animals studied to date, ants rely on bacteria for a number of functions [3]. Bacterial mutualists aid in nutrient acquisition and protection of colony resources for their ant hosts. Nevertheless, ants remain understudied in terms of their associated bacterial diversity, how those bacterial communities differ across different ant species, and the function of many of the bacteria found in and on ants.

1.1 Bacterial symbionts and their role in ant nutrition

For arthropods in the family Formicidae, living in tropical trees poses certain challenges, some unique to the arboreal lifestyle, while others are more universally present in animals. Work in the last 40 years has demonstrated that ants are not only more dominant in the tropical tree canopy than previously thought, but that they also outnumber their supposed prey items [1, 4]. The large numbers of ants in this ecosystem suggests that they occupy a lower trophic level than previously assumed, as carnivory is unlikely to be supported due to insufficient prey [1, 4]. Therefore, many arboreal ants are likely functional herbivores that do not rely on prey to support their nutritional needs [1, 5].

This hypothesis is supported by arboreal ants, as a whole, having nitrogen isotope levels consistent with herbivores [1, 6]. Although few ants are observed consuming foliage, they are often observed visiting extrafloral nectaries (EFN), which is likely to be one of their primary food sources [5]. Some of these ant species are also known to harbor phloem-feeding Hemiptera in their nests, which produce a carbohydrate rich food source, honeydew, for the ants [7]. In exchange, many ant species provide these food sources with protection. EFN producing plants

are often protected from herbivores by ant colonies that utilize the nectar [8, 9]. Likewise, ant colonies provide their honeydew producing Hemiptera protection [10–12].

The paradox remains for how these functional herbivores, which consume nutrient-poor diets, acquire the necessary levels of nitrogen to support growth. EFN and honeydew both provide sufficient carbohydrates for ant nutritive needs, but are often low in other nutrients, including biologically available nitrogen [2, 5, 13]. Unlike predatory ants, which are thought to absorb needed nitrogen-containing compounds from their prey items, ants that subsist on EFN and honey dew must procure nitrogen by other means [1, 14]. Davidson *et al.* raised the possibility that this may occur by microbial symbionts, which has been shown to occur in a handful of ant species [1]. *Camponotus floridanus* ants that harbor the bacterium *Blochmannia floridanus* are able to use the ammonia that is produced by the bacterium from recycling urea [15]. Additional work suggests that this means of nitrogen acquisition might be present in additional species of ants in the tribe Camponotini [16]. Similarly, the bacterial species in the genera *Pantoea* and *Klebsiella* fix free nitrogen in the fungus gardens of attine ants, releasing usable ammonia that is later detectable in the ants themselves [17]. Putative nitrogen-fixing and -recycling bacteria have been found in other ant species and are thought to provide nitrogen to their hosts. Numerous bacteria related to known nitrogen fixers were isolated from *Pseudomyrmex ferrugineus* ants living on *Acacia hindsii* trees [18]. Russell *et al.* also proposed that Rhizobiales in herbivorous ants may play a role in nitrogen fixation [19]. Likewise, putative nitrogen fixing bacteria have been found in a number of different *Tetraponera* species [20, 21].

The *Pantoea* and *Klebsiella* found in the fungus gardens of attine ants are but two community members in a complex system [22–24]. The fungus found in the gardens of attine ants is well understood to serve as the primary food source within the colony, but the role of

bacteria in these gardens is only now coming to light. In addition to nitrogen fixation, bacterial community members in these gardens are thought to be vital in aiding nutrient acquisition for the fungus [24]. This bacterial community may also play a role in the breakdown of plant biomass that is not otherwise accessible to the ants [22, 23]. The release of nutrients is thought to aid in fungal growth, which then supports colony growth. Beyond this and nitrogen acquisition, bacterial nutritional symbionts in ants are largely unknown.

1.2 Bacterial symbionts and their role in ant protection

In additional to nutritional roles, some bacteria serve protective roles in ants. As mentioned above, attine ants farm fungi that serve as their primary food source. These fungal gardens can be parasitized by a specialist fungal pathogen, *Escovopsis* spp. In addition to mechanical weeding of the coevolved garden pathogen these ants are also thought to apply metapleural gland secretions to the garden to aid in pathogen suppression [25, 26]. Fungus growing ants also utilize secondary metabolites produced by *Pseudonocardia* to deal with *Escovopsis* [27–29]. This bacterium produces antifungal compounds that have high inhibitory activity toward *Escovopsis* while not harming the fungus garden itself.

A similar defensive role for Actinobacteria has been suggested in at least one other ant system. The arboreal ant species *Allomerus* maintains a fungus that aids them in ambush hunting [30]. These ants build a lattice like structure along plant stems and branches, using trichomes as support for fungal growth, then hide inside to ambush insects when they land on the plant [30]. *Streptomyces*, a genus known for producing antimicrobial compounds, are associated with these ants [31]. While it has yet to be shown experimentally that these ants are using the *Streptomyces* to produce protective compounds, protective Actinobacteria are in a number of other insects beyond ants. Digger wasps, southern pine beetles, and spruce beetles use Actinobacteria in a

protective manner [32–34]. This role has also been proposed in a number of other arthropod systems [35–39].

1.3 *Wolbachia* in ants

Despite the ubiquity of *Wolbachia* in many insect genera, the role of *Wolbachia* in ants has yet to be fully elucidated. In some insects, *Wolbachia* are known to manipulate reproduction via one of four methods, including feminization of males, male-killing, cytoplasmic incompatibility, and induction of parthenogenesis [40]. Despite this work in other insects, no one has conclusively shown that *Wolbachia* manipulates reproduction in ants.

Wolbachia has been found in most subfamilies of Formicidae, but not all genera, or species, are equally infected [21, 40–45]. In *Formica truncorum, Wolbachia* may affect reproduction by reducing the number of reproductives produced in a colony [46]. However, most of the known roles for how *Wolbachia* changes reproduction are unlikely in ants because of how they reproduce. Wenseleers *et al.* surmised that cytoplasmic incompatibility was unlikely because while *Wolbachia* infection was even across *Formica truncorum* colonies, sex ratios in colonies were not [46]. Feminization of males and induction of parthenogenesis are also unlikely [40]. Whether the other form of reproductive manipulation, male-killing, occurs remains to be shown.

Recent work in the attine ant *Acromyrmex octospinosus* suggests that *Wolbachia* might actually have a nutritional role for these ants [41]. Researchers found *Wolbachia* extracellularly, an anomaly thus far in arthropods, and predominately in the gut. Traditionally *Wolbachia* is thought to be an intracellular bacterium often associated with the ovaries of arthropods. While this paper did not demonstrate a nutritional role, the location and density of *Wolbachia* in these ants suggests a nutritional role [41].

1.4 Other bacteria found associated with ants

In addition to *Wolbachia*, nitrogen-acquiring bacteria, and protective Actinobacteria discussed above, a number of other bacteria have been found in ants. However, due to experimental constraints, and until recently, lack of knowledge that these bacteria were even present, little research has been done to establish the role of these bacteria in ant physiology.

Russell *et al.* found in lab-maintained colonies of *Cephalotes* sp. ants that in addition to the Rhizobiales mentioned earlier, these ants also maintained Burkholderiales, Xanthomonadales, Pseudomonadales and Verrucomicrobiales [19]. They suggested that these bacteria are not transmitted transovarially, but within the colony itself, as these bacteria were not found in early instar larvae, but were present by later instars. They also point out that one must consider the developmental cycle of ants. The bacteria acquired as larvae may be shed during the final molt, only to be reacquired as a newly emerged worker.

Eilmus and Heil, in addition to potential nitrogen fixing bacteria, found a number of other bacteria associated with *P. ferrugineus* ants [18]. These included bacteria in the phyla Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes, Proteobacteria, and Spirochaetes. In addition to putative nitrogen fixers and *Wolbachia*, *Tetraponera* also harbor bacteria similar to *Bartonella, Sodalis* and *Pantoea* [21]. More specifically, *Tetraponera binghami*, also harbored *Rhizobium*, *Methylobacterium*, *Burkholderia*, *Pseudomonas*, and *Flavobacteria* in specialized pouches located just off the gut [20]. While Feldhaar *et al.* state that *Camponotus* ants have only *Blochmannia* in their guts, He *et al.* found three other types of bacteria [15, 47]. However, experimental limitations from older techniques (DGGE and tRFLP) make it likely that there are other bacterial community members that have not yet been detected in many ants.

1.5 Arboreal ants and the role of microbes in these systems

As conspicuous members of tropical ecosystems, ant-plant mutualisms have held the fascination of numerous scientists for several hundred years [48, 49]. Pioneering observations by many naturalists in the late 19th century and early 20th century raised the idea that tropical plants enlisted the aid of certain ant species to protect against other animals that might harm the host plant [48–55]. Despite many observations of the presence of fungi in these systems, little research was done on the role microbes played in these symbiotic systems [50, 56, 57].

Recently, fungus found in the domatia of plant-ants were shown to be maintained by resident ants, in some cases fed to larvae, and involved in provisioning nitrogen to the plant [58–62]. Additional ant species are also known to use fungi for structural needs, rather than nutritional [30, 62–65]. The growing body of evidence suggests that some of these ants may fulfill the characteristics necessary to deem them fungus-farmers, adding to our understanding of the relationships between Formicidae and beneficial fungi [64].

In the last decade, there has been renewed interest in arboreal ants, specifically the roles bacteria may play. With the advent of next-generation sequencing technologies, we now have the ability to not only sequence more samples, but to also sequence them to a greater depth. As a result, larger scale comparative studies are now possible on many niches, including ants. Despite this, and the numerous species of arboreal ants, only two papers have addressed whole bacterial community comparisons in arboreal ants. Kautz *et al.* found that four colonies of *Cephalotes varians*, an exclusively arboreal turtle ant, harbored similar bacterial communities, and that many of the taxa identified were similar to bacteria previously found in ants [66]. They speculated that much of what they found were likely nutritional symbionts. Similarly, Seipke *et al.* found that two geographically disparate plant-ants harbored similar ectobiont communities at the phyla

level [67]. Unfortunately, phyla level comparisons are not very indicative of bacterial community differences, as most insects harbor bacteria in the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria [68]. Likewise, experimental design problems, namely vortexing ants in glycerol for removal of ectobionts for community assessment, make the conclusions of this paper questionable.

1.6 Central objectives of this dissertation

My research focuses on the microbial communities of multiple tropical arboreal ant species. As discussed above, bacteria are known or presumed to have many roles in ants. The work presented in this thesis aims to increase our knowledge about (a) the presence and potential roles of Actinobacteria in fungus-ant-plant systems (Chapter 2), (b) how microbial communities in *Cecropia*-obligate *Azteca* ants compare across species pairings and geographic location (Chapter 3), and (c) how microbial communities differ between arboreal ants with different life histories (Chapter 4).

These objectives are addressed using thirteen different arboreal ant species from the Paleotropics and the Neotropics, summarized in Table 1.1. Three ant-plant systems recently discovered to maintain fungus in their domatia, *Petalomyrmex phylax* in *Leonardoxa africana*, *Pseudomyrmex penetrator* in *Tachigali* sp., and *Crematogaster margaritae* in *Keetia hispida*, were used to address the question of whether other fungus-associated ants associate with protective or nutritive Actinobacteria, akin to what is found in attine ants and other arthropods (Chapter 2). We used selective media and traditional culture based techniques to isolate Actinobacteria that were then 16S rRNA gene sequenced and compared to other known Actinobacteria. *Cecropia*-obligate *Azteca ants* were used to characterize and compare the microbiota of different ant-tree species pairings and to explore whether host bacterial

communities varied across geographic distance (Chapter 3). These colonies were collected from

Costa Rica and subjected to 16S rRNA amplicon pyrosequencing for microbial community

comparisons. Lastly, seven arboreal ant species from three different subfamilies in Formicidae

were used to assess the effect of host species, diet, and life history on microbial community

composition (Chapter 4). These samples were also subjected to 16S rRNA amplicon

pyrosequencing.

Table 1.1 Ant species used in this dissertation.

Ant species	Subfamily	Nest location	Country of Origin	Ch.
Azteca alfari	Dolichoderinae	*Cecropia* sp.	Costa Rica	3
Azteca coerulepennis	Dolichoderinae	*Cecropia* sp.	Costa Rica	3
Azteca constructor	Dolichoderinae	*Cecropia* sp.	Costa Rica	3
Azteca xanthochroa	Dolichoderinae	*Cecropia* sp.	Costa Rica	3, 4
Crematogaster longispina	Myrmicinae	Carton nest	Costa Rica	4
Crematogaster margaritae	Myrmicinae	*Keetia hispida*	Cameroon	2
Myrmelachista flavocotea	Formicinae	*Ocotea*	Costa Rica	4
Nylanderia caeciliae	Pseudomyrmecinae	Carton nest	Costa Rica	4
Petalomyrmex phylax	Formicinae	*Leonardoxa africana*	Cameroon	2
Pheidole bicornis	Formicinae	*Piper*	Costa Rica	4
Pheidole fiorii	Formicinae	Carton nest	Costa Rica	4
Pseudomyrmex penetrator	Pseudomyrmecinae	*Tachigali* sp.	French Guiana	2
Tapinoma ramulorum inrectum	Dolichoderinae	Carton nest	Costa Rica	4

1.7 References

1.   Davidson DW, Cook SC, Snelling RR, Chua TH (2003) Explaining the abundance of ants in lowland tropical rainforest canopies. Science 300: 969–972. doi:10.1126/science.1082074

2.   Holldobler B, Wilson EO (1990) The Ants. The Belknap Press of Harvard University Press, Cambridge, Massachusetts.

3.   Moya A, Peretó J, Gil R, Latorre A (2008) Learning how to live together: genomic insights into prokaryote-animal symbioses. Nature Reviews Genetics 9: 218–29. doi:10.1038/nrg2319

4.   Tobin JE (1991) A neotropical rainforest canopy, ant community: some ecological considerations. In: Huxley CR, Cutler DF (eds) Ant-Plant Interactions. Oxford University Press, Oxford, UK, pp 536–538

5.   Blüthgen N et al. (2000) How plants shape the ant community in the Amazonian rainforest canopy: the key role of extrafloral nectaries and homopteran honeydew. Oecologia 125: 229–240. doi:10.1007/s004420000449

6. Blüthgen N, Gebauer G, Fiedler K (2003) Disentangling a rainforest food web using stable isotopes: dietary diversity in a species-rich ant community. Oecologia 137: 426–435. doi:10.1007/s00442-003-1347-8

7. Blüthgen N, Gottsberger G, Fiedler K (2004) Sugar and amino acid composition of ant-attended nectar and honeydew sources from an Australian rainforest. Austral Ecology 29: 418–429

8. De la Fuente MAS, Marquis RJ (1999) The role of ant-tended extrafloral nectaries in the protection and benefit of a Neotropical rainforest tree. Oecologia 118: 192–202

9. Heil M, McKey D (2003) Protective ant-plant interactions as model systems in ecological and evolutionary research. Annual Review of Ecology, Evolution, and Systematics 34: 425–453. doi:10.1146/annurev.ecolsys.34.011802.132410

10. Way MJ (1963) Mutualism between ants and honeydew-producing Homoptera. Annual Review of Entomology 8: 307–344. doi:10.1146/annurev.en.08.010163.001515

11. Pringle EG, Dirzo R, Gordon DM (2011) Indirect benefits of symbiotic coccoids for an ant-defended myrmecophytic tree. Ecology 92: 37–46

12. Styrsky JD, Eubanks MD (2007) Ecological consequences of interactions between ants and honeydew-producing insects. Proceedings of the Royal Society - Biological Sciences 274: 151–164. doi:10.1098/rspb.2006.3701

13. Rico-Gray V (1993) Use of plant-derived food resources by ants in the dry tropical lowlands of coastal Veracruz, Mexico. Biotropica 25: 301–315

14. Cook SC, Davidson DW (2006) Nutritional and functional biology of exudate-feeding ants. Entomologia Experimentalis et Applicata 118: 1–10. doi:10.1111/j.1570-7458.2006.00374.x

15. Feldhaar H et al. (2007) Nutritional upgrading for omnivorous carpenter ants by the endosymbiont Blochmannia. BMC Biology 5: 48. doi:10.1186/1741-7007-5-48

16. Wernegreen JJ, Kauppinen SN, Brady SG, Ward PS (2009) One nutritional symbiosis begat another: Phylogenetic evidence that the ant tribe Camponotini acquired Blochmannia by tending sap-feeding insects. BMC Evolutionary Biology 9: 292. doi:10.1186/1471-2148-9-292

17. Pinto-Tomás AA et al. (2009) Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. Science 326: 1120–1123. doi:10.1126/science.1173036

18. Eilmus S, Heil M (2009) Bacterial associates of arboreal ants and their putative functions in an obligate ant-plant mutualism. Applied and Environmental Microbiology 75: 4324–4332. doi:10.1128/AEM.00455-09

19. Russell JA et al. (2009) Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. Proceedings of the National Academy of Sciences 106: 21236–21241. doi:10.1073/pnas.0907926106

20. Van Borm S, Buschinger A, Boomsma JJ, Billen J (2002) Tetraponera ants have gut symbionts related to nitrogen-fixing root-nodule bacteria. Proceedings of the Royal Society - Biological Sciences 269: 2023–2027. doi:10.1098/rspb.2002.2101

21. Stoll S, Gadau J, Gross R, Feldhaar H (2007) Bacterial microbiota associated with ants of the genus Tetraponera. Biological Journal of the Linnean Society 90: 399–412. doi:10.1111/j.1095-8312.2006.00730.x

22.   Aylward FO et al. (2012) Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus gardens. The ISME journal 1–14. doi:10.1038/ismej.2012.10

23.   Suen G et al. (2010) An insect herbivore microbiome with high plant biomass-degrading capacity. PLoS Genetics 6: e1001129. doi:10.1371/journal.pgen.1001129

24.   Scott JJ et al. (2010) Microbial community structure of leaf-cutter ant fungus gardens and refuse dumps. PLoS ONE 5: e9922. doi:10.1371/journal.pone.0009922

25.   Currie CR, Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants. Proceedings of the Royal Society - Biological Sciences 268: 1033–1039. doi:10.1098/rspb.2001.1605

26.   Fernández-Marín H, Zimmerman JK, Rehner SA, Wcislo WT (2006) Active use of the metapleural glands by ants in controlling fungal infection. Proceedings of the Royal Society - Biological Sciences 273: 1689–1695. doi:10.1098/rspb.2006.3492

27.   Currie CR, Scott JA, Summerbell RA, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398: 701–704

28.   Currie CR, Bot ANM, Boomsma JJ (2003) Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. Oikos 101: 91–102

29.   Currie CR, Poulsen M, Mendenhall J, Boomsma JJ, Billen J (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 311: 81–83. doi:10.1126/science.1119744

30.   Dejean A et al. (2010) Arboreal ants use the "Velcro(R) principle" to capture very large prey. PLoS ONE 5: e11331. doi:10.1371/journal.pone.0011331

31.   Seipke RF et al. (2012) Fungus-growing Allomerus ants are associated with antibiotic-producing actinobacteria. Antonie van Leeuwenhoek 101: 443–447. doi:10.1007/s10482-011-9621-y

32.   Scott JJ et al. (2008) Bacterial protection of beetle-fungus mutualism. Science 322: 63. doi:10.1126/science.1160423

33.   Kaltenpoth M et al. (2006) "Candidatus Streptomyces philanthi", an endosymbiotic streptomycete in the antennae of Philanthus digger wasps. International Journal of Systematic and Evolutionary Microbiology 56: 1403–1411

34.   Cardoza YJ, Klepzig KD, Raffa KF (2006) Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. Ecological Entomology 31: 636–645. doi:10.1111/j.1365-2311.2006.00829.x

35.   Visser AA, Nobre T, Currie CR, Aanen DK, Poulsen M (2012) Exploring the potential for Actinobacteria as defensive symbionts in fungus-growing termites. Microbial Ecology 63: 975–985. doi:10.1007/s00248-011-9987-4

36.   Hulcr J et al. (2011) Presence and diversity of Streptomyces in Dendroctonus and sympatric bark beetle galleries across North America. Microbial Ecology 61: 759–768. doi:10.1007/s00248-010-9797-0

37.   Grubbs KJ et al. (2011) Genome sequence of Streptomyces griseus Strain XyelbKG-1, an ambrosia beetle-associated Actinomycete. Journal of Bacteriology 193: 2890–2891. doi:10.1128/JB.00330-11

38.   Adams AS et al. (2011) Cellulose-degrading bacteria associated with the invasive woodwasp Sirex noctilio. The ISME Journal 5: 1323–1331. doi:10.1038/ismej.2011.14

39.   Poulsen M, Oh D-C, Clardy J, Currie CR (2011) Chemical analyses of wasp-associated Streptomyces bacteria reveal a prolific potential for natural products discovery. PLoS ONE 6: e16763. doi:10.1371/journal.pone.0016763

40.   Russell JA (2012) The ants (Hymenoptera: Formicidae) are unique and enigmatic hosts of prevalent Wolbachia (Alphaproteobacteria) symbionts. Myrmecological News 16: 7–23

41.   Andersen SB, Boye M, Nash DR, Boomsma JJ (2012) Dynamic Wolbachia prevalence in Acromyrmex leaf-cutting ants: potential for a nutritional symbiosis. Journal of Evolutionary Biology 25: 1340–1350. doi:10.1111/j.1420-9101.2012.02521.x

42.   Sebastien A, Gruber MAM, Lester PJ (2012) Prevalence and genetic diversity of three bacterial endosymbionts (Wolbachia, Arsenophonus, and Rhizobiales) associated with the invasive yellow crazy ant (Anoplolepis gracilipes). Insectes Sociaux 59: 33–40. doi:10.1007/s00040-011-0184-8

43.   Russell JA et al. (2009) Specialization and geographic isolation among Wolbachia symbionts from ants and lycaenid butterflies. Evolution 63: 624–640. doi:10.1111/j.1558-5646.2008.00579.x

44.   Russell JA et al. (2012) A Veritable Menagerie of Heritable Bacteria from Ants, Butterflies, and Beyond: Broad Molecular Surveys and a Systematic Review. PLoS ONE 7: e51027. doi:10.1371/journal.pone.0051027

45.   Kautz S, Rubin BER, Moreau CS (2013) Bacterial infections across the ants: frequency and prevalence of Wolbachia, Spiroplasma, and Asaia. Psyche 2013: 1–11. doi:10.1155/2013/936341

46.   Wenseleers T, Sundström L, Billen J (2002) Deleterious Wolbachia in the ant Formica truncorum. Proceedings of the Royal Society - Biological Sciences 269: 623–629. doi:10.1098/rspb.2001.1927

47.   He H, Chen Y, Zhang Y, Wei C (2011) Bacteria Associated with Gut Lumen of Camponotus japonicus Mayr. Environmental Entomology 40: 1405–9. doi:10.1603/EN11157

48.   Belt T (1874) The Naturalist in Nicaragua. The University of Chicago Press, Chicago.

49.   Wheeler WM (1912) Observations of the Central American Acacia Ants. The Transactions of the Second Entomological Congress 109–139

50.   Bailey IW (1922) Notes on Neotropical Ant-Plants. I. Cecropia angulata, sp. nov. Botanical Gazette 74: 369–391

51.   Wheeler WM (1942) Studies of Neotropical Ant-Plants and Their Ants. Bulletin of the Museum of Comparative Zoology, Harvard.

52.   Wheeler WM, Bequaert JC (1929) Amazonian Myrmecophytes and their Ants. Zoologischer Anzeiger 82: 10–39

53.   Wheeler WM (1928) Two Interesting Neotropical Myrmecophytes (Cordia nodos and C. alliodora). International Congress of Entomology II: 342–353

54.   Bailey IW (1924) Notes on Neotropical Ant-Plants. III. Cordia nodosa Lam. Botanical Gazette 77: 32–49

55.   Muller F (1874) The Habits of Various Insects. Nature 10: 102–103

56.   Miehe H (1911) Untersuchungen über die javanische Myrmecodia. Abhandlungen der Mathematisch-Physischen Klasse der Königlich-Sächsischen Gesellschaft der Wissenschaften 312–361

57.   Bailey IW (1920) Some Relations between Ants and Fungi. Ecology 1: 174–189

58.   Defossez E et al. (2009) Ant-plants and fungi: a new threeway symbiosis. New Phytologist 182: 942–949

59.   Defossez E, Djiéto-Lordon C, McKey D, Selosse M-A, Blatrix R (2010) Plant-ants feed their host plant, but above all a fungal symbiont to recycle nitrogen. Proceedings of the Royal Society - Biological Sciences 278: 1419–1426. doi:10.1098/rspb.2010.1884

60.   Voglmayr H et al. (2011) The diversity of ant-associated black yeasts: Insights into a newly discovered world of symbiotic interactions. Fungal Biology 115: 1077–1091

61.   Blatrix R, Bouamer S, Morand S, Selosee M-A (2009) Ant-plant mutualisms should be viewed as symbiotic communities. Plant Signaling & Behavior 4: 554–556. doi:10.1111/j.1469-8137.2009.02793.x.publications

62.   Leroy C et al. (2011) Trophic mediation by a fungus in an ant-plant mutualism. Journal of Ecology 99: 583–590. doi:10.1111/j.1365-2745.2010.01763.x

63.   Mayer VE, Voglmayr H (2009) Mycelial carton galleries of Azteca brevis (Formicidae) as a multi-species network. Proceedings of the Royal Society - Biological Sciences 276: 3265–3273. doi:10.1098/rspb.2009.0768

64.   Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR (2005) The evolution of agriculture in insects. Annual Review of Ecology, Evolution, and Systematics 36: 563–595. doi:10.1146/annurev.ecolsys.36.102003.152626

65.   Ruiz-González MX et al. (2011) Specific, non-nutritional association between an ascomycete fungus and Allomerus plant-ants. Biology Letters 7: 475–479. doi:10.1098/rsbl.2010.0920

66.   Kautz S, Rubin BER, Russell JA, Moreau CS (2013) Surveying the microbiome of ants: Comparing 454 pyrosequencing with traditional methods to uncover bacterial diversity. Applied and Environmental Microbiology 79: 525–534. doi:10.1128/AEM.03107-12

67.   Seipke RF, Barke J, Heavens D, Yu DW, Hutchings MI (2013) Analysis of the bacterial communities associated with two ant-plant symbioses. Microbiology Open 1–8. doi:10.1002/mbo3.73

68.   Jones RT, Sanchez LG, Fierer N (2013) A cross-taxon analysis of insect-associated bacterial diversity. PLoS ONE 8: e61218. doi:10.1371/journal.pone.0061218

Chapter 2. Characterization of Actinobacteria associated with three ant-plant mutualisms

Manuscript in preparation: Alissa S. Hanshew, Bradon R. McDonald, Carol Díaz Díaz, Champlain Djiéto-Lordon, Rumsaïs Blatrix, Cameron R. Currie

Coauthors contributed in the following ways: ASH, CDD, and CRC designed the experiments. CDL collected the samples. ASH performed the bacterial isolations. ASH and CDD performed the sequencing. CDD and ASH performed the fungal and cellulase bioassays. BRM performed phylogenetic analyses. CRC provided lab material and support. ASH wrote the paper.

2.1 Abstract

Ant-plant mutualisms are conspicuous and ecologically important members of tropical ecosystems yet remain largely unexplored in terms of insect-associated microbial communities. Recent work has revealed that ants in some ant-plant systems cultivate fungi (Chaetothyriales) within their domatia that are fed to larvae and obtain nitrogen from ant activity. Using *Pseudomyrmex penetrator/Tachigali* sp. from French Guiana, *Petalomyrmex phylax/Leonardoxa africana* and *Crematogaster margaritae/Keetia hispida*, both from Cameroon, as models we tested the hypothesis that ant-plant-fungus mutualisms co-occur with culturable Actinobacteria. Using selective media, we isolated 861 putative Actinobacteria from the three systems. All *C. margaritae/K. hispida* samples had culturable Actinobacteria with an average of 9.73 colony forming units (CFUs) per sample, while 27% of *P. penetrator/Tachigali* samples (average CFUs 1.14) and 55% of *P. phylax/L. africana* samples (average CFUs 3.57) yielded Actinobacteria. The most CFUs were obtained from *P. penetrator* workers, *P. phylax* alates, and *C. margaritae* pupae. 16S rRNA gene sequencing and phylogenetic analysis revealed the presence of four main clades of *Streptomyces* and one clade of *Nocardioides* within these symbioses. *Streptomyces* with antifungal properties were associated with all three systems, suggesting that they could serve as protective symbionts, as found in other insects. In addition, a number of isolates from a

clade of *Streptomyces* associated with *P. phylax*/*L. africana* and *C. margaritae*/*K. hispida* are capable of degrading cellulose, suggesting that *Streptomyces* in these systems may serve a nutritional role. Repeated isolation of particular clades of Actinobacteria from two geographically distant locations supports the role of these isolates as residents in ant-plant-fungi niches and adds to the growing body of evidence suggesting widespread use of Actinobacteria by insects as protective and nutritional symbionts.

2.2 Introduction

Ant-plant mutualisms are pervasive and important components of tropical ecosystems, and represent unique arboreal niches in which to study microbial ecology. In these symbioses, plant hosts generally provide their ant symbionts with two main resources, a nutrient-rich food source in the form of extrafloral nectar secretions or specialized plant structures (e.g. Müllerian bodies from *Cecropia* trees or Beltian bodies from *Acacia* trees), and nesting space in hollowed out plant structures termed domatia [1]. In return, the ants protect the plants from herbivores and remove encroaching vegetation. Additional work suggests that these ants may also provide nutrients to their host plant [2–9]. Both plant hosts and resident ants are phylogenetically diverse and found globally throughout the tropics [10]. Fungi have long been observed in the domatia in ant-plant mutualisms, but the mutualistic nature of this interaction has been demonstrated only recently [11, 12]. The fungi in these patches were identified as black yeasts in the ascomycete order Chaetothyriales, found in a wide range of ant-plant symbioses, observed to be fed to larvae, and shown to obtain nitrogen from ant activity [11, 13–15]. Although fungal hyphae in these ant-plant systems are found in contact with plant material inside the domatia, they were not observed to grow into the plant tissue, suggesting that these fungal symbionts are likely not pathogenic to

the tree [11, 15, 16]. This recent work suggests that the mutualism extends beyond the ant-plant interaction and that these systems include aspects of insect-fungal agriculture [16, 17].

Fungiculture is characterized by four tenants: inoculation of the fungal cultivar into new growth substrate for crop propagation, maintenance of the appropriate growth conditions for the fungal crop, use of the fungal crop as a major source of sustenance, and an obligate relationship with the fungal crop [18]. Three taxonomically distinct groups of arthropods, including attine ants, ambrosia beetles, and fungus-farming termites, are known to fulfill these requirements [18–21]. Fungiculture is likely practiced by additional insects, including ants in the genera *Allomerus* and *Lasius*, species in several tribes of gall midges, the southern pine beetle *Dendroctonus frontalis*, the lizard beetle *Doubledaya bucculenta*, the leaf rolling weevil *Euops chinesis*, and the woodwasp *Sirex noctilio*, all of which meet several of the requirements to be deemed fungus-farmers [17, 18, 22–29]. Symbioses with beneficial fungi provide access to an otherwise unexplored niche for the insect host by making stored carbon in recalcitrant plant material more available and thus reducing competition with other foraging arthropods for food. In return, insects may increase the fitness of their fungal symbionts by expanding its geographic distribution and protecting it from parasites and pathogens.

Insect fungiculture requires that the host protects both itself and its fungal mutualist from pathogens [18]. While it is likely that all fungus-farmers are threatened by potentially virulent pathogens, attine ants remain the best studied example. Infections by the co-evolved mycoparasitic fungus *Escovopsis* have been documented in at least one third of attine ant colonies, with infection rates varying by genus, species, and age of the colony [30, 31]. Colonies control infection using at least three different tactics: mechanical removal of the pathogen by garden weeding, application of antifungal metabolites produced by Actinobacteria maintained on

the ants' cuticle, and potentially by application of secretions from the metapleural glands [32–35]. Likewise, antibiotic-producing Actinobacteria have been isolated from fungus-growing termites and are speculated to protect their fungal gardens from the fungal pathogen *Pseudoxylaria* [36]. Other insects also use secondary metabolites produced by Actinobacteria. Beewolves, which do not cultivate fungus, use several antimicrobials produced by the bacterium '*Candidatus* Streptomyces philanthi' to protect their overwintering brood and its food source from fungal attack [37–39]. Actinobacteria with potentially similar roles have been found in other insect systems, including bark beetles, ambrosia beetles, *Allomerus* ants, *S. noctilio*, and two species of solitary mud dauber wasps [40–44].

In addition to producing numerous secondary metabolites, Actinobacteria are capable of degrading a variety of substrates [45]. Therefore it is perhaps not surprising that Actinobacteria associated with arthropods have recently been shown to fulfill host nutritive roles. Plant associated insects such as *S. noctilio*, emerald ash borer (*Agrilus planipennis)*, and higher termites may use the metabolic potential of cellulose-degrading Actinobacteria to gain access to nutrients in recalcitrant plant material [43, 46–48]. *Streptomyces* associated with these three insects degrade cellulose *in vitro*, and may serve this role in the host or in their feeding galleries [43, 46–48]. Likewise, firebugs and cotton stainers require two actinobacterial symbionts to exploit Malvale seeds as novel food sources [49].

We hypothesized that Actinobacteria may similarly serve as defensive or nutritional symbionts in ant-plant symbioses, as has been found in other insect systems. In addition to potentially protecting the black yeast from pathogens as in the symbioses described above, Actinobacteria might also serve in a number of roles such as protecting the ants, their brood, or the host plant from infection, or aiding in host nutrition. We assessed three ant-plant symbioses

known to harbor fungi in their domatia (*Pseudomyrmex penetrator*/*Tachigali* sp. from French Guiana, *Petalomyrmex phylax*/*Leonardoxa africana* and *Crematogaster margaritae*/*Keetia hispida*, both from Cameroon) as potential niches for Actinobacteria, and began testing the possible function of these isolates as protective and nutritional symbionts. Bacterial isolations were performed on samples from each system and subjected to 16S rRNA gene sequencing. Antifungal bioassays and filter paper degradation assays were performed on a subset of isolates to assess their potential as protective or nutritional symbionts.

2.3 Methods

2.3.1 Colony sampling

Domatia containing ants were cut from trees, placed in sterile containers, and shipped to the laboratory at the University of Wisconsin-Madison. *Pseudomyrmex penetrator* workers, queens, pupae, larvae and scale insects in *Tachigali* sp. were collected in French Guiana in Oct 2009 (Tables 2.1 and 2.2). Fungal patches from 10 *Tachigali* domatia were also collected as scrapings into empty tubes or tubes containing either 1X phosphate buffered saline (PBS) or 10% glycerol (Table 2.2). Four *Leonardoxa africana* domatia inhabited by *Petalomyrmex phylax* were similarly collected in Cameroon in Jan 2010 including workers, alates, larvae, pupae, and fungal patch scrapings, along with two *Keetia hispida* domatia with *Crematogaster margaritae* including workers, larvae, pupae, and scale insects (Tables 2.1 and 2.2).

2.3.2 Microbial Isolations

For all samples, Actinobacteria were cultured using the spread plating technique with 100 μl of inoculum on chitin medium supplemented with the antifungals nystatin and cyclohexamide (per liter: 5.33 g chitin, 0.767 g K_2HPO_4, 0.367 g KH_2PO_4, 0.244 g $MgSO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4 \cdot 7H_2O$, 0.001 g $MnCl_2 \cdot 4H_2O$, 20 g of agar, 0.0427 g nystatin, and 0.0667 g

cyclohexamide). To prepare inocula, *P. penetrator* workers, pupae, and queens were washed in 1 ml 1X PBS, placed in 500 µl 1X PBS, and shaken for 4 min in a Mini-beadbeater (Biospec Products, Bartlesville, OK) with 0.25 g of 1:1 400:800 µm beads. Except for fungal patches, all remaining samples were placed in 500 µl 1X PBS with beads and shaken for 2 min twice in a beadbeater and used as inocula (Tables 2.1 and 2.2). Fungal patches in PBS and glycerol were homogenized with mini-pestles, while dry fungal patches were shaken in a beadbeater for 2 min with 500 µl of 1% tween 20 in 1X PBS with beads, centrifuged for 3.5 min, homogenized with a minipestle, and used as inocula. Plates were incubated at 28°C for up to 4 months, and checked regularly for growth morphologically consistent with Actinobacteria. Putative Actinobacteria colonies were counted and all morphologically distinct colonies were streaked for isolation first using chitin media followed by yeast malt extract agar medium containing antifungals (YMEA) (per liter: 4 g yeast extract, 10 g malt extract, 4 g dextrose, 15 g agar, 40 mg nystatin, and 0.05 g cyclohexamide). To determine if colony morphology could serve as a way to group strains, all isolates were grouped based on colony morphology, and representatives of each morphotype from each ant-plant system were subjected to 16S rRNA gene sequencing and phylogenetic analysis. Strain names correspond to the ant-plant system of origin, PsTa for *P. penetrator/Tachigali*, LaPp for *P. phylax/L. africana*, and KhCr for *C. margaritae/K. hispida*.

2.3.3 DNA extraction, 16S rRNA gene amplification and sequencing

To extract genomic DNA from pure cultures, a loopful of actinobacterial growth was added to 250 µl of 2X cetyltrimethylammonium bromide extraction buffer and lysed by triplicate bead beating for 2 min followed by incubation at -80°C for 2.5 min [50]. One volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, vortexed, and centrifuged for 5 min at 13,000g. The upper phase was mixed with one volume of chloroform-isoamyl alcohol (24:1) in a

new tube, vortexed, and centrifuged for 5 min at 13,000g. The upper phase was then mixed with one volume of cold isopropanol, incubated at -20°C overnight, and centrifuged for 15 min at 13,000g at 4°C. The resulting DNA pellet was washed once with 70% ethanol, dried, and resuspended in 50 μl Tris-EDTA (pH 8.0; Fisher, Fair Lawn, NJ).

The universal bacterial primers 27F (5' AGAGTTTGATCNTGGCTCAG) and 1492R (5' TACGGYTACCTTGTTACG) were used to amplify the near full-length 16S rRNA gene [51]. Each 25 μl reaction included: 12.5 μl EconoTaq Plus Green (Lucigen, Middleton, WI), 200 nM of each primer, and 10 ng of template DNA. The thermocycling program was: initial denaturation at 95°C for 2 min, 35 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 2 min, and a final extension of 72°C for 3 min. PCR products were visualized on a 2% agarose gel in 1X TAE buffer to confirm the presence of the expected single band at ~1465 bp.

PCR products were sequenced using the BigDye Terminator mix v. 3.1 (Applied Biosystems, Carlsbad, CA) separately using primers 27F and 1492R. Each 10 μl reaction included 1 μl BigDye mix, 1.5 μl BigDye buffer, 100 nM 27F or 1492R, 0.5 μl DMSO, and 0.5 μl PCR product. The thermocycling program was: initial denaturation at 96°C for 2 min, 35 cycles of 96°C for 10 s, 52°C for 15 s, and 60°C for 3 min, and a final extension of 72°C for 1 min. PCR products were purified using CleanSeq magnetic beads (Agencourt Biosciences, Brea, CA) as per the manufacturer's protocol, and sequenced using an ABI 3730x sequencer at the University of Wisconsin-Madison Biotechnology Center. Sequences were deposited in Genbank under accession numbers xxxx-xxxx (see also Supplemental Table 2.1).

2.3.4 Phylogenetic tree construction

16S rRNA gene sequence contigs were aligned and edited using Sequencher v 4.5 (Gene Codes Corporation, Ann Arbor, MI). Sequences with more than 5 ambiguous bases were

discarded. Actinobacterial sequences were identified using SeqMatch from the Ribosomal Database Project (RDP), release 10, update 31 [52]. Non-Actinobacteria sequences were not used in further analyses. Reference actinobacterial 16S rRNA gene sequences, including taxa previously isolated from insects [36, 38, 41, 43, 53, 54], were downloaded from the Genbank and RefSeq databases [55, 56]. The 16S rRNA gene sequences of *Slackia exigua* (accession AF101240) and *Atopobium fossor* (accession L34620) were used as a basal Actinobacteria outgroup, based on Gao and Gupta [57]. All sequences were aligned using the G-INS-i algorithm implemented in MAFFT v7.017b [58], trimmed at the 5' and 3' ends to remove trailing ends of longer sequences, and then realigned. Where individual samples had two or more identical sequences, only one sequence was used for the tree (henceforth referred to as duplicate sequences). This alignment was then used to generate a phylogenetic tree using RAxML 7.2.6 with the GTRGAMMA nucleotide substitution model and 1000 rapid bootstraps [59, 60]. The tree was visualized using the web version of iTOL [61, 62].

2.3.5 Actinobacteria-fungal Petri plate bioassay

Nineteen isolates were chosen for fungal bioassays based on initial morphological diversity. They were challenged with two strains of entomopathogens, *Fusarium oxysporum* and *Metarhizium anisopliae*, in addition to the general fungi *Aspergillus niger* and *Trichoderma reesei*, as per Cafaro *et al*. and Poulsen *et al*. [63, 64]. A small loopful of each Actinobacteria isolate was point inoculated in quadruplicate into the center of 8.5 cm Petri dishes containing YMEA without antifungals and incubated for 10 days at 28°C. Test fungi were then point inoculated 2.5 cm away from the edge of the bacterial colony, alongside control plates without Actinobacteria. Bioassays were incubated at 28°C and inspected daily until the fungus in the

fungus-only control plates had grown to completely to the opposite side of the plate. Each bioassay plate was photographed and their zones of inhibition (ZOI) were measured.

2.3.6 Cellulose degradation assay

The same nineteen isolates described above were also tested for their ability to degrade cellulose. Isolates were inoculated in duplicate into tubes containing 5 ml of autoclaved M63 medium and 5 mL of trace elements solution (SPV-4) [65]. A strip of 1 cm x 10 cm Whatman #1 filter paper was added to each tube as a cellulose source. Tubes were incubated with constant agitation at 331 RPM at 28°C for a maximum of 22 days and examined daily for visual evidence of filter paper degradation. An additional 25 isolates having the same morphology on YMEA as *Streptomyces* sp KhCrAH244 were subsequently assayed for cellulose degradation, and their 16S rRNA genes sequenced.

2.4 Results

In total, we screened for Actinobacteria in 96 samples from *P. penetrator/Tachigali*, 99 samples from *P. phylax/L. africana*, and 44 samples from *C. margaritae/K. hispida*. From all three ant-plant mutualisms, Actinobacteria were isolated from all types of insect samples, including workers, pupae, larvae, queens, and alates (Table 2.2). Actinobacteria were cultured from all fungal patches from *P. phylax/L. africana* including all three storage techniques, and from *P. penetrator/Tachigali* fungal patches stored in PBS (Table 2.2). In total, Actinobacteria were isolated from 27% of samples from *P. penetrator/Tachigali*, 55% of *P. phylax/L. africana* samples and 100% of *C. margaritae/K. hispida* samples (Table 2.1). Average putative Actinobacteria CFUs per sample varied across the three systems; *P. penetrator/Tachigali* samples contained 1.1 CFUs, *P. phylax/L. africana* samples contained 3.6 CFUs, and *C. margaritae/K. hispida* samples contained 9.7 CFUs (Table 2.1). *P. phylax* alates, and *P.*

penetrator and *C. margaritae* workers yielded the most CFUs, 8.3, 3.1, and 10.4, respectively (Table 2.2).

A portion of the isolates morphologically identified as Actinobacteria were chosen for 16S rRNA gene sequencing: 60 out of 109 isolates from *P. penetrator*/*Tachigali*, 104 out of 353 isolates from *P. phylax*/*L. africana*, and 121 out of 399 isolates from *C. margaritae*/*K. hispida*. The remaining isolates were not sequenced or used for assays. After removing non-Actinobacteria, low quality, and duplicate sequences (see Supplemental Table 2.1 for list of duplicate sequences), 46 *P. penetrator*/*Tachigali*, 69 *P. phylax*/*L. africana*, and 107 *C. margaritae*/*K. hispida* sequences were used for phylogenetic analysis (Figure 2.1, Supplemental Table 2.1), revealing five major clades of Actinobacteria isolated from these ant-plant associations. Clade I comprises 36, 14, and 68 of the actinobacterial 16S rRNA gene sequences from *P. penetrator*/*Tachigali*, *P. phylax*/*L. africana*, and *C. margaritae*/*K. hispida*, respectively, and form a clade with the *Streptomyces albidoflavus* species group (Figure 2.1). Clade I contains the majority of ant-plant sequences in this tree and is the only well-supported clade associated with isolates from all three sampled ant-plant mutualisms. Clade II includes 10 isolates from *P. phylax*/*L. africana,* 21 from *C. margaritae*/*K. hispida,* the *S. griseus* species group, and the insect isolates *S. griseus* XylebKG1 (isolated from an ambrosia beetle)*, Streptomyces* sp AV109 (from a fungus-farming termite), and *Streptomyces* sp SA3 actE (from *S. noctilio*) [36, 41, 43]. Clade III comprises 2 isolates from *P. phylax*/*L. africana*, 17 isolates from *C. margaritae*/*K. hispida* and the type strain *S. drozdowiczii*. Clade IV comprises 10 isolates from *P. phylax*/*L. africana*, and the type strains *S. recifensis*, *S. griseoluteus* and *S. seoulensis*. Lastly, clade V contains 20 *P. phylax*/*L. africana* isolates and is sister to *Nocardioidies luteus* and *N. albus*. Clades I-V are supported by bootstrap values of 70%, 80%, 94%, 52%, and 93%, respectively. The remaining

14 isolates represent singletons, those infrequently isolated, and minor clades. Only clade II contained Actinobacteria isolated from other insects (as described above), as did a minor clade containing 2 isolates from *P. phylax*/*L. africana* that were related to isolates from *D. frontalis* and *S. noctilio* [43, 53].

All 19 strains tested for antifungal activity decreased the growth of at least one of the tested fungi, but only eight isolates exhibited distinct ZOI (Table 2.3). *Streptomyces* sp PsTaAH124, *Streptomyces* sp PsTaAH130, and *Streptomyces* sp LaPpAH322 inhibited all four fungi tested, while *Streptomyces* sp KhCrAH320 inhibited *M. anisopliae* and *T. reesei*. *Streptomyces* sp PsTaAH5 and *Streptomyces* sp KhCrAH316 inhibited only *T. reesei*, while *Streptomyces* sp KhCrAH308 and *Streptomyces* sp LaPpAH224 inhibited only *M. anisopliae*. Of the tested fungi, *M. anisopliae* was inhibited the least with only one of four replicates from six strains exhibiting a clear ZOI, while *T. reesei* was inhibited in all replicates from six isolates.

Only one of the nineteen original isolates tested, strain *Streptomyces* sp KhCrAH244, degraded cellulose averaging 9 days until filter paper breakage (Figure 2.2). Of the eighteen additional isolates with the same culture morphology as *Streptomyces* sp KhCrAH244 tested to further confirm this phenotype, nine isolates degraded filter paper, as did all of the seven additional isolates from clade III. Only isolates from clade III degraded cellulose, taking between 7 and 22 days (Figure 2.2). On average, isolates capable of degrading cellulose took 9.26 days to break the filter paper.

Colony morphology was found to be a poor predictor of phylogenetic placement, especially for *Streptomyces* strains. This was particularly evident in the additional isolates screened for cellulose degradation, described above. Of the eighteen, only nine were identified as members of clade III after 16S rRNA gene sequencing. The eleven remaining strains, despite

having similar colony morphology to *Streptomyces* sp KhCrAH244, did not degrade cellulose and were not members of clade III.

2.5 Discussion

We investigated three ant-plant mutualisms as niches for Actinobacteria and their potential roles as nutritional or defensive symbionts within these symbioses. Our results show that Actinobacteria with the ability to degrade cellulose and inhibit fungi were obtained from the following: 1) three phylogenetically disparate ant species, 2) from two geographically distant locations, 3) from all three ant-plant symbioses explored, and 4) from all types of samples of each system examined. These Actinobacteria may play a variety of roles including aiding in acquisition of nutrients and mediating host/niche defense.

Isolation of 861 putative Actinobacteria from *P. penetrator*/*Tachigali*, *P. phylax*/*L. africana*, and *C. margaritae*/*K. hispida* suggests that these symbiotic systems are indeed niches for Actinobacteria. Indeed, all *C. margaritae*/*K. hispida* samples contained culturable Actinobacteria while a portion of *P. penetrator*/*Tachigali* and *P. phylax*/*L. africana* contained culturable Actinobacteria (Tables 2.1 and 2.2). Every life stage across all three systems were also associated with Actinobacteria, including workers, alates, larvae, pupae, and queens (Table 2.2). The range of Actinobacteria CFUs found here is consistent with those found in other insect-Actinobacteria association studies. *S. noctilio* were found to have an average of 11.5 CFUs, which dropped to 3.12 when two outliers were removed [43]. Similarly, most *D. frontalis* were associated with at least one CFU of *Streptomyces*, with an average of 7.69 CFUs across 110 beetles sampled [53]. Additionally, solitary mud daubers (*Sceliphron caementarium* and *Chalybion californicum*) were found to average 0.78-3.1 Actinobacteria morphotypes based on whole insect, or specific body parts [44].

Isolation of Actinobacteria from fungal patches was more inconsistent than from other material types, though this may have been partially a factor of storage technique. All three storage techniques for the fungal patches from *P. phylax/L. africana* yielded Actinobacteria, while only fungal patches stored in PBS from *P. penetrator/Tachigali* did. Similarly, Seipke *et al*. also surmised that storage and transport conditions likely biased their results in looking for Actinobacteria associated with *Allomerus* [42]. The possibility remains that fungal patches from *P. penetrator/Tachigali* were more fastidious and did not survive transport in glycerol or with no storage solution.

Our results suggest that the three symbioses explored here associate with only a few species of culturable Actinobacteria. Clades I-IV were identified as *Streptomyces* while clade V was identified as *Nocardioides* (Figure 2.1). Clade I was the only clade isolated from all three ant-plant symbioses explored, and could represent a *Streptomyces* symbiont important to the tripartite symbiosis between ants, plants, and fungi, regardless of geographic location [11]. Two clades (II and III) were associated with both of the systems from Cameroon, while the remaining two clades (IV and V) were specific for *P. phylax/L. africana*. This arrangement of clade association could indicate geographic (clades II and III), host (clades IV and V), and ant-plant-niche specificity (clade I).

With one exception, clade II, our isolates represent clades that were not previously known to include strains from other insects. Interestingly, other insect isolates in clade II are also from insect systems that associate with fungus, including an ambrosia beetle, a fungus-farming termite, and *S. noctilio* (Figure 2.1) [36, 41, 43]. However, because clade II groups with the *S. griseus* species group, a group of *Streptomyces* that has been isolated from many different niches, this may be more indicative of the ubiquity, and lack of 16S rRNA gene resolution, of

this species group [66]. Likewise, other insect isolates might clade with our strains, but the lack of near full-length 16S rRNA gene sequences from those isolates in public databases precluded their inclusion in our phylogenetic tree. Including those shorter sequences would likely have required trimming our sequences to a length where most of the phylogenetic resolution would have been lost. Additionally, while other genera from the phylum Actinobacteria are represented in our dataset, isolates were predominately identified as *Streptomyces*. Frequent isolation of *Streptomyces* may be due to bias in culturing technique and media choice, so may not be indicative of the absence of other types of Actinobacteria from these systems.

A wide range of antifungal activity was found in bioassays challenging potentially pathogenic fungi with phylogenetically diverse Actinobacteria isolated from all three systems (Table 2.3). All of the tested Actinobacteria isolates exhibited some level of antifungal capacity, with the *Streptomyces* strains performing better than the *Nocardioides* isolates. However, only eight isolates produced measurable ZOIs (Table 2.3). *T. reesei*, a generalist fungal pathogen, was the most inhibited fungus, with three isolates from clade I and three singletons producing large ZOIs. *M. anisopliae*, the only strict insect pathogen used here, was the least inhibited, with one isolate each from clades I and II, and four singletons, producing small ZOIs. While *in vitro* activity is not indicative of *in vivo* activity, *Streptomyces* are particularly well known producers of antimicrobials and the activity of these strains likely differs between artificial bioassays in Petri dishes and challenges with pathogens in the host system [67]. Additionally, *in vivo* activity can be difficult to demonstrate. To date, the only reports showing *in vivo* antifungal activity of Actinobacteria in insects include attine ants and beewolves [33, 68]. Lastly, while *M. anisopliae*

is a known entomopathogen, the fungi tested here are not specific to these ant-plant systems, as no work has been done on pathogens, fungal or otherwise, in these systems.

Unlike antifungal activity, the ability to degrade cellulose was found in only one clade of isolates from the ant-plant systems from Cameroon (clade III; Figures 2.1 and 2.2). As these ants are arboreal, these cellulose-degrading bacteria could be playing any of several roles. Cellulose-degrading bacteria have been isolated from other plant associated insects and have been shown to contribute nutritionally to their host [43, 46–49]. While the fungi in these ant-plant systems was not found to penetrate the plant tissue inside the domatia [11], cellulose-degrading bacteria could still co-occur with these ants and may be releasing by-products from cellulose degradation that are used by the ants. There is also the possibility that these cellulose degrading isolates could represent pathogens to a component of the Cameroonian systems, taking advantage of the access to a new niche opened by the ants to internal plant structures.

Taxonomically diverse insects have convergently evolved similar solutions for nutrient acquisition by associating with fungal symbionts [18]. Likewise, insects have also evolved similar mechanisms for the protection of themselves, their brood, and their food source [69]. Many parallels can be drawn between the recently recognized fungal cultivating ant-plant mutualisms, other Hymenoptera, and other fungus-farming insects. Attine ants are perhaps the most explored and understood in this regard, hosting unique clades of *Pseudonocardia* that produce antifungal compounds that aid in the protection of the fungal cultivar [32]. Likewise, female beewolves (Hymenoptera) apply '*Candidatus* Streptomyces philanthi' to brood cells to aid in protection of their offspring [37]. Consistent co-occurrence of isolates from clade I, and others, suggests that the ant-plant-fungus systems explored here associate with more than one clade of Actinobacteria and may also use the metabolic potential found in this phylum of

bacteria. Our work describes the isolation of Actinobacteria from *P. penetrator/Tachigali*, *P. phylax/L. africana*, and *C. margaritae/K. hispida,* and demonstrates the potential roles for these bacteria. Future work is needed to determine if the strains isolated here are novel Actinobacteria species, and to determine if these bioactive Actinobacteria isolates serve the roles described here *in vivo*.

2.6 Acknowledgements

2.7 References

1.   Rico-Gray V, Oliveira PS (2007) The Ecology and Evolution of Ant-Plant Interactions. The University of Chicago Press, Chicago.

2.   Beattie A (1989) Myrmecotrophy: Plants fed by ants. Trends in Ecology & Evolution 4: 172–176

3.   Sagers CL, Ginger SM, Evans RD (2000) Carbon and nitrogen isotopes trace nutrient exchange in an ant-plant mutualism. Oecologia 123: 582–586

4.   Fischer RC, Wanek W, Richter A, Mayer V (2003) Do ants feed plants? A 15N labelling study of nitrogen fluxes from ants to plants in the mutualism of *Pheidole* and *Piper*. Journal of Ecology 91: 126 – 134

5.   Janzen DH (1974) Epiphytic myrmecophytes in Sarawak: mutualism through the feeding of plants by ants. Biotropica 6: 237–259

6.   Rickson FR (1979) Absorption of animal tissue breakdown products into a plant stem-the feeding of a plant by ants. American Journal of Botany 66: 87–90. doi:10.2307/2442629

7.   Treseder KK, Davidson DW, Ehleringer JR (1995) Absorption of ant-provided carbon dioxide and nitrogen by a tropical epiphyte. Nature 375: 137–139

8.   Solano PJ, Dejean A (2004) Ant-fed plants: comparison between three geophytic myrmecophytes. Biological Journal of the Linnean Society 83: 433–439. doi:10.1111/j.1095-8312.2004.00381.x

9.   Watkins JE, Cardelús CL, Mack MC (2008) Ants mediate nitrogen relations of an epiphytic fern. The New Phytologist 180: 5–8. doi:10.1111/j.1469-8137.2008.02606.x

10.   Davidson DW, Mckey D (1993) The evolutionary ecology of symbiotic ant-plant relationships. Journal of Hymenoptera Research 2: 13–83

11.   Defossez E et al. (2009) Ant-plants and fungi: a new threeway symbiosis. New Phytologist 182: 942–949

12.   Miehe H (1911) Untersuchungen über die javanische Myrmecodia. Abhandlungen der Mathematisch-Physischen Klasse der Königlich-Sächsischen Gesellschaft der Wissenschaften 312–361

13.   Voglmayr H et al. (2011) The diversity of ant-associated black yeasts: Insights into a newly discovered world of symbiotic interactions. Fungal Biology 115: 1077–1091

14.   Blatrix R et al. (2012) Plant-ants use symbiotic fungi as a food source: new insight into the nutritional ecology of ant–plant interactions. Proceedings of the Royal Society - Biological Sciences 279: 3940–3947. doi:10.1098/rspb.2012.1403

15.   Defossez E, Djiéto-Lordon C, McKey D, Selosse M-A, Blatrix R (2010) Plant-ants feed their host plant, but above all a fungal symbiont to recycle nitrogen. Proceedings of the Royal Society - Biological Sciences 278: 1419–1426. doi:10.1098/rspb.2010.1884

16.   Blatrix R, Bouamer S, Morand S, Selosee M-A (2009) Ant-plant mutualisms should be viewed as symbiotic communities. Plant Signaling & Behavior 4: 554–556. doi:10.1111/j.1469-8137.2009.02793.x.publications

17.   Lauth J, Ruiz-González MX, Orivel J (2011) New findings in insect fungiculture. Communicative & Integrative Biology 4: 728–730. doi:10.4161/cib.4.6.17590

18.   Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR (2005) The evolution of agriculture in insects. Annual Review of Ecology, Evolution, and Systematics 36: 563–595. doi:10.1146/annurev.ecolsys.36.102003.152626

19.   Mikheyev AS, Mueller UG, Abbot P (2010) Comparative dating of attine ant and lepiotaceous cultivar phylogenies reveals coevolutionary synchrony and discord. The American Naturalist 175: E126–E133. doi:10.1086/652472

20.   Six DL, Klepzig KD (2004) *Dendroctonus* bark beetles as model systems for studies on symbiosis. Symbiosis 37: 207–232

21.   Farrell BD et al. (2001) The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). Evolution 55: 2011–2027

22.   Toki W, Tanahashi M, Togashi K, Fukatsu T (2012) Fungal farming in a non-social beetle. PLoS ONE 7: e41893. doi:10.1371/journal.pone.0041893

23.   Rohfritsch O (2008) Plants, gall midges, and fungi: a three-component system. Entomologia Experimentalis et Applicata 128: 208–216. doi:10.1111/j.1570-7458.2008.00726.x

24.   Dejean A, Solano PJ, Ayroles J, Corbara B, Orivel JJ (2005) Arboreal ants build traps to capture prey. Nature 434: 973

25. Mueller UG, Schultz TR, Currie CR, Adams RMM, Malloch D (2001) The origin of the attine ant-fungus. Quarterly Review of Biology 76: 169–197

26. Hajek AE, Nielsen C, Kepler RM, Long SJ, Castrillo L (2013) Fidelity among sirex woodwasps and their fungal symbionts. Microbial Ecology 65: 753–762. doi:10.1007/s00248-013-0218-z

27. Li X, Guo W, Ding J (2012) Mycangial fungus benefits the development of a leaf-rolling weevil, *Euops chinesis*. Journal of Insect Physiology 58: 867–873. doi:10.1016/j.jinsphys.2012.03.011

28. Klepzig KD, Wilkens RT (1997) Competitive interactions among symbiotic fungi of the southern pine beetle. Applied and Environmental Microbiology 63: 621–627

29. Leroy C et al. (2011) Trophic mediation by a fungus in an ant-plant mutualism. Journal of Ecology 99: 583–590. doi:10.1111/j.1365-2745.2010.01763.x

30. Currie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens. Proceedings of the National Academy of Science 96: 7998–8002

31. Currie CR (2001) Prevalence and impact of a virulent parasite on a tripartite mutualism. Oecologia 128: 99–106. doi:10.1007/s004420100630

32. Currie CR, Scott JA, Summerbell RA, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398: 701–704

33. Currie CR, Bot ANM, Boomsma JJ (2003) Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. Oikos 101: 91–102

34. Fernández-Marín H, Zimmerman JK, Rehner SA, Wcislo WT (2006) Active use of the metapleural glands by ants in controlling fungal infection. Proceedings of the Royal Society - Biological Sciences 273: 1689–1695. doi:10.1098/rspb.2006.3492

35. Currie CR, Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants. Proceedings of the Royal Society - Biological Sciences 268: 1033–1039. doi:10.1098/rspb.2001.1605

36. Visser AA, Nobre T, Currie CR, Aanen DK, Poulsen M (2012) Exploring the potential for Actinobacteria as defensive symbionts in fungus-growing termites. Microbial Ecology 63: 975–985. doi:10.1007/s00248-011-9987-4

37. Kaltenpoth M, Schmitt T, Polidori C, Koedam D, Strohm E (2010) Symbiotic streptomycetes in antennal glands of the South American digger wasp genus *Trachypus* (Hymenoptera, Crabronidae). Physiological Entomology 35: 196–200. doi:10.1111/j.1365-3032.2010.00729.x

38. Kaltenpoth M et al. (2006) "Candidatus Streptomyces philanthi", an endosymbiotic streptomycete in the antennae of *Philanthus* digger wasps. International Journal of Systematic and Evolutionary Microbiology 56: 1403–1411

39. Kaltenpoth M, Yildirim E, Gürbüz MF, Herzner G, Strohm E (2012) Refining the roots of the beewolf-*Streptomyces* symbiosis: antennal symbionts in the rare genus *Philanthinus* (Hymenoptera, Crabronidae). Applied and Environmental Microbiology 78: 822–827. doi:10.1128/AEM.06809-11

40.   Hulcr J et al. (2011) Presence and diversity of *Streptomyces* in *Dendroctonus* and sympatric bark beetle galleries across North America. Microbial Ecology 61: 759–768. doi:10.1007/s00248-010-9797-0

41.   Grubbs KJ et al. (2011) Genome sequence of *Streptomyces griseus* Strain XyelbKG-1, an ambrosia beetle-associated Actinomycete. Journal of Bacteriology 193: 2890–2891. doi:10.1128/JB.00330-11

42.   Seipke RF et al. (2012) Fungus-growing *Allomerus* ants are associated with antibiotic-producing actinobacteria. Antonie van Leeuwenhoek 101: 443–447. doi:10.1007/s10482-011-9621-y

43.   Adams AS et al. (2011) Cellulose-degrading bacteria associated with the invasive woodwasp *Sirex noctilio*. The ISME Journal 5: 1323–1331. doi:10.1038/ismej.2011.14

44.   Poulsen M, Oh D-C, Clardy J, Currie CR (2011) Chemical analyses of wasp-associated *Streptomyces* bacteria reveal a prolific potential for natural products discovery. PLoS ONE 6: e16763. doi:10.1371/journal.pone.0016763

45.   Miao V, Davies J (2010) Actinobacteria: the good, the bad, and the ugly. Antonie van Leeuwenhoek 98: 143–150. doi:10.1007/s10482-010-9440-6

46.   Vasanthakumar A, Handelsman J, Schloss PD, Bauer LS, Raffa KF (2008) Gut microbiota of an invasive subcortical beetle, *Agrilus planipennis* Fairmaire, across various life stages. Environmental Entomology 37: 1344–1353

47.   Pasti MB, Pometto III AL, Nuti MP, Crawford DL (1990) Lignin-solubilizing ability of Actinomycetes isolated from termite (Termitidae) Gut. Applied and Environmental Microbiology 56: 2213–2218

48.   Pasti MB, Belli ML (1985) Cellulolytic activity of Actinomycetes isolated from termites (Termitidae) gut. FEMS Microbiology Letters 26: 107–112. doi:10.1111/j.1574-6968.1985.tb01574.x

49.   Salem H, Kreutzer E, Sudakaran S, Kaltenpoth M (2013) Actinobacteria as essential symbionts in firebugs and cotton stainers (Hemiptera, Pyrrhocoridae). Environmental Microbiology 15: 1956–1968. doi:10.1111/1462-2920.12001

50.   Hillis DM, Marble BK, Larson A, David SK, Zimmer EA (1996) Nucleic Acids IV: Sequencing and Cloning. In: Hillis DM, Moritz C, Mable BK (eds) Molecular Systematics. 2nd ed. Sinauer Associates, Inc., Sunderland, MA, pp 321–381

51.   Lane DJ (1991) 16S/23S rRNA Sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, Ltd, New York, pp 115–175

52.   Cole JR et al. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research 37: D141–145. doi:10.1093/nar/gkn879

53.   Scott JJ et al. (2008) Bacterial protection of beetle-fungus mutualism. Science 322: 63. doi:10.1126/science.1160423

54.   Cafaro MJ, Currie CR (2005) Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. Canadian Journal of Microbiology 51: 441–446. doi:10.1139/W05-023

55.   Benson DA et al. (2013) GenBank. Nucleic Acids Research 41: D36–42. doi:10.1093/nar/gks1195

56.   Pruitt KD, Tatusova T, Brown GR, Maglott DR (2012) NCBI Reference Sequences (RefSeq): current status, new features and genome annotation policy. Nucleic Acids Research 40: D130–135. doi:10.1093/nar/gkr1079

57.   Gao B, Gupta RS (2012) Phylogenetic framework and molecular signatures for the main clades of the phylum Actinobacteria. Microbiology and Molecular Biology Reviews 76: 66–112. doi:10.1128/MMBR.05011-11

58.   Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30: 772–780. doi:10.1093/molbev/mst010

59.   Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690. doi:10.1093/bioinformatics/btl446

60.   Stamatakis A, Hoover P, Rougemont J (2008) A Rapid bootstrap algorithm for the RAxML web servers. Systematic Biology 57: 758–771. doi:10.1080/10635150802429642

61.   Letunic I, Bork P (2007) Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics 23: 127–128. doi:10.1093/bioinformatics/btl529

62.   Letunic I, Bork P (2011) Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. Nucleic Acids Research 39: W475–478. doi:10.1093/nar/gkr201

63.   Cafaro MJ et al. (2011) Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. Proceedings of the Royal Society - Biological Sciences 278: 1814–1822. doi:10.1098/rspb.2010.2118

64.   Poulsen M et al. (2009) Variation in *Pseudonocardia* antibiotic defence helps govern parasite-induced morbidity in *Acromyrmex* leaf-cutting ants. Environmental Microbiology Reports 2: 534–540. doi:10.1111/j.1758-2229.2009.00098.x

65.   Takasuka TE, Book AJ, Lewin GR, Currie CR, Fox BG (2013) Aerobic deconstruction of cellulosic biomass by an insect-associated *Streptomyces*. Scientific Reports 3: 1–10. doi:10.1038/srep01030

66.   Guo Y, Zheng W, Rong X, Huang Y (2008) A multilocus phylogeny of the *Streptomyces griseus* 16S rRNA gene clade: use of multilocus sequence analysis for streptomycete systematics. International Journal of Systematic and Evolutionary Microbiology 58: 149–159. doi:10.1099/ijs.0.65224-0

67.   Waksman SA, Schatz A, Reynolds DM (2010) Production of antibiotic substances by actinomycetes. Annals of the New York Academy of Sciences 1213: 112–124. doi:10.1111/j.1749-6632.2010.05861.x

68.   Kaltenpoth M, Go W, Herzner G, Strohm E, Gottler W (2005) Symbiotic bacteria protect wasp larvae from fungal infestation. Current Biology 15: 475–479. doi:10.1016/j

69.   Kaltenpoth M (2009) Actinobacteria as mutualists: general healthcare for insects? Trends In Microbiology 17: 529–535. doi:10.1016/j.tim.2009.09.006

Table 2.1. General collection and bacterial isolate information.

Ant/Plant	*P. penetrator/* *Tachigali* sp.	*P. phylax/* *L. africana*	*C. margaritae/* *K. hispida*
Country of Origin	French Guiana	Cameroon	Cameroon
Total number of samples	96	99	41
Samples with Actinobacteria (percent)	26 (27%)	54 (55%)	41 (100%)
Average Actinobacteria CFUs per sample[a]	1.14	3.57	9.73
Total putative Actinobacteria isolates obtained	109	353	399
Total isolates 16S rRNA gene sequenced	66	117	128
Total isolates identified as Actinobacteria	60	104	121
Total sequences represented in Fig. 2.1	45	69	105

[a]Average putative Actinobacteria CFUs per sample, including samples that produced no Actinobacteria colonies on culture media. See Table 2.2 for a breakdown by sample type.

Table 2.2. Summary of sampled ant-plants and Actinobacteria isolation rate.

Ant/Plant	*P. penetrator*/*Tachigali* sp.			*P. phylax*/*L. africana*			*C. margaritae*/*K. hispida*		
Sample	# samples	CFUs	Avg CFUs[a]	# samples	CFUs	Avg CFUs[a]	# samples	CFUs	Avg CFUs[a]
Workers	23	71	3.09	61	162	2.66	34	346	10.18
Alates	-	-	-	7	58	8.29	-	-	-
Pupae	9	18	2.00	5	21	4.20	3	32	10.67
Larvae	25	3	0.12	12	65	5.42	2	3	1.50
Queens	3	2	0.67	-	-	-	-	-	-
Scale Insects	7	2	0.29	-	-	-	2	18	9.00
Fungal patch – PBS	10	13	1.3	5	19	3.8	-	-	-
Fungal patch – Gly[b]	9	0	0	5	14	2.8	-	-	-
Fungal patch – dry	10	0	0	4	13	3.25	-	-	-

[a] Average Actinobacteria CFUs per sample type including samples that yielded no Actinobacteria colonies on culture media. [b] Glycerol.

Table 2.3. Bioassay evaluation of the antifungal activity of the Actinobacteria isolates from ant-plant symbioses.

	Clade	*A. niger*	*F. oxysporum*	*M. anisopliae*	*T. reesei*
Streptomyces sp PsTaAH5	I				black
Streptomyces sp KhCrAH316	I				black
Streptomyces sp KhCrAH320	I			medium gray	black
Streptomyces sp KhCrAH246	II				
Streptomyces sp KhCrAH308	II			light gray	
Streptomyces sp KhCrAH244	III				
Streptomyces sp LaPpAH163	IV				
Streptomyces sp LaPpAH180	IV				
Nocardioides sp LaPpAH12	V				
Nocardioides sp LaPpAH20	V				
Nocardioides sp LaPpAH209	V				
Nocardioides sp LaPpAH242	V				
Streptomyces sp PsTaAH124	na	black		light gray	black
Streptomyces sp PsTaAH130	na	dark gray	black	light gray	black
Streptomyces sp PsTaAH137	na				
Streptomyces sp PsTaAH146	na				
Actinobacteria sp LaPpAH190	na				
Streptomyces sp LaPpAH224	na			light gray	
Streptomyces sp LaPpAH322	na	dark gray	black	light gray	black

Antifungal properties of Actinobacteria in petri plate bioassays against four fungi. Boxes represent average ZOI (n=4) of a given pairing and different symbols indicate the degree of inhibition. White no inhibition, light gray 0.01-1.0 mm, medium gray 2.01-3.0 mm, dark gray 3.01-4.0 mm, black >4.01 mm. Major clade designation, if there is one, is included in the column next to each isolate name (na: no major clade).
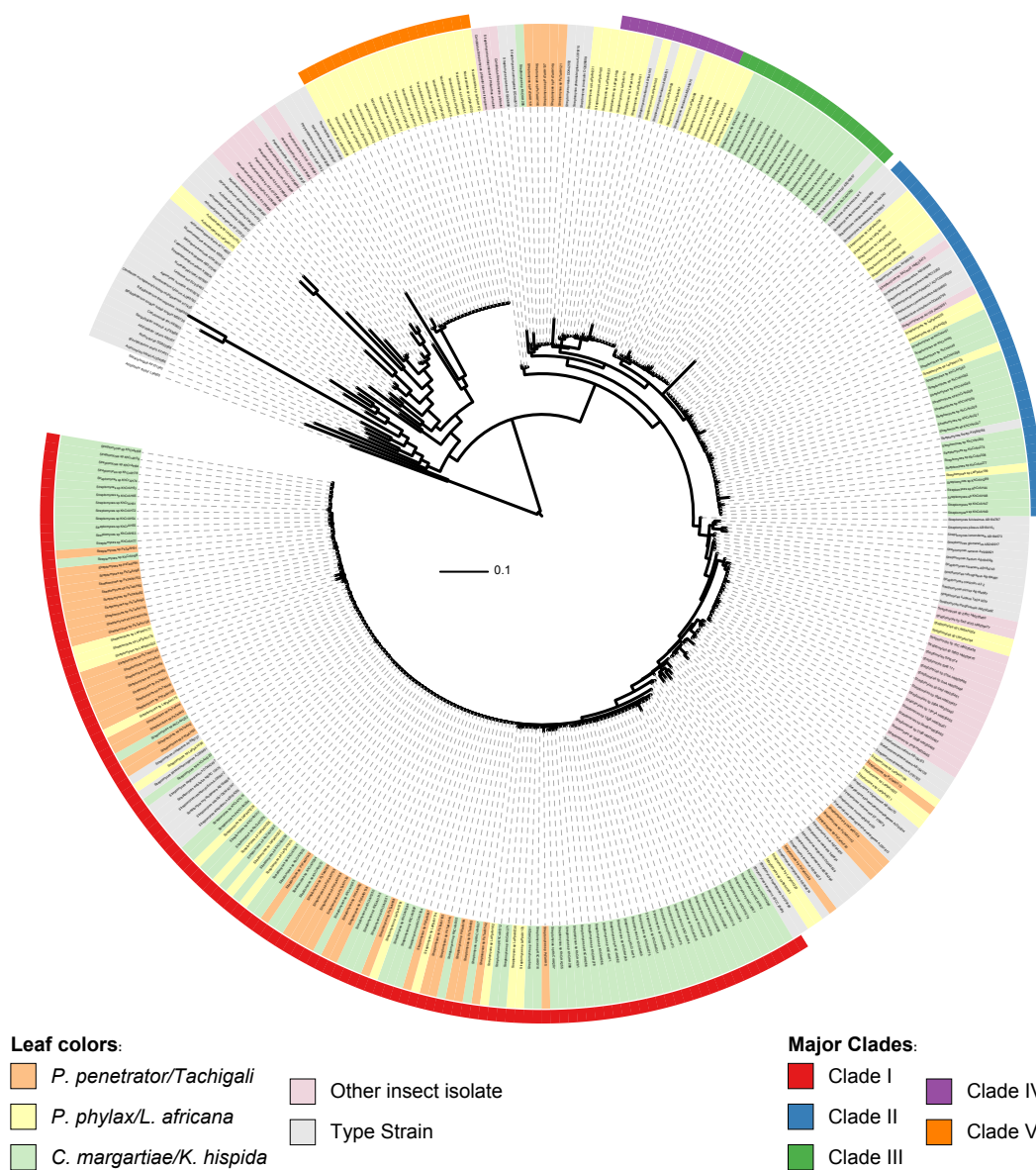
Figure 2.1. Phylogenetic tree of Actinobacteria isolated from three ant-plant systems, related type strains, and other Actinobacteria isolated from arthropods. Leaves are color-coded based on isolate origin, including *P. penetrator/Tachigali* (orange), *P. phylax/L. africana* (yellow), *C. margaritae/K. hispida* (green), other insects (pink), and type strains (grey). The outer color stripes represent the five major clades of isolates associated with the three ant-plant systems explored here, including type strains and other insect isolates that fall within these clades. From left to right these include clade I (red), clade II (blue), clade III (green), clade IV (purple), and clade V (orange).
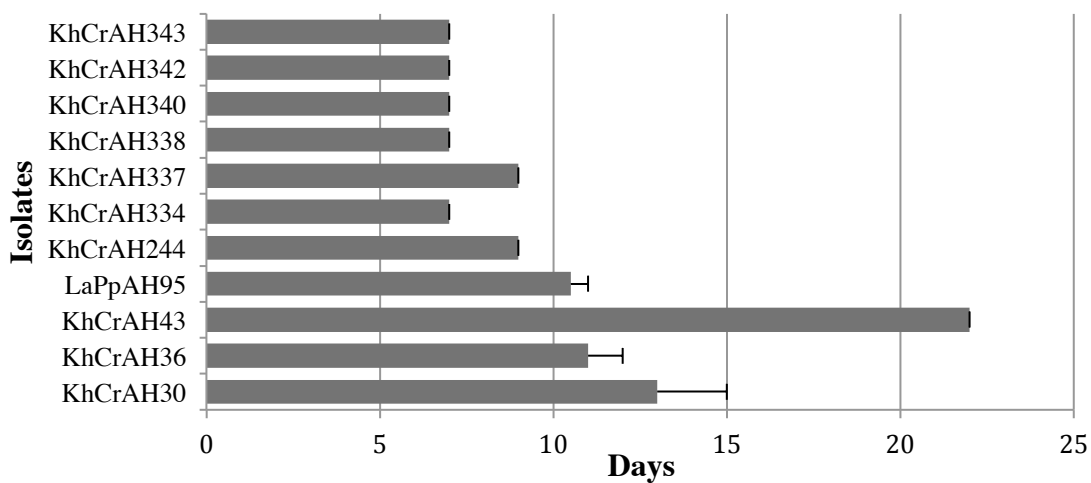
Figure 2.2. Average number of days until evidence of filter paper degradation. Forty-four isolates were tested for their ability to degrade filter paper; isolates that did not degrade filter paper are not included here. Likewise, isolates that did degrade cellulose but were found to be duplicates from the same sample by 16S rRNA gene sequencing were removed. All the isolates represented are *Streptomyces* from clade III. Error bars represent standard error.

Supplemental Table 2.1A. Isolates included in Figure 2.1. GenBank accession numbers will be added after sequences are submitted.

Isolate Name	Full name	Component	Clade	Fungal bioassay	Cellulase assay	GenBank ID
C. margaritae/K. hispida from Cameroon						
Streptomyces sp KhCrAH30	30.KhCrWA7-3-5	worker	III		positive	
Streptomyces sp KhCrAH33	33.KhCrWA13-1-7	worker	III			
Streptomyces sp KhCrAH35	35.KhCrWA15-1-3	worker	III			
Streptomyces sp KhCrAH36	36.KhCrWA17-3-3	worker	III		positive	
Streptomyces sp KhCrAH40	40.KhCrWA23-3-3	worker	III			
Streptomyces sp KhCrAH41	41.KhCrWD28-1-5	worker	III			
Streptomyces sp KhCrAH42	42.KhCrWD31-2-8	worker	III			
Streptomyces sp KhCrAH43	43.KhCrWD37-1-1	worker	III		positive	
Streptomyces sp KhCrAH44	44.KhCrWA5-3-3	worker	II			
Streptomyces sp KhCrAH45	45.KhCrWA6-2-1	worker	II			
Streptomyces sp KhCrAH46	46.KhCrWA7-2-1	worker	II			
Streptomyces sp KhCrAH47	47.KhCrWA12-3-3	worker	II			
Streptomyces sp KhCrAH48	48.KhCrWA14-3-4	worker	II			
Streptomyces sp KhCrAH49	49.KhCrWA18-3-5	worker	II			
Streptomyces sp KhCrAH51	51.KhCrWA23-2-1	worker	II			
Streptomyces sp KhCrAH54	54.KhCrWA5-1-2	worker	I			
Streptomyces sp KhCrAH55	55.KhCrWA7-3-8	worker	I			
Streptomyces sp KhCrAH57	57.KhCrWA11-3-2	worker	I			
Streptomyces sp KhCrAH58	58.KhCrWA13-3-5	worker	I			
Streptomyces sp KhCrAH59	59.KhCrWA14-3-2	worker	I			
Streptomyces sp KhCrAH61	61.KhCrWA17-3-4	worker	I			
Streptomyces sp KhCrAH62	62.KhCrWA18-3-4	worker	I			
Streptomyces sp KhCrAH63	63.KhCrWA19-3-2	worker	I			
Streptomyces sp KhCrAH64	64.KhCrWA20-1-4	worker	I			
Streptomyces sp KhCrAH65	65.KhCrWA22-2-1	worker	I			
Streptomyces sp KhCrAH67	67.KhCrWD27-2-3	worker	I			

Streptomyces sp KhCrAH68	68.KhCrWD28-1-6	worker	I		
Streptomyces sp KhCrAH69	69.KhCrWD29-2-6	worker	I		
Streptomyces sp KhCrAH70	70.KhCrWD30-1-5	worker	I		
Streptomyces sp KhCrAH71	71.KhCrWD31-2-7	worker	I		
Streptomyces sp KhCrAH72	72.KhCrWD33-1-5	worker	I		
Streptomyces sp KhCrAH74	74.KhCrWD35-2-3	worker	I		
Streptomyces sp KhCrAH75	75.KhCrWD37-2-6	worker	I		
Streptomyces sp KhCrAH76	76.KhCrWD38-2-2	worker	I		
Streptomyces sp KhCrAH77	77.KhCrP40-2-8	pupae	I		
Streptomyces sp KhCrAH78	78.KhCrP41-1-2	pupae	I		
Streptomyces sp KhCrAH79	79.KhCrWD51-1-4	worker	I		
Streptomyces sp KhCrAH243	243.KhCrL43-1-3	larvae	I		
Streptomyces sp KhCrAH244	244.KhCrP39-1-1A	pupae	III	negative	positive
Streptomyces sp KhCrAH246	246.KhCrP40-1-1	pupae	II	negative	negative
Streptomyces sp KhCrAH247	247.KhCrP40-1-3	pupae	II		
Streptomyces sp KhCrAH249	249.KhCrP40-1-8	pupae	I		
Streptomyces sp KhCrAH250	250.KhCrP40-2-7	pupae	I		
Streptomyces sp KhCrAH251	251.KhCrS44-1-1	scale insect	I		
Streptomyces sp KhCrAH253	253.KhCrS50-2-6	scale insect	I		
Streptomyces sp KhCrAH254	254.KhCrWA5-1-1	worker	II		
Streptomyces sp KhCrAH255	255.KhCrWA5-1-4	worker	I		
Streptomyces sp KhCrAH256	256.KhCrWA5-1-5	worker	III		
Streptomyces sp KhCrAH257	257.KhCrWA5-3-2	worker	I		
Streptomyces sp KhCrAH258	258.KhCrWA6-1-1	worker	I		
Streptomyces sp KhCrAH259	259.KhCrWA6-3-5	worker	II		
Streptomyces sp KhCrAH260	260.KhCrWA7-2-2	worker	I		
Streptomyces sp KhCrAH261	261.KhCrWA7-2-3A	worker	I		
Streptomyces sp KhCrAH263	263.KhCrWA7-3-9	worker	I		
Streptomyces sp KhCrAH264	264.KhCrWA9-1-2	worker	I		
Streptomyces sp KhCrAH265	265.KhCrWA9-1-3	worker	I		

Streptomyces sp KhCrAH266	266.KhCrWA10-2-5	worker	I
Streptomyces sp KhCrAH267	267.KhCrWA11-3-3	worker	I
Streptomyces sp KhCrAH269	269.KhCrWA13-1-4	worker	II
Streptomyces sp KhCrAH270	270.KhCrWA13-2-3	worker	I
Streptomyces sp KhCrAH271	271.KhCrWA13-3-6	worker	I
Streptomyces sp KhCrAH274	274.KhCrWA14-3-7	worker	II
Streptomyces sp KhCrAH275	275.KhCrWA15-1-2	worker	I
Streptomyces sp KhCrAH276	276.KhCrWA15-3-3A	worker	I
Streptomyces sp KhCrAH277	277.KhCrWA15-3-4	worker	II
Streptomyces sp KhCrAH278	278.KhCrWA16-1-8	worker	I
Streptomyces sp KhCrAH279	279.KhCrWA17-1-7	worker	I
Streptomyces sp KhCrAH280	280.KhCrWA17-3-5	worker	II
Streptomyces sp KhCrAH281	281.KhCrWA17-3-6	worker	I
Streptomyces sp KhCrAH282	282.KhCrWA18-1-1	worker	I
Streptomyces sp KhCrAH283	283.KhCrWA18-1-4	worker	II
Streptomyces sp KhCrAH284	284.KhCrWA19-1-4	worker	I
Streptomyces sp KhCrAH285	285.KhCrWA19-3-3	worker	no clade
Streptomyces sp KhCrAH287	287.KhCrWA20-1-3	worker	I
Streptomyces sp KhCrAH288	288.KhCrWA21-3-1	worker	I
Streptomyces sp KhCrAH289	289.KhCrWA22-1-2	worker	II
Streptomyces sp KhCrAH290	290.KhCrWA22-2-2	worker	III
Streptomyces sp KhCrAH291	291.KhCrWA22-2-3	worker	I
Streptomyces sp KhCrAH292	292.KhCrWA22-3-6	worker	II
Streptomyces sp KhCrAH293	293.KhCrWA22-3-4	worker	I
Streptomyces sp KhCrAH294	294.KhCrWA23-1-1	worker	I
Streptomyces sp KhCrAH296	296.KhCrWA23-1-4	worker	I
Streptomyces sp KhCrAH297	297.KhCrWA23-3-1	worker	I
Streptomyces sp KhCrAH298	298.KhCrWA23-3-4	worker	II
Streptomyces sp KhCrAH301	301.KhCrWD28-2-2	worker	I
Streptomyces sp KhCrAH303	303.KhCrWD30-1-3	worker	I

Species	Code	Caste	Group		
Streptomyces sp KhCrAH304	304.KhCrWD30-2-7	worker	I		
Streptomyces sp KhCrAH305	305.KhCrWD30-2-8	worker	I		
Streptomyces sp KhCrAH307	307.KhCrWD31-2-4	worker	I		
Streptomyces sp KhCrAH308	308.KhCrWD32-1-1	worker	II	positive	negative
Streptomyces sp KhCrAH309	309.KhCrWD33-1-3	worker	I		
Streptomyces sp KhCrAH312	312.KhCrWD33-2-9	worker	I		
Streptomyces sp KhCrAH313	313.KhCrWD33-2-10	worker	I		
Streptomyces sp KhCrAH314	314.KhCrWD34-1-5	worker	I		
Streptomyces sp KhCrAH315	315.KhCrWD35-2-4	worker	I		
Streptomyces sp KhCrAH316	316.KhCrWD36-1-4	worker	I	positive	negative
Streptomyces sp KhCrAH317	317.KhCrWD38-1-1	worker	I		
Streptomyces sp KhCrAH318	318.KhCrWD38-1-3	worker	I		
Streptomyces sp KhCrAH319	319.KhCrWD39-1-1	worker	I		
Streptomyces sp KhCrAH320	320.KhCrWD47-1-4	worker	I	positive	negative
Streptomyces sp KhCrAH321	321.KhCrWD51-1-6	worker	II		
Streptomyces sp KhCrAH334	334.KhCrWA11-1-2	worker	III		positive
Streptomyces sp KhCrAH337	337.KhCrWA17-1-6	worker	III		positive
Streptomyces sp KhCrAH338	338.KhCrWA15-2-2	worker	III		positive
Streptomyces sp KhCrAH340	340.KhCrWA11-3-1	worker	III		positive
Streptomyces sp KhCrAH342	342.KhCrWD28-2-4	worker	III		positive
Streptomyces sp KhCrAH343	343.KhCrWD32-1-8B	worker	III		positive
P. phylax/L. africana from Cameroon					
Nocardioides sp LaPpAH9	9.LaPpWD8-1-3	worker	V		
Nocardioides sp LaPpAH10	10.LaPpWA69-3-2	worker	V		
Nocardioides sp LaPpAH11	11.LaPpWD71-2-1	worker	V		
Nocardioides sp LaPpAH12	12.LaPpWA76-3-3	worker	V	negative	negative
Nocardioides sp LaPpAH13	13.LaPpWA77-3-4	worker	V		
Nocardioides sp LaPpAH14	14.LaPpWA78-2-2	worker	V		
Nocardioides sp LaPpAH18	18.LaPpWA92-2-1	worker	V		
Nocardioides sp LaPpAH20	20.LaPpWA94-3-2	worker	V	negative	negative

Nocardioides sp LaPpAH21	21.LaPpWA97-3-3	worker	V		
Nocardioides sp LaPpAH22	22.LaPpWA100-3-1	worker	V		
Nocardioides sp LaPpAH25	25.LaPpL106-2-1	larvae	V		
Nocardioides sp LaPpAH28	28.LaPpWA110-1-1	worker	V		positive
Streptomyces sp LaPpAH95	95.LaPpA3-1-10	alate	III		
Streptomyces sp LaPpAH97	97.LaPpWD15-2-1	worker	IV		
Streptomyces sp LaPpAH98	98.LaPpWD16-2-2	worker	IV		
Streptomyces sp LaPpAH99	99.LaPpWD17-1-1	worker	IV		
Streptomyces sp LaPpAH105	105.LaPpA47-1-5	alate	no clade		
Streptomyces sp LaPpAH109	109.LaPpWD7-2-1	worker	IV		
Streptomyces sp LaPpAH110	110.LaPpL75-2-1	larvae	IV		
Streptomyces sp LaPpAH161	161.LaPpA2-1-5	alate	no clade		
Streptomyces sp LaPpAH162	162.LaPpA2-1-6	alate	I		
Streptomyces sp LaPpAH163	163.LaPpA3-1-1	alate	IV	negative	negative
Streptomyces sp LaPpAH164	164.LaPpA3-1-7	alate	no clade		
Streptomyces sp LaPpAH165	165.LaPpA3-2-6	alate	III		
Streptomyces sp LaPpAH166	166.LaPpA47-2-1	alate	II		
Streptomyces sp LaPpAH169	169.LaPpA47-3-5	alate	II		
Streptomyces sp LaPpAH170	170.LaPpA47-3-6	alate	I		
Nocardioides sp LaPpAH172	172.LaPpA81-1-4	alate	V		
Actinobacteria sp LaPpAH173	173.LaPpA81-3-1	alate	no clade		
Streptomyces sp LaPpAH174	174.LaPpEmp1-2-7	fungal patch (dry)	I		
Streptomyces sp LaPpAH175	175.LaPpEmp4-2-2	fungal patch (dry)	I		
Streptomyces sp LaPpAH176	176.LaPpGly4-1-3	fungal patch (glycerol)	II		
Streptomyces sp LaPpAH177	177.LaPpGly5-5-2	fungal patch (glycerol)	I		
Streptomyces sp LaPpAH178	178.LaPpGly5-1-4	fungal patch (glycerol)	I		
Streptomyces sp LaPpAH180	180.LaPpL24-1-4	larvae	IV	negative	negative
Streptomyces sp LaPpAH181	181.LaPpL24-2-2	larvae	IV		
Streptomyces sp LaPpAH185	185.LaPpL37-3-4	larvae	no clade		
Streptomyces sp LaPpAH186	186.LaPpL52-1-1	larvae	II		

Species	ID	Type	Clade		
Streptomyces sp LaPpAH187	187.LaPpL52-3-1	larvae	II		
Streptomyces sp LaPpAH188	188.LaPpL67-1-3	larvae	no clade		
Actinobacteria sp LaPpAH190	190.LaPpL106-1-2	larvae	no clade	positive	negative
Streptomyces sp LaPpAH193	193.LaPpP19-1-5	pupae	IV		
Streptomyces sp LaPpAH198	198.LaPpPBS1-5-2	fungal patch (PBS)	I		
Streptomyces sp LaPpAH199	199.LaPpPBS2-5-4	fungal patch (PBS)	II		
Streptomyces sp LaPpAH200	200.LaPpPBS4-1-1	fungal patch (PBS)	I		
Streptomyces sp LaPpAH201	201.LaPpPBS4-1-3D	fungal patch (PBS)	I		
Streptomyces sp LaPpAH202	202.LaPpPBS4-1-3B	fungal patch (PBS)	I		
Streptomyces sp LaPpAH205	205.LaPpWA57-2-3	worker	II		
Nocardioides sp LaPpAH209	209.LaPpWA91-1-1	worker	V	negative	negative
Streptomyces sp LaPpAH211	211.LaPpWA93-2-1B	worker	no clade		
Streptomyces sp LaPpAH218	218.LaPpWA107-1-2	worker	I		
Nocardioides sp LaPpAH219	219.LaPpWA107-3-2	worker	V		
Nocardioides sp LaPpAH220	220.LaPpWA108-2-1	worker	V		
Streptomyces sp LaPpAH223	223.LaPpWD7-2-3	worker	no clade		
Streptomyces sp LaPpAH224	224.LaPpWD11-2-2	worker	no clade	positive	negative
Streptomyces sp LaPpAH225	225.LaPpWD12-1-1	worker	I		
Streptomyces sp LaPpAH226	226.LaPpWD13-2-4	worker	IV		
Streptomyces sp LaPpAH229	229.LaPpWD17-1-2	worker	I		
Streptomyces sp LaPpAH230	230.LaPpWD18-2-3	worker	I		
Streptomyces sp LaPpAH231	231.LaPpWD27-1-2	worker	no clade		
Streptomyces sp LaPpAH233	233.LaPpWD31-1-1	worker	no clade		
Streptomyces sp LaPpAH234	234.LaPpWD62-1-5	worker	II		
Streptomyces sp LaPpAH235	235.LaPpWD62-2-1	worker	II		
Streptomyces sp LaPpAH236	236.LaPpWD62-2-6	worker	II		
Nocardioides sp LaPpAH237	237.LaPpWD73-2-1	worker	V		
Nocardioides sp LaPpAH239	239.LaPpWD87-1-3	worker	V		
Nocardioides sp LaPpAH241	241.LaPpWD102-1-1	worker	V		
Nocardioides sp LaPpAH242	242.LaPpWD105-2-3	worker	V	negative	negative

			no clade	positive	negative
Streptomyces sp LaPpAH322	322.LaPpWA93-2-3B	worker	no clade		negative
P. penetrator/Tachigali sp. from French Guiana					
Streptomyces sp PsTaAH1	1.PsTaP2-3-1	pupae	I		
Streptomyces sp PsTaAH2	2.PsTaP5-2-1	pupae	I		
Streptomyces sp PsTaAH3	3.PsTaWA2-5-W-2	worker	I		
Streptomyces sp PsTaAH4	4.PsTaWA4-4-3	worker	I		
Streptomyces sp PsTaAH5	5.PsTaWA5-4-W-7	worker	I	positive	negative
Streptomyces sp PsTaAH7	7.PsTaWD3-1-6	worker	I		
Streptomyces sp PsTaAH8	8.PsTaWD10-1-W-1	worker	I		
Streptomyces sp PsTaAH80	80.PsTaP2-1-1	pupae	I		
Streptomyces sp PsTaAH81	81.PsTaQ-6-1	queen	I		
Streptomyces sp PsTaAH82	82.PsTaWA2-5-W-1 #1	worker	I		
Streptomyces sp PsTaAH83	83.PsTaWA4-4-5	worker	I		
Streptomyces sp PsTaAH84	84.PsTaWA4-4-6	worker	I		
Streptomyces sp PsTaAH85	85.PsTaWA5-3-1	worker	I		
Streptomyces sp PsTaAH86	86.PsTaWA6-3-W-3	worker	I		
Streptomyces sp PsTaAH87	87.PsTaWA7-2-3	worker	I		
Streptomyces sp PsTaAH88	88.PsTaWA9-2-W-2	worker	I		
Streptomyces sp PsTaAH89	89.PsTaWA9-2-W-6	worker	I		
Streptomyces sp PsTaAH90	90.PsTaWA10-4-2	worker	I		
Streptomyces sp PsTaAH91	91.PsTaWD3-1-3 #3	worker	I		
Streptomyces sp PsTaAH92	92.PsTaWD3-1-5	worker	I		
Streptomyces sp PsTaAH93	93.PsTaWD10-2-2	worker	I		
Streptomyces sp PsTaAH94	94.PsTaWD13-1-2	worker	I		
Streptomyces sp PsTaAH112	112.PsTaT12-PBS-6-4	fungal patch (PBS)	no clade		
Streptomyces sp PsTaAH113	113.PsTaLL1-1-1	larvae	no clade		
Streptomyces sp PsTaAH118	118.PsTaP2-3-2 #3	pupae	no clade		
Streptomyces sp PsTaAH119	119.PsTaP2-3-3	pupae	I		
Streptomyces sp PsTaAH121	121.PsTaP8-2-W-1 #1	pupae	no clade		
Streptomyces sp PsTaAH124	124.PsTaS2-4-2	scale insect	no clade	positive	negative

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Streptomyces sp PsTaAH126	126.PsTaT12-PBS-4-2	fungal patch (PBS)	no clade		
Streptomyces sp PsTaAH130	130.PsTaT12-PBS-5-2	fungal patch (PBS)	no clade	positive	negative
Streptomyces sp PsTaAH135	135.PsTaWA4-4-W-1	worker	I		
Streptomyces sp PsTaAH136	136.PsTaWA5-3-W-3 #1	worker	I		
Streptomyces sp PsTaAH137	137.PsTaWA5-4-W-2	worker	no clade	positive	negative
Streptomyces sp PsTaAH138	138.PsTaWA6-1-W-1	worker	I		
Streptomyces sp PsTaAH139	139.PsTaWA7-2-2	worker	I		
Streptomyces sp PsTaAH140	140.PsTaWA7-2-4	worker	I		
Streptomyces sp PsTaAH141	141.PsTaWA7-2-5	worker	I		
Streptomyces sp PsTaAH142	142.PsTaWA7-3-2	worker	I		
Streptomyces sp PsTaAH143	143.PsTaWA7-3-3	worker	I		
Streptomyces sp PsTaAH146	146.PsTaWA7-2-W-4 #4	worker	no clade	positive	negative
Streptomyces sp PsTaAH149	149.PsTaWA9-1-W-2 #2	worker	no clade		
Streptomyces sp PsTaAH150	150.PsTaWD3-1-1	worker	I		
Streptomyces sp PsTaAH152	152.PsTaWD3-1-3	worker	I		
Streptomyces sp PsTaAH154	154.PsTaWD3-1-3 #4	worker	I		
Streptomyces sp PsTaAH155	155.PsTaWD3-1-4	worker	I		
Streptomyces sp PsTaAH158	158.PsTaWD10-1-2	worker	I		

Supplemental Table 2.1B. Isolates not included in Figure 2.1.

Isolate Name	Full name	Reason not in Fig 1	Component	Cellulase assay
C. margaritae/K. hispida from Cameroon				
Streptomyces sp KhCrAH31	31.KhCrWA11-2-1	duplicate of *Streptomyces* sp KhCrAH340	worker	
Streptomyces sp KhCrAH32	32.KhCrWA12-3-1	poor sequence quality	worker	
Streptomyces sp KhCrAH38	38.KhCrWA21-2-2	poor sequence quality	worker	positive
Streptomyces sp KhCrAH39	39.KhCrWA22-3-1	duplicate of *Streptomyces* sp KhCrAH290	worker	positive
Streptomyces sp KhCrAH50	50.KhCrWA22-1-1	duplicate of *Streptomyces* sp KhCrAH289	worker	
Streptomyces sp KhCrAH52	52.KhCrWD30-1-2	poor sequence quality	worker	
Streptomyces sp KhCrAH56	56.KhCrWA9-3-3	poor sequence quality	worker	
Streptomyces sp KhCrAH60	60.KhCrWA15-3-2	poor sequence quality	worker	
Streptomyces sp KhCrAH66	66.KhCrWA23-2-2	duplicate of *Streptomyces* sp KhCrAH297	worker	
Streptomyces sp KhCrAH73	73.KhCrWD34-1-1	duplicate of *Streptomyces* sp KhCrAH314	worker	
Streptomyces sp KhCrAH245	245.KhCrP39-1-1B	duplicate of *Streptomyces* sp KhCrAH244	pupae	
Streptomyces sp KhCrAH248	248.KhCrP40-1-4A	duplicate of *Streptomyces* sp KhCrAH246	pupae	
Streptomyces sp KhCrAH268	268.KhCrWA12-1-3	poor sequence quality	worker	
Streptomyces sp KhCrAH272	272.KhCrWA14-2-6	duplicate of *Streptomyces* sp KhCrAH274	worker	
Streptomyces sp KhCrAH273	273.KhCrWA14-2-7B	duplicate of *Streptomyces* sp KhCrAH274	worker	
Streptomyces sp KhCrAH286	286.KhCrWA20-1-2	poor sequence quality	worker	
Streptomyces sp KhCrAH299	299.KhCrWA23-3-6	duplicate of *Streptomyces* sp KhCrAH51	worker	
Streptomyces sp KhCrAH300	300.KhCrWD27-1-2B	poor sequence quality	worker	
Streptomyces sp KhCrAH302	302.KhCrWD29-1-6	poor sequence quality	worker	
Streptomyces sp KhCrAH310	310.KhCrWD33-1-6	duplicate of *Streptomyces* sp KhCrAH309	worker	
Streptomyces sp KhCrAH336	336.KhCrWA17-2-5	duplicate of *Streptomyces* sp KhCrAH337	worker	positive
Streptomyces sp KhCrAH339	339.KhCrWD32-1-8A	duplicate of *Streptomyces* sp KhCrAH343	worker	positive
P. phylax/L. africana from Cameroon				
Nocardioides sp LaPpAH15	15.LaPpA81-1-3	duplicate of *Nocardioides* sp LaPpAH172	alate	
Nocardioides sp LaPpAH16	16.LaPpWD87-1-1	duplicate of *Nocardioides* sp LaPpAH239	worker	
Nocardioides sp LaPpAH17	17.LaPpWA91-3-1	duplicate of *Nocardioides* sp LaPpAH209	worker	

Species	ID	Note	Caste	
Nocardioides sp LaPpAH23	23.LaPpWD102-2-1	duplicate of *Nocardioides* sp LaPpAH241	worker	
Nocardioides sp LaPpAH26	26.LaPpWA107-1-1	duplicate of *Nocardioides* sp LaPpAH219	worker	
Nocardioides sp LaPpAH27	27.LaPpWA108-1-1	duplicate of *Nocardioides* sp LaPpAH220	worker	
Streptomyces sp LaPpAH96	96.LaPpWD13-1-1	poor sequence quality	worker	
Streptomyces sp LaPpAH100	100.LaPpWD18-2-2	poor sequence quality	worker	
Streptomyces sp LaPpAH101	101.LaPpP19-1-3B	duplicate of *Streptomyces* sp LaPpAH193	pupae	
Streptomyces sp LaPpAH103	103.LaPpL24-1-1	duplicate of *Streptomyces* sp LaPpAH180	larvae	
Streptomyces sp LaPpAH106	106.LaPpWA57-3-1	poor sequence quality	worker	
Streptomyces sp LaPpAH107	107.LaPpWA100-1-1	poor sequence quality	worker	positive
Streptomyces sp LaPpAH108	108.LaPpA3-1-4	duplicate of *Streptomyces* sp LaPpAH163	alate	
Streptomyces sp LaPpAH159	159.LaPpA2-1-1	poor sequence quality	alate	
Streptomyces sp LaPpAH160	160.LaPpA2-1-3	poor sequence quality	alate	
Streptomyces sp LaPpAH167	167.LaPpA47-2-5	duplicate of *Streptomyces* sp LaPpAH105	alate	
Methylobacterium sp LaPpAH171	171.LaPpA56-1-1	not Actinobacteria	alate	
Streptomyces sp LaPpAH182	182.LaPpL24-2-1E	duplicate of *Streptomyces* sp LaPpAH180	larvae	
Streptomyces sp LaPpAH183	183.LaPpL24-3-4	poor sequence quality	larvae	
Streptomyces sp LaPpAH184	184.LaPpL37-1-3	duplicate of *Streptomyces* sp LaPpAH185	larvae	
Streptomyces sp LaPpAH189	189.LaPpL75-2-4	duplicate of *Streptomyces* sp LaPpAH110	larvae	
Nocardioides sp LaPpAH191	191.LaPpL106-1-3	duplicate of *Nocardioides* sp LaPpAH25	larvae	
Streptomyces sp LaPpAH192	192.LaPpP19-1-3A	duplicate of *Streptomyces* sp LaPpAH193	pupae	
Streptomyces sp LaPpAH194	194.LaPpP19-1-6	duplicate of *Streptomyces* sp LaPpAH193	pupae	
Streptomyces sp LaPpAH195	195.LaPpP19-2-3B	poor sequence quality	pupae	
Streptomyces sp LaPpAH196	196.LaPpP19-3-3	duplicate of *Streptomyces* sp LaPpAH193	pupae	
Methylobacterium sp LaPpAH203	203.LaPpWA41-1-2	not Actinobacteria	worker	
Nocardioides sp LaPpAH206	206.LaPpWA76-2-1	duplicate of *Nocardioides* sp LaPpAH12	worker	
Nocardioides sp LaPpAH207	207.LaPpWA77-1-1	duplicate of *Nocardioides* sp LaPpAH13	worker	
Nocardioides sp LaPpAH208	208.LaPpWA78-3-1	duplicate of *Nocardioides* sp LaPpAH14	worker	
Nocardioides sp LaPpAH210	210.LaPpWA92-1-1	duplicate of *Nocardioides* sp LaPpAH18	worker	
Nocardioides sp LaPpAH213	213.LaPpWA94-2-3	duplicate of *Nocardioides* sp LaPpAH20	worker	
Nocardioides sp LaPpAH214	214.LaPpWA97-1-5	duplicate of *Nocardioides* sp LaPpAH21	worker	

Streptomyces sp LaPpAH215	215.LaPpWA97-3-2	poor sequence quality	worker	
Nocardioides sp LaPpAH217	217.LaPpWA100-2-2	duplicate of *Nocardioides* sp LaPpAH22	worker	
Streptomyces sp LaPpAH228	228.LaPpWD17-2-2	duplicate of *Streptomyces* sp LaPpAH99	worker	
Streptomyces sp LaPpAH238	238.LaPpWD86-2-1	poor sequence quality	worker	
Streptomyces sp LaPpAH240	240.LaPpWD88-2-4	poor sequence quality	worker	
Streptomyces sp LaPpAH323	323.LaPpA3-2-2	duplicate of *Streptomyces* sp LaPpAH163	alate	negative
Streptomyces sp LaPpAH324	324.LaPpL75-3-1	duplicate of *Streptomyces* sp LaPpAH110	larvae	negative
Streptomyces sp LaPpAH325	325.LaPpL75-1-7	duplicate of *Streptomyces* sp LaPpAH110	larvae	negative
Streptomyces sp LaPpAH326	326.LaPpL75-1-5	duplicate of *Streptomyces* sp LaPpAH110	larvae	negative
Streptomyces sp LaPpAH327	327.LaPpL75-1-2	duplicate of *Streptomyces* sp LaPpAH110	larvae	negative
Streptomyces sp LaPpAH329	329.LaPpL75-2-4	duplicate of *Streptomyces* sp LaPpAH110	larvae	negative
Streptomyces sp LaPpAH330	330.LaPpA3-1-5	duplicate of *Streptomyces* sp LaPpAH163	alate	negative
Streptomyces sp LaPpAH331	331.LaPpL75-1-1	duplicate of *Streptomyces* sp LaPpAH110	larvae	negative
Streptomyces sp LaPpAH332	332.LaPpL75-2-3	duplicate of *Streptomyces* sp LaPpAH110	larvae	negative
Streptomyces sp LaPpAH333	333.LaPpA3-1-2	duplicate of *Streptomyces* sp LaPpAH163	alate	negative
P. penetrator/Tachigali sp. from French Guiana				
Streptomyces sp PsTaAH6	6.PsTaWA7-3-1	poor sequence quality	worker	
Streptomyces sp PsTaAH111	111.PsTaT12-PBS-5-3	duplicate of *Streptomyces* sp PsTaAH130	fungal patch (PBS)	
Streptomyces sp PsTaAH114	114.PsTaP2-2-1 #1	duplicate of *Streptomyces* sp PsTaAH118	pupae	
Streptomyces sp PsTaAH115	115.PsTaP2-2-1 #2	duplicate of *Streptomyces* sp PsTaAH118	pupae	
Streptomyces sp PsTaAH116	116.PsTaP2-3-2 #1	duplicate of *Streptomyces* sp PsTaAH118	pupae	
Methylobacterium sp PsTaAH120	120.PsTaP7-3-W-3	not Actinobacteria	pupae	
Streptomyces sp PsTaAH122	122.PsTaP8-2-W-1 #3	duplicate of *Streptomyces* sp PsTaAH121	pupae	
Streptomyces sp PsTaAH125	125.PsTaT12-PBS-4-1	duplicate of *Streptomyces* sp PsTaAH126	fungal patch (PBS)	
Streptomyces sp PsTaAH127	127.PsTaT12-PBS-4-3	duplicate of *Streptomyces* sp PsTaAH126	fungal patch (PBS)	
Streptomyces sp PsTaAH128	128.PsTaT12-PBS-4-4	duplicate of *Streptomyces* sp PsTaAH126	fungal patch (PBS)	
Streptomyces sp PsTaAH129	129.PsTaT12-PBS-5-1	duplicate of *Streptomyces* sp PsTaAH130	fungal patch (PBS)	
Streptomyces sp PsTaAH131	131.PsTaT12-PBS-6-3	duplicate of *Streptomyces* sp PsTaAH112	fungal patch (PBS)	
Methylobacterium sp PsTaAH132	132.PsTaT13-PBS-4-1	not Actinobacteria	fungal patch (PBS)	
Methylobacterium sp PsTaAH133	133.PsTaWA2-4-1	not Actinobacteria	worker	

Streptomyces sp PsTaAH134	134.PsTaWA2-5-W-1 #2	poor sequence quality	worker
Streptomyces sp PsTaAH144	144.PsTaWA7-2-W-4 #1	duplicate of *Streptomyces* sp PsTaAH146	worker
Streptomyces sp PsTaAH145	145.PsTaWA7-2-W-4 #2	duplicate of *Streptomyces* sp PsTaAH146	worker
Streptomyces sp PsTaAH147	147.PsTaWA9-1-W-2	duplicate of *Streptomyces* sp PsTaAH149	worker
Streptomyces sp PsTaAH148	148.PsTaWA9-1-W-2 #1	duplicate of *Streptomyces* sp PsTaAH149	worker
Streptomyces sp PsTaAH156	156.PsTaWD3-1-7	poor sequence quality	worker
Methylobacterium sp PsTaAH157	157.PsTaWD8-3-1	not Actinobacteria	worker

Chapter 3. The microbiota of *Cecropia*-obligate *Azteca* ants in Costa Rica

Manuscript in preparation: Alissa S. Hanshew and Cameron R. Currie

Coauthors contributed in the following ways: ASH and CRC designed experiments. ASH carried out the experiments and wrote the paper. CRC provided lab material and support.

3.1 Abstract

The factors determining the structure of microbial communities in social insects across different host species in the same genera and across geographic distance remain poorly understood. Here we characterize and compare the microbiota of *Cecropia*-obligate *Azteca* ants. Despite this symbiotic system's ubiquity in Neotropical environments and status as a model for animal-plant mutualisms, no study has investigated the microbial community of this symbiotic system. Using 454 pyrosequencing of the 16S rRNA gene (V5-V8 regions) we obtained 165,570 high quality bacterial sequences from 36 samples derived from 18 colonies representing 4 species of *Azteca*, 2 species of *Cecropia*, from 2 geographically distant locations in Costa Rica. Proteobacteria were dominant in nearly every sample, while Actinobacteria, Bacteroidetes, and Firmicutes were also consistently found. The most commonly found bacterial orders in the microbiota of *Azteca* ants regardless of host ant or tree species were Actinomycetales, Burkholderiales, Enterobacteriales, Pseudomonadales, Rhizobiales, and Xanthomonadales. Our results indicated that bacterial communities in these ants are not correlated with geographic location within Costa Rica, *Cecropia* species, or *Azteca* species. *Azteca* queens were more likely to have similar bacterial communities to each other, while the communities associated with *Azteca* workers were more diffuse. Only two colonies had high abundances of *Wolbachia*, while *Ralstonia* and an unidentified Enterobacteriaceae were found in nearly every sample. This description of the bacteria associated with *Cecropia*-obligate *Azteca* ants adds to the growing body of research

demonstrating that insects rely on symbiotic bacteria for a number of functions, and that geographic location may not be an important factor contributing to the structure of the microbial communities harbored within this symbiotic system.

3.2 Introduction

With more than 12,000 currently described species, ants are ubiquitous and often dominant arthropods in many ecosystems, yet our knowledge of their biology remains incomplete. Indeed, it is impossible to fully understand the biology of ants without comprehensive knowledge of their associated microorganisms (11). It is generally assumed that bacterial mutualists found associated with ants fall into one of two functional categories, nutritional or protective, but most microbes associated with ants currently have unknown roles.

Many ants consume nutrient poor diets that are thought to be insufficient to meet their nitrogen needs (8). To overcome this, many ants are thought to associate with nitrogen-fixing or -recycling bacteria (8). For example, the bacteria *Pantoea* and *Klebsiella* actively fix nitrogen in fungus-growing ant gardens (31). Likewise, the bacteria *Blochmannia floridanus* engages in urea recycling, which makes nitrogen biologically available in the ant *Camponotus floridanus* (15). Bacteria in the order Rhizobiales are also thought to aid in nitrogen acquisition in *Cephalotes* ants, though while the presence of *nifH* genes seems to support this hypothesis, acetylene reduction assays failed to demonstrate nitrogen fixation in these ants (36).

In addition to nutritional challenges, ants are vulnerable, by virtue of their social lifestyle, to infection by pathogens, as ants often live in tight quarters with genetically homogenous nestmates in humid conditions (20). Some Hymenoptera, including ants, have co-opted the production of secondary metabolites by Actinobacteria as defense against microbial pathogens (20, 22). Ectosymbiotic bacteria in the genus *Pseudonocardia* play an active role in protecting

the fungus gardens of fungus-growing ants from attack from the coevolved fungal pathogen *Escovopsis* (6, 7). A similar role for *Streptomyces* has been proposed in *Allomerus* ants, though as yet, only the presence of bacteria is known, not their specific function (41). Indeed, to date most bacteria found associated with ants have no currently-described role, including *Wolbachia,* which is known to affect sex ratios in other insects (37).

Modern molecular techniques have added greatly to our understanding of the microbial community composition associated with ants. The recent application of next generation sequencing techniques to elucidate microbial communities has led to a significant increase in our knowledge of ant-associated bacteria. The bacterial community of invasive *Solenopsis* ants in Texas was found to be incredibly similar to that found in native *Solenopsis* ants (18). Similarly, differences in bacterial communities associated with *Cephalotes varians* were minimal and the identified bacterial taxa tended to cluster with previously found ant-specific lineages (2, 21). Likewise, at the phylum level, similar bacterial communities were found on the cuticles of *Allomerus* and *Tetraponera* ants despite originating from two different continents (40). This is perhaps not surprising, as many insect-associated microbial communities are dominated by taxa from only a handful of bacterial phyla (19).

Much remains to be determined about the bacteria associated with ants, including their ecological role and their importance in ant-plant mutualisms. Ant-plant mutualisms are conspicuous members of tropical ecosystems, yet despite a long history of interest from natural historians and, for systems such as the mutualism between *Cecropia* trees and *Azteca* ants, a wide body of literature on their natural history, they remain under-explored in terms of microbial associations (3). *Cecropia* are conspicuous members of forest gaps, secondary forests, and roadside flora in the Neotropics (25). In their native range, which extends from Mexico to

Argentina, *Cecropia* inhabited by *Azteca* represent seven tree species and thirteen ant species

(23, 26, 27). Different *Azteca* species are not tied to any specific *Cecropia* species as host trees,

and the pairings between ant and tree species appear to be more limited by local climate than by

any barrier between the ant and the tree (25, 27). While these trees can be inhabited by other ant

species, *Cecropia* trees with active *Azteca* colonies grow taller, show fewer signs of herbivore

attack, and survive longer than uninhabited trees (9, 39, 43). In return for hollow trunks and

branches that serve as domatia (nesting space), and glycogen-rich Müllerian bodies, created by

the tree as a primary food source for the ants, *Azteca* colonies protect their host trees from

encroaching vegetation and herbivores, particularly the dominant herbivore in the Neotropics,

leaf-cutting ants (23, 33). Additional work suggests that resident *Azteca* also remove fungal

spores from their host tree and that the trichilia (the patch where Müllerian bodies are produced)

of unoccupied *Cecropia* are often covered in fungal growth (34, 35).

We used 454 pyrosequencing of the 16S rRNA gene to elucidate the bacterial community

associated with four species of *Azteca* ants inhabiting two species of *Cecropia* trees across a

geographic gradient in Costa Rica. This report of the bacteria associated with *Cecropia*-obligate

Azteca ants serves to highlight the importance of specific bacterial community members in

Neotropical ant-plant symbioses. We describe and compare the microbiota of (i) *A. xanthochroa*

in *C. obtusifolia*, (ii) different *Azteca-Cecropia* species pairing, (iii) multiple life stages of

Azteca, and (iv) *Azteca* colonies over geographic distance.

3.3 Materials and Methods

3.3.1 Ant colony collection and identification

Eighteen colonies of *Cecropia*-obligate *Azteca* ants were collected in May 2011 in Costa

Rica in one of three locations, specifically La Selva Biological Station (La Selva), Carara

Biological Reserve (Carara), and along Highway 27 near the town of Orotina (Figure 3.1, Table 3.1). Samples were collected from C*ecropia* trees 1-3 m tall with externally active *Azteca* colonies. Tree sections roughly 0.25 m in length were placed into zip-top bags, transported at ambient temperature, and dissected in sterile conditions. The following sample types were collected from each domatia (when present): workers, larvae, pupae, and queens. Due to the colony structure of *Azteca*, finding this material was not possible for all colonies. *Azteca* species were identified based on queen morphology (29). For colonies where the queens were not located, colonies were identified based on worker morphology (28). *Cecropia* species were determined based on leaf morphology (24). In two cases, the *Cecropia* species was not identified and the colonies from these trees were marked as coming from "*Cecropia* sp.". Pools of five workers, five larvae, or five pupae, with queens kept as single samples, were placed in 95% ethanol for transportation to the laboratory at the University of Wisconsin-Madison for DNA extraction and amplicon pyrosequencing. Samples were named based on colony number and life stage (Table 3.2). For example, the pools of larvae, pupae and workers from colony 31 were named '31larvae', '31pupae', and '31workers'.

3.3.2 DNA extraction

All samples were removed from ethanol and dried briefly at 37°C to complete the removal of ethanol. Whole insects were placed in 2 ml screw-top tubes with a single 3 mm steel bead and 500 μl of extraction buffer from an EpiCenter MasterPure Complete DNA and RNA Purification Kit (Illumina, Madison, WI). Samples were shaken in a Mini-beadbeater (Biospec Products, Bartlesville, OK) for 2.5 min. After bead beating, the samples were processed following the remainder of the DNA extraction kit protocol.

3.3.3 Amplicon preparation and 454 pyrosequencing

Preparation of 16S rRNA bacterial amplicons for 454 pyrosequencing were performed following the method of Hanshew *et al.* with the following parameters: forward primer 799F-mod6 at 250 nM final concentration, reverse primer 1392R at 250 nM final concentration, 35 ng of DNA for each sample, and both rounds of PCR performed using Herculase II DNA polymerase (Agilent Technologies, Santa Clara, CA) (17). PCR was performed in two rounds, the first with regular primers, and the second with primers with the 454 pyrosequencing A- and B-adapters and multiplex identifiers (MIDs) for each sample (Roche, Indianapolis, IN). 454 pyrosequencing with Lib-L Titanium chemistry was conducted on a Roche GS Junior following manufacturer's guidelines for amplicon sequencing with shotgun processing, with modifications given in (17).

3.3.4 Data processing and analysis

Sequence processing was performed using the bioinformatics program suite mothur (v 1.31.1) with standard amplicon processing pipeline with default parameters (38). Alignment of processed sequences was done against the combined bacterial, archaeal, and eukaryotic Silva-derived database implemented in mothur, and chimeras were removed using the program UCHIME (13, 32). Sequences were binned into operational taxonomic units (OTUs) at 95% similarity. OTUs identified as chloroplasts, eukaryotes, or unclassifiable at the kingdom level were removed after initial data assessment. Rare reads were defined as OTUs comprising less than 1% of total sequences in a sample, and removed for downstream analysis. Clearcut, implemented in mothur, was used to create a relaxed neighbor joining tree of community structure and for the creation of UniFrac distance matrices (14, 30). Beta diversity was assessed

using non-metric multidimensional scaling (NMDS) analysis in PRIMER (v 6) using UniFrac and Bray-Curtis distance matrices (4, 30).

3.4 Results and Discussion

3.4.1 Analysis of 454 pyrosequencing data

A total of 165,785 16S rRNA gene sequences were obtained from 36 samples from 18 colonies of Costa Rican *Cecropia*-obligate *Azteca* ants after all quality filtering steps (Tables 3.1 and 3.2; Figure 3.1). Despite the presumed herbivorous nature of these ants, which would be expected to result in a high percentage of chloroplast-derived sequences, we detected only 202 chloroplast sequences in total, with less than 4% of sequences for any given sample (Table 3.2); the low percentage of chloroplast contamination is assumed to be due to the usage of the 799F-mod6 primer shown previously to reduce chloroplast amplification (17). 165,570 sequences remained after removal of chloroplast and other non-bacterial sequences (Table 3.2). The number of sequences per sample ranged from 724 to 18,255 (mean 4,599.2 ± 702.9 SE; Table 3.2) before the removal of rare reads. The average sequence length was 267.1 bp after quality trimming and removal of primer sequences and MIDs.

At the phylum level, samples had little diversity, with 4 phyla and 7 classes representing at least 87.8% of reads in each sample: Actinobacteria (Actinobacteria), Proteobacteria (Alpha-, Beta-, and Gammaproteobacteria), Firmicutes (Bacilli), and Bacteroidetes (Flavobacteria and Sphingobacteria) (Figure 3.2). These results are similar to those found in other studies comparing bacterial communities in multiple insect taxa suggesting that arthropod bacterial communities are often represented by these particular bacterial phyla (12, 19).

Between 1 and 25 OTUs were identified at the 95% similarity level in individual samples after removal of rare sequences (12.6 ± 1.0 SE; Table 3.2), representing a total of 85 OTUs

(Figure 3.3). Eight OTUs were found in more than half of all *Azteca* samples (Table 3.3).

These common OTUs represented 1.2-100% of the sequences (55.1% ± 5.0 SE), with some

samples dominated by single uncommon OTUs. For example, all of the samples from colony 46

were dominated by a single OTU identified as *Wolbachia*. This OTU was only found in 2 other

samples (39workers, 92.3%; and 47queen, 2.2%). While *Wolbachia* has previously been found in

ants, research is still inconclusive on the effects of this bacterium (1, 16, 37, 44, 45). The typical

mechanisms by which *Wolbachia* influences sex ratios are thought to be unlikely in ants (37, 45).

Additionally, recent work in *Acromyrmex octospinosus* suggests that *Wolbachia* might even play

a nutritional role based on its location and density in the ants (1).

3.4.2 *A. xanthochroa* workers and the effect of host plant species

One of our primary aims was to explore the presence of a core microbial community

associated with *A. xanthochroa* workers. After rare sequences were removed, sequences from *A.*

xanthochroa workers were binned into 67 OTUs at 95% similarity, representing at least 92% of

total sequences. On average, individual samples contained 17.0 ± 1.5 (SE) OTUs (Figure 3.3).

There were eleven common OTUs in more than half of *A. xanthochroa* worker samples,

representing 22.4-84.7**%** of total sequences in each sample (Table 3.3). All *A. xanthochroa*

workers contained one OTU in common (Table 3.3). This OTU represented 2.4-12.9% of

sequences within each of the eleven *A. xanthochroa* worker samples.

Within the eleven common OTUs, three were in the order Rhizobiales, three were

Burkholderiales, two were Actinomycetales, and all with one OTU each, Enterobacteriales,

Sphingobacteriales, and Xanthomonadales. The presence of Rhizobiales was previously

correlated with herbivory in ants (36). Every *A. xanthochroa* worker had at least one OTU

classified as a Rhizobiales, while six of the eleven were associated with all three Rhizobiales

OTUs. Only one of these common OTUs was confidently identified at the genus level as *Methylobacterium*, bacteria thought to be ubiquitous on leaf surfaces, but also found previously in ants and implicated in nitrogen fixation (10, 42). Neither of the two remaining common Rhizobiales OTUs could confidently be classified beyond the family level. These bacteria could be true members of the *A. xanthochroa* microbial community as suggested in previous herbivorous ant work (36), or they could be transients picked up by ants from their environment or diet.

Surprisingly, *Ralstonia* was one of the OTUs found in all but one *A. xanthochroa* worker samples. While species of *Ralstonia* can be isolated from soil and water, this genus also includes many serious plant pathogens, yet here, the host trees were not under obvious pathogen attack (5). While we cannot eliminate the possibility that the *Ralstonia* found here are indeed pathogens, we cannot also rule out that they might be playing some other nonpathogenic role in this system.

Nonmetric multidimensional scaling (NMDS) plots show that the microbiota of *A. xanthochroa* workers are not correlated with their host tree species (Figure 3.4A). The bacterial community associated with 49workers, a colony found in *C. insignis*, is more similar to 40workers, a colony found in *C. obtusifolia*, than 34workers, also in *C. insignis*. Overall, the bacterial communities associated with *A. xanthochroa*, while they share some similarities, do not cluster tightly. Unweighted UniFrac, which measures community membership by presence versus absence, showed the dissimilarity between most *A. xanthochroa* worker samples, and again that host tree species was not a factor in what bacterial OTUs were or were not present (Figure 3.4B). This is likely due to the large number of OTUs that are not shared between

samples. In weighted UniFrac, which takes into account the abundance of all community members, the sample pattern remained diffuse (Figure 3.4C).

3.4.3 *Azteca* **workers and (the lack of) geographic signal**

Across all *Azteca* workers, 77 OTUs were identified representing at least 68.5% of total sequences after removal of rare sequences. Surprisingly, the driving force behind the microbiota of these ants did not appear to be the *Azteca* or *Cecropia* species, nor was it the geographic location. Indeed, in some cases, two samples from both different *Azteca* and *Cecropia* species were more similar to each other than two samples from the same *Azteca* and the same *Cecropia* species (Figure 3.5). For example, 51workers, despite being an *A. constructor* colony in *C. insignis* from Carara was more similar to many of the *A. xanthochroa/C. obtusifolia* communities from La Selva, and less similar to 52workers, which was also an *A. constructor* colony in *C. insignis* from Carara (Figure 3.5). When examined by geographic location, similar numbers of OTUs were found in samples from La Selva as those found on the Pacific coast (Figure 3.3). Likewise, some samples from the Pacific coast were actually more similar to samples from La Selva than were samples from the same location within LSBS, despite being different *Azteca* species inhabiting different *Cecropia* species (Figure 3.5).

More than half of the worker samples contained seven OTUs in common, representing 2.2-76.8% of total sequences in each sample (Table 3.3). Of these seven OTUs, two were found in almost all of the workers, regardless of *Azteca* species, as had been found in *A. xanthochroa* workers, including *Ralstonia,* and Rhizobiales (*Ochrobactrum).*

3.4.4 *Azteca* **life stages and (the lack of) geographic signal**

One difficulty in studying microbial community composition across life stages in *Cecropia*-obligate *Azteca* ants is the difficulty in locating all the life stages in a single tree. We

targeted trees 1-3 meters tall in an attempt to get most of the colony components for each colony. However, this sampling technique proved to be insufficient, as we were able to locate all life stages (workers, larvae, pupae, and the queen) in only four colonies. This may have been due to the fact that different *Azteca* species have different internal structuring of their colonies; some *Azteca* species are more likely to concentrate their queen and brood in one internal, well protected, location (23). Other *Azteca* species are more likely to disperse their brood away from the domatia containing the queen (23). It is possible that we missed collection of some life stages due to not sampling these young trees close enough to the ground.

We found nine colonies with at least one life stage in addition to workers. Like much of the rest of the comparative work here, there seemed to be only a few common themes across life stages. Workers on average had more OTUs than pupae, larvae, or queens (Figure 3.3). The increased diversity is possibly a factor of the workers contacting the outside of the tree, while larvae, pupae, and queens are kept sequestered inside the domatia. Additionally, larvae, pupae, and queens from the same colony were not necessarily more similar to their worker nestmates, and sometimes workers from other colonies were more similar to other life stages from other colonies (Figure 3.6). This held true regardless of *Azteca* species (Figure 3.6A), *Cecropia* species (Figure 3.6B), or geographic location (Figure 3.6C).

However, queens, regardless of *Azteca* species, *Cecropia* species, or location, were more similar to each other than other life stages (Figure 3.6D). Similarly some of the brood samples clustered closer to queens than to their worker nestmates. Workers on the other hand appear to have more diffuse relationships, which may be explained by the fact that workers on average carried more OTUs, as described above.

3.5 Summary

Here we characterized and compared the bacterial communities in 18 colonies of *Cecropia*-obligate *Azteca* ants. Surprisingly, the composition and abundance of community members was not correlated with any specific *Azteca* species, *Cecropia* species, nor geographic location. However, *Azteca* queens, regardless of the previously mentioned factors, were more similar to each other than any other life stage. A few common taxa were found in nearly all samples including *Ralstonia* and a few Rhizobiales OTUs. While *Ralstonia* has no known function in ants, Rhizobiales are postulated to be important in herbivorous ants. Bacteria in this order are thought to be involved in nitrogen acquisition in other species of ants, and could be serving in that role here as well. The possibility remains that *Azteca* may not have a single core microbial community, or that it is dependent on some other factor that we cannot account for here. While *Azteca* are generally known to be herbivorous, insect parts have been found in their domatia, and perhaps could represent occasional additions to their diet. This could have an effect on the microbial community of the colony, and may account for the variation seen here.

3.6 Acknowledgements

3.7 References

1.   **Andersen SB, Boye M, Nash DR, Boomsma JJ**. 2012. Dynamic *Wolbachia* prevalence in *Acromyrmex* leaf-cutting ants: potential for a nutritional symbiosis. Journal of Evolutionary Biology **25**:1340–1350.

2.   **Anderson KE, Russell JA, Moreau CS, Kautz S, Sullam KE, Hu Y, Basinger U, Mott BM, Buck N, Wheeler DE**. 2012. Highly similar microbial communities are shared among related and trophically similar ant species. Molecular Ecology **21**:2282–2296.

3.   **Belt T**. 1874. The Naturalist in Nicaragua. The University of Chicago Press, Chicago.

4.   **Clarke K, Gorley R**. 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth, UK.

5.   **Coenye T, Goris J, De Vos P, Vandamme P, LiPuma JJ**. 2003. Classification of *Ralstonia pickettii*-like isolates from the environment and clinical samples as *Ralstonia insidiosa* sp. nov. International Journal of Systematic and Evolutionary Microbiology **53**:1075–1080.

6.   **Currie CR, Scott JA, Summerbell RA, Malloch D**. 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature **398**:701–704.

7.   **Currie CR, Wong B, Stuart AE, Schultz TR, Rehner SA, Mueller UG, Sung G-H, Spatafora JW, Straus NA**. 2003. Ancient tripartite coevolution in the attine ant-microbe symbiosis. Science **299**:386–388.

8.   **Davidson DW, Cook SC, Snelling RR, Chua TH**. 2003. Explaining the abundance of ants in lowland tropical rainforest canopies. Science **300**:969–972.

9.   **Davidson D**. 2005. *Cecropia* and its biotic defenses, p. 214–226. *In* Berg, CC, Rosselli, P (eds.), *Cecropia*. The New York Botanical Garden, Bronx, NY.

10.   **Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R, von Mering C, Vorholt JA**. 2009. Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. Proceedings of the National Academy of Science **106**:16428–16433.

11.   **Dillon RJ, Dillon VM**. 2004. The gut bacteria of insects: nonpathogenic interactions. Annual Review of Entomology **49**:71–92.

12.   **Douglas AE**. 2009. The microbial dimension in insect nutritional ecology. Functional Ecology **23**:38–47.

13.   **Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R**. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics **27**:2194–2200.

14.   **Evans J, Sheneman L, Foster J**. 2006. Relaxed neighbor joining: a fast distance-based phylogenetic tree construction method. Journal of Molecular Evolution **62**:785–792.

15.   **Feldhaar H, Straka J, Krischke M, Berthold K, Stoll S, Mueller MJ, Gross R**. 2007. Nutritional upgrading for omnivorous carpenter ants by the endosymbiont *Blochmannia*. BMC Biology **5**:48.

16.   **Fernando de Souza R, Daivison Silva Ramalho J, Santina de Castro Morini M, Wolff JLC, Araújo RC, Mascara D**. 2009. Identification and characterization of *Wolbachia* in *Solenopsis saevissima* fire ants (Hymenoptera: Formicidae) in southeastern Brazil. Current Microbiology **58**:189–194.

17.   **Hanshew AS, Mason CJ, Raffa KF, Currie CR**. 2013. Minimization of chloroplast contamination in 16S rRNA gene pyrosequencing of insect herbivore bacterial communities. In review.

18. **Ishak HD, Plowes R, Sen R, Kellner K, Meyer E, Estrada DA, Dowd SE, Mueller UG**. 2011. Bacterial diversity in *Solenopsis invicta* and *Solenopsis geminata* ant colonies characterized by 16S amplicon 454 pyrosequencing. Microbial Ecology **61**:821–831.

19. **Jones RT, Sanchez LG, Fierer N**. 2013. A cross-taxon analysis of insect-associated bacterial diversity. PLoS ONE **8**:e61218.

20. **Kaltenpoth M, Engl T**. 2013. Defensive microbial symbionts in Hymenoptera. Functional Ecology **Early Edit**.

21. **Kautz S, Rubin BER, Russell JA, Moreau CS**. 2013. Surveying the microbiome of ants: Comparing 454 pyrosequencing with traditional methods to uncover bacterial diversity. Applied and Environmental Microbiology **79**:525–534.

22. **Kroiss J, Kaltenpoth M, Schneider B, Schwinger M-G, Hertweck C, Maddula RK, Strohm E, Svatos A**. 2010. Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. Nature Chemical Biology **6**:261–263.

23. **Longino JT**. 1991. Taxonomy of the *Cecropia*-inhabiting *Azteca* ants. Journal of Natural History **25**:1571–1602.

24. **Longino JT**. 2005. *Cecropia* species of Costa Rica.

25. **Longino JT**. 1989. Geographic variation and community structure in an ant-plant mutualism: *Azteca* and *Cecropia* in Costa Rica. Biotropica **21**:126–132.

26. **Longino JT**. 2007. A taxonomic review of the genus *Azteca* (Hymenoptera: Formicidae) in Costa Rica and a global revision of the *aurita* group. Zootaxa **1491**:1–62.

27. **Longino JT**. 1991. *Azteca* ants in *Cecropia* trees: taxonomy, colony structure, and behaviour, p. 271–288. *In* Huxley, CR, Cutler, DF (eds.), Ant-Plant Interactions. Oxford University Press, Oxford.

28. **Longino JT**. 2007. Key to *Azteca* of Costa Rica.

29. **Longino JT**. 2007. Key to *Azteca* of Costa Rica - Queens.

30. **Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R**. 2011. UniFrac: an effective distance metric for microbial community comparison. The ISME Journal **5**:169–172.

31. **Pinto-Tomás AA, Anderson MA, Suen G, Stevenson DM, Chu FST, Cleland WW, Weimer PJ, Currie CR**. 2009. Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. Science **326**:1120–1123.

32. **Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO**. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research **35**:7188–7196.

33. **Rickson FR**. 1971. Glycogen plastids in müllerian body cells of *Cecropia peltata* - a higher green Plant. Science **173**:344–347.

34. **Rico-Gray V, Oliveira PS**. 2007. The Ecology and Evolution of Ant-Plant Interactions. The University of Chicago Press, Chicago.

35. **Roux O, Céréghino R, Solano PJ, Dejean A**. 2011. Caterpillars and fungal pathogens: two co-occurring parasites of an ant-plant mutualism. PLoS ONE **6**:e20538.

36. **Russell JA, Moreau CS, Goldman-Huertas B, Fujiwara M, Lohman DJ, Pierce NE**. 2009. Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. Proceedings of the National Academy of Sciences **106**:21236–21241.

37. **Russell JA**. 2012. The ants (Hymenoptera: Formicidae) are unique and enigmatic hosts of prevalent *Wolbachia* (Alphaproteobacteria) symbionts. Myrmecological News **16**:7–23.

38. **Schloss PD, Gevers D, Westcott SL**. 2011. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS ONE **6**:e27310.

39. **Schupp EW**. 1986. *Azteca* protection of *Cecropia*: ant occupation benefits juvenile trees. Oecologia **70**:379–385.

40. **Seipke RF, Barke J, Heavens D, Yu DW, Hutchings MI**. 2013. Analysis of the bacterial communities associated with two ant-plant symbioses. Microbiology Open 1–8.

41. **Seipke RF, Barke J, Ruiz-Gonzalez MX, Orivel J, Yu DW, Hutchings MI**. 2012. Fungus-growing *Allomerus* ants are associated with antibiotic-producing actinobacteria. Antonie van Leeuwenhoek **101**:443–447.

42. **Van Borm S, Buschinger A, Boomsma JJ, Billen J**. 2002. *Tetraponera* ants have gut symbionts related to nitrogen-fixing root-nodule bacteria. Proceedings of the Royal Society - Biological Sciences **269**:2023–2027.

43. **Vasconcelos HL, Casimiro AB**. 1997. Influence of *Azteca alfari* ants on the exploitation of *Cecropia* trees by a leaf-cutting ant. Biotropica **29**:84–92.

44. **Wenseleers T, Ito F, Van Borm S, Huybrechts R, Volckaert F, Billen J**. 1998. Widespread occurrence of the micro-organism *Wolbachia* in ants. Proceedings of the Royal Society - Biological Sciences **265**:1447–52.

45. **Wenseleers T, Sundström L, Billen J**. 2002. Deleterious *Wolbachia* in the ant *Formica truncorum*. Proceedings of the Royal Society - Biological Sciences **269**:623–629.

Table 3.1. General collection information for the 18 colonies collected for this study.
Included are colony codes, identification information for both *Azteca* and *Cecropia* species,
collection location, and a plus sign for the component types collected from each colony.

Colony code	*Azteca* species	*Cecropia* species	Location	Workers	Larvae	Pupae	Queen
31	*alfari*	*obtusifolia*	A	+	+	+	+
32	*xanthochroa*	*Cecropia* sp.	A	+			
33	*xanthochroa*	*obtusifolia*	A	+	+		
34	*xanthochroa*	*insignis*	A	+			+
37	*xanthochroa*	*obtusifolia*	A	+			+
38	*xanthochroa*	*obtusifolia*	A	+			
39	*constructor*	*obtusifolia*	A	+			+
40	*xanthochroa*	*obtusifolia*	A	+			
42	*xanthochroa*	*obtusifolia*	A	+			
43	*xanthochroa*	*obtusifolia*	A	+			
44	*xanthochroa*	*obtusifolia*	A	+			
46	*constructor*	*Cecropia* sp.	A	+	+	+	+
47	*xanthochroa*	*obtusifolia*	A	+		+	+
49	*xanthochroa*	*insignis*	A	+	+	+	+
50	*coerulepennis*	*insignis*	B	+	+	+	+
51	*constructor*	*insignis*	B	+			
52	*constructor*	*insignis*	B	+			
53	*alfari*	*insignis*	C	+			

A: La Selva Biological Station; B: Carara Biological Reserve; C: Highway 27 near Orotina.

Table 3.2. General sequence metrics for each sample. Included are the total number of sequences in each sample, percent chloroplast in each sample, total bacterial sequences in each sample, number of observed OTUs, and sequences remaining after removal of rare sequences.

Sample	# sequences	% chloroplast	bacterial sequences	S_{obs}	Abundant sequences
31workers	4203	0	4203	12	3825
31larvae	2901	3.65	2795	12	2669
31pupae	1573	0	1573	15	1472
31queen	2745	0	2745	11	2447
32workers	1265	0	1265	8	1146
33workers	1438	0	1438	10	1325
33larvae	1385	0	1385	1	1379
34workers	6988	0.01	6987	15	5650
34queen	1242	0	1242	13	1167
37workers	1605	0	1605	15	1382
37queen	2077	3.90	1996	12	1894
38workers	11218	0	11218	16	9667
39workers	1867	0	1867	4	1726
39queen	1555	0.13	1553	14	1418
40workers	10839	0.01	10838	21	8932
42workers	2586	0	2586	17	1988
43workers	11354	0	11346	25	7772
44workers	728	0.55	724	20	655
46workers	933	0	933	2	908
46larvae	7982	0	7982	4	7505
46pupae	4333	0	4333	3	4246
46queen	1601	0	1601	2	1570
47workers	18261	0.01	18255	20	13441
47pupae	6614	0	6614	20	4849
47queen	2259	0	2259	12	2084
49workers	5341	0.06	5338	20	4791
49larvae	2277	0	2277	11	2136
49pupae	2082	0.05	2081	11	1828
49queen	2800	0	2800	9	2477
50workers	1304	0	1304	10	970
50larvae	3663	0	3663	12	3094
50pupae	1370	0	1370	16	1181
50queen	5953	0	5953	11	5582
51workers	12168	0	12168	21	11370
52workers	9005	0	9005	16	7005
53workers	10270	0.02	10268	14	9415
total	165785		165570	85	
average	4605.1	0.23	4599.2	12.64	3915.7
SE	702.6	0.15	702.9	1.00	562.0

Table 3.3. Common OTUs found in at least half of all samples, half of *Azteca* workers, and half of *A. xanthochroa* workers. The highest taxonomic identification of each OTU possible is included, the proportion of samples in which these common OTUs appeared, and the average percent of sequences represented by each OTU.

Order, Family, *Genus*	All samples		*Azteca* Workers		*A. xanthochroa* workers	
	Out of 36	Avg. % (SE)	Out of 18	Avg. % (SE)	Out of 11	Avg. % (SE)
Actinomycetales	-------	-------	-------	-------	6	5.4 (1.9)
Actinomycetales, Nocardiaceae (87), *Gordonia* (87)	23	6.5 (1.7)	11	7.6 (3.3)	7	3.8 (0.9)
Actinomycetales, Propionibacteriaceae, *Propionibacterium*	19	4.2 (0.7)	-------	-------	-------	-------
Burkholderiales, Burkholderiaceae, *Ralstonia*	33	27.6 (3.8)	16	15.3 (3.3)	10	18.0 (4.7)
Burkholderiales, Comamonadaceae	-------	-------	-------	-------	7	4.8 (2.2)
Burkholderiales, Oxalobacteraceae	-------	-------	10	2.3 (0.3)	7	1.9 (0.2)
Enterobacteriales, Enterobacteriaceae, Enteric Bacteria cluster	20	18.9 (5.8)	12	18.4 (5.7)	9	20.1 (7.3)
Rhizobiales, Bradyrhizobiaceae, *Bradyrhizobium* (75)	21	4.1 (0.4)	-------	-------	6	2.7 (0.4)
Rhizobiales, Brucellaceae (60), *Ochrobactrum* (58)	32	6.4 (0.6)	17	5.7 (0.7)	11	6.6 (1.0)
Rhizobiales, Methylobacteriaceae (96), *Methylobacterium* (96)	19	3.9 (0.6)	10	3.8 (0.6)	8	4.2 (0.7)
Sphingomonadales, Sphingomonadaceae, *Sphingomonas* (77)	-------	-------	-------	-------	6	2.4 (0.2)
Xanthomonadales, Xanthomonadaceae, *Pseudoxanthomonas* (65)	20	5.0 (1.1)	14	4.0 (0.7)	10	4.1 (0.9)

Only confidence values under 100 are shown. Cells marked with dashes indicate that the OTU was not common for that group of samples.

Figure 3.1. Map of collection locations in Costa Rica. A: La Selva Biological Station; B: Carara Biological Reserve; C: Highway 27 near Orotina.
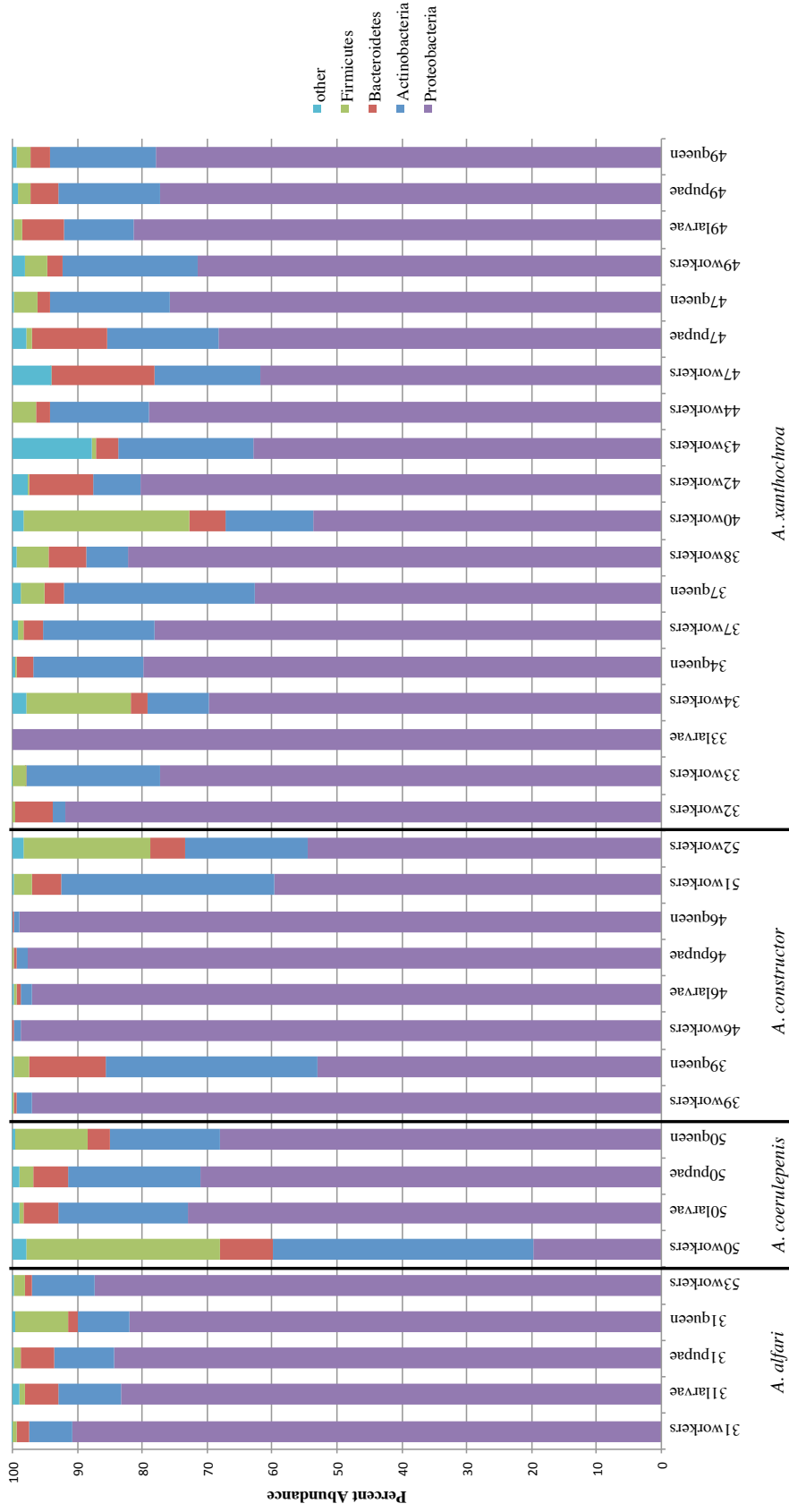
Figure 3.2. Classification of bacterial community members to the phylum level for each *Azteca* sample.
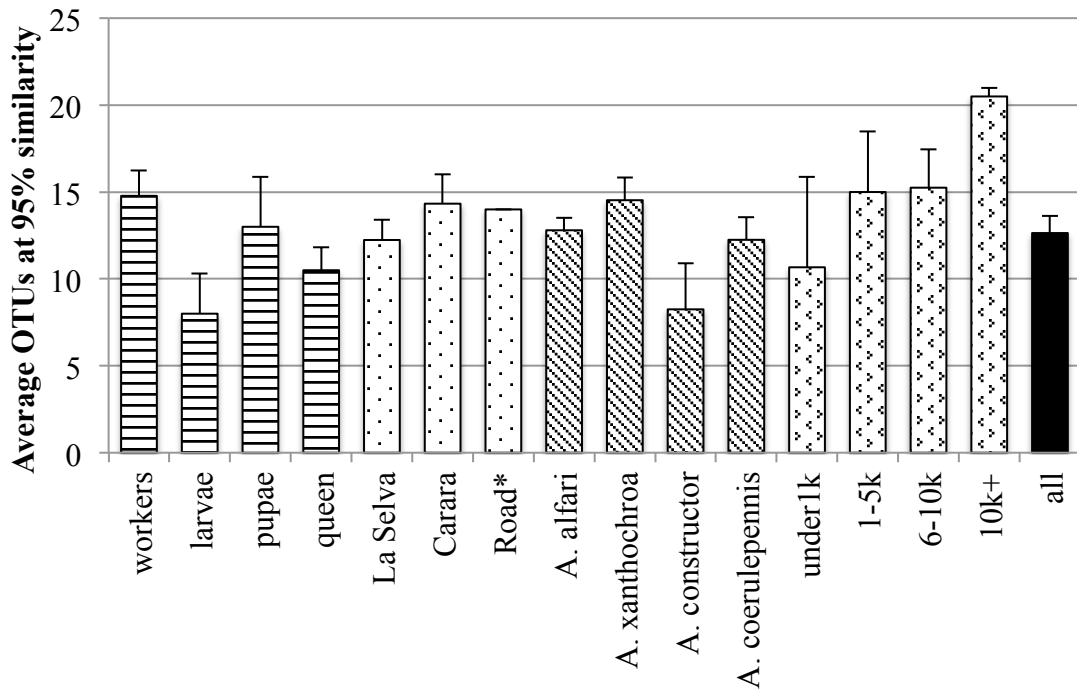
*n=1 for the Road sample.

Figure 3.3. Average number of abundant OTUs in various comparisons. This includes across various life stages, between locations, across different *Azteca* species, based on total number of sequences for samples, and across all samples. Error bars represent standard error.

A.

B.

C.
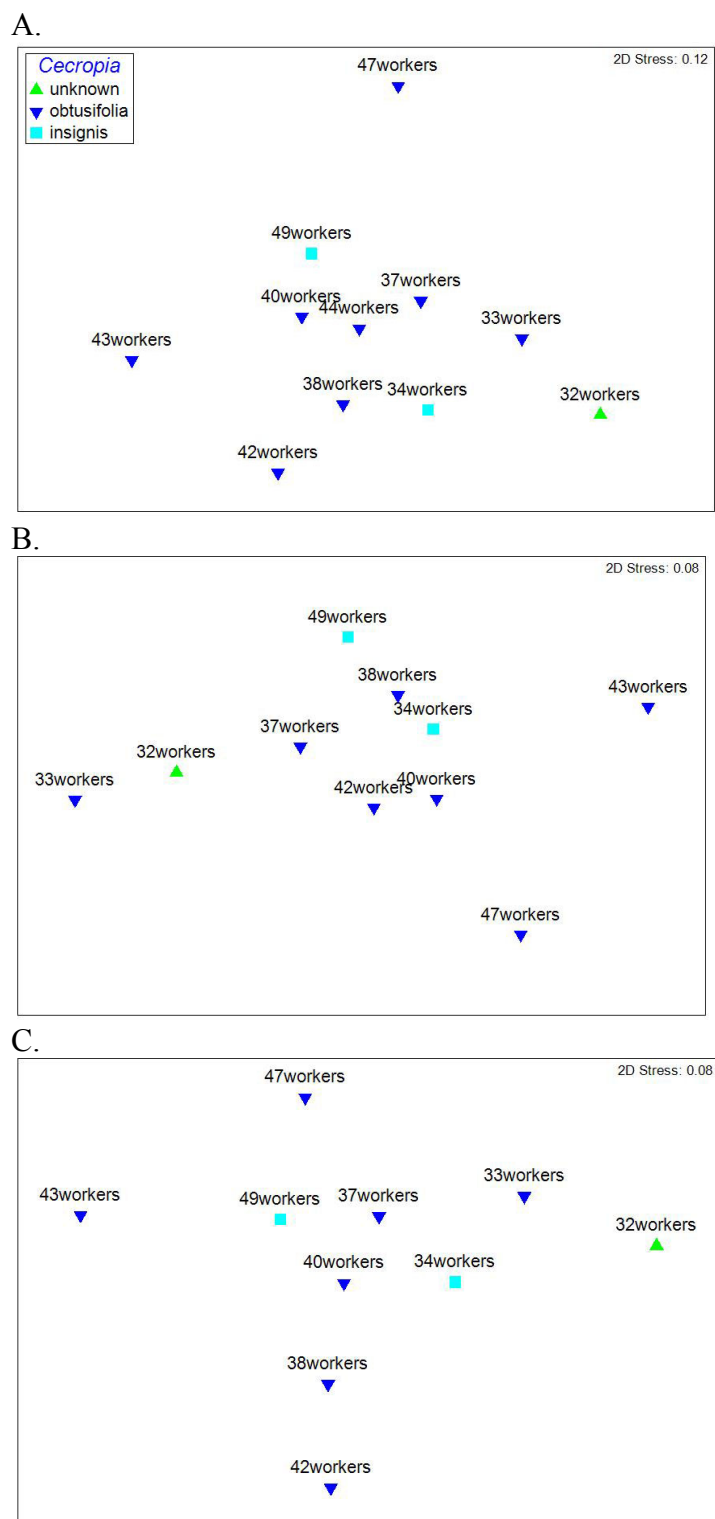
Figure 3.4. NMDS ordinations of the bacterial communities associated with *A. xanthochroa* workers. NMDS analyses based on (A) Bray-Curtis similarity matrix, (B) unweighted Unifrac (B) and (C) weighted Unifrac. The key in (A) is applicable for (B) and (C).

A.

B.

C.
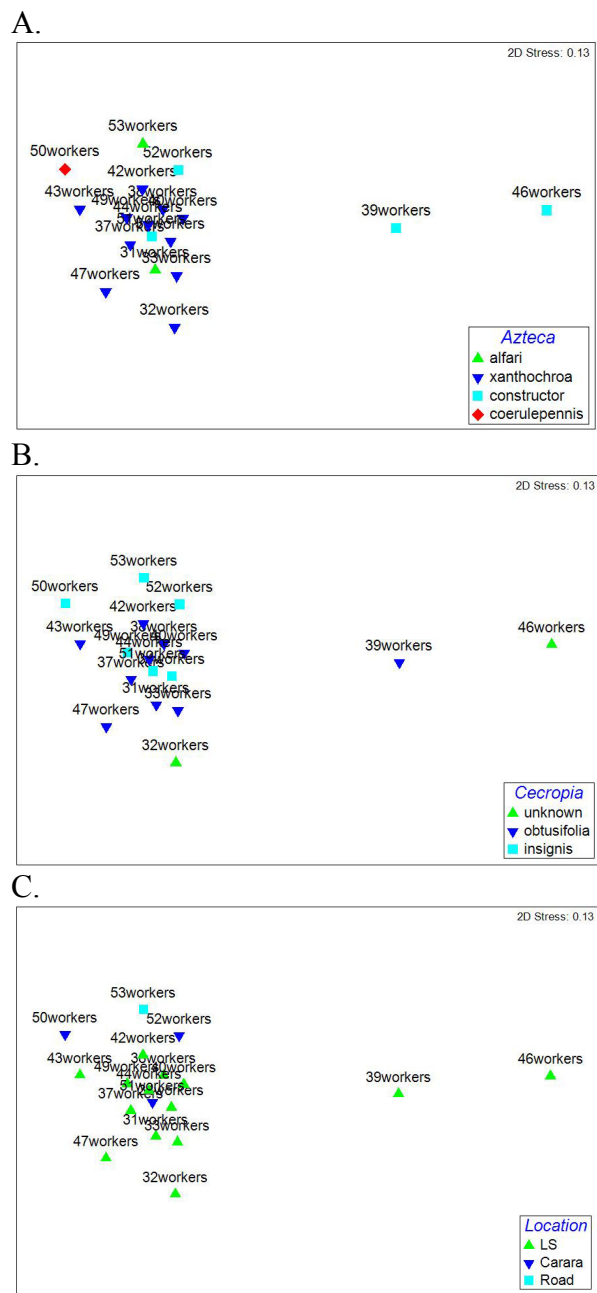
Figure 3.5. NMDS ordinations of the bacterial communities associated with *Azteca* workers.
NMDS analyses based on Bray-Curtis similarity matrix, viewed by *Azteca* species (A), *Cecropia* species (B), and location (C).
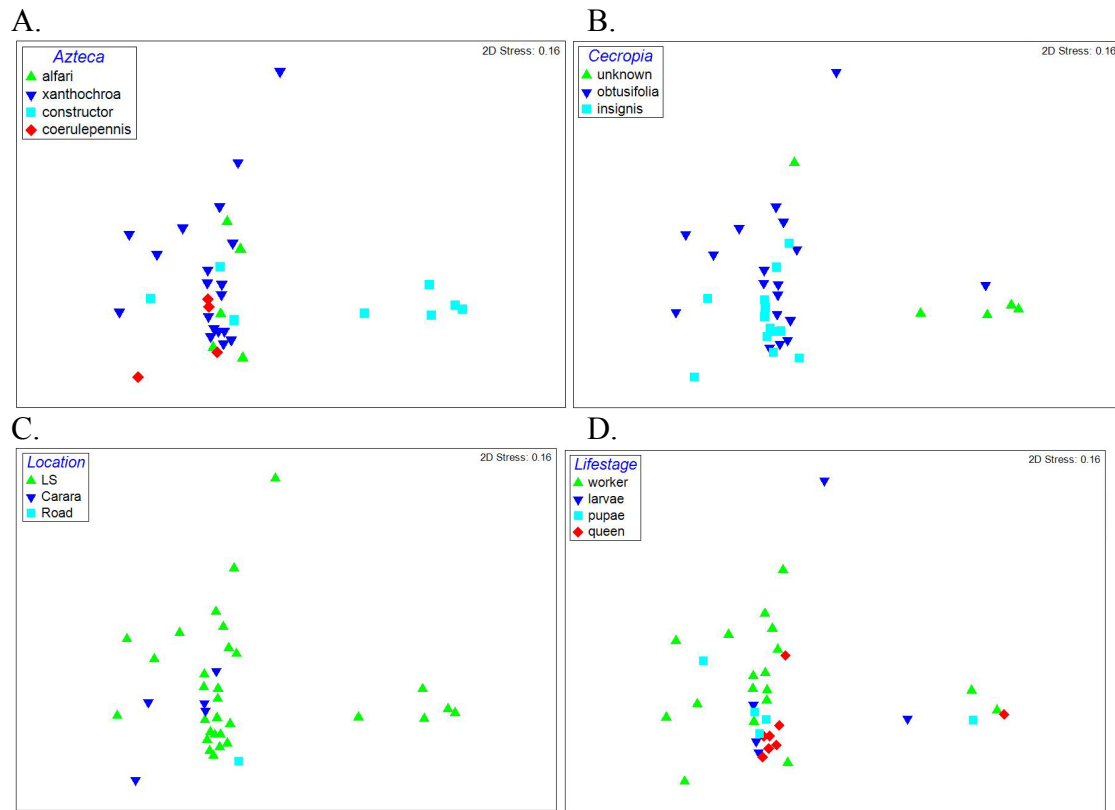
Figure 3.6. NMDS ordinations of the bacterial communities associated with all *Azteca* samples. NMDS analyses based on Bray-Curtis similarity matrix, viewed by *Azteca* species (A), *Cecropia* species (B), location (C), and life stage (D). Labels have been removed for the ease of seeing all data points.

Chapter 4. Host species and diet of arboreal ants shape their microbial communities

Manuscript in preparation: Alissa S Hanshew and Cameron R Currie

Coauthors contributed in the following ways: ASH designed and carried out the experiments, and wrote the paper. CRC provided lab material and support.

4.1 Abstract

Host factors, including phylogeny and trophic level, are thought to drive microbial community composition in animals. These factors influence many insect-associated microbial communities, but comparisons amongst ant species with different diets and life histories remains understudied. We collected 73 samples representing seven species of ants spanning three subfamilies of Formicidae, two different nesting strategies, and various trophic levels. We used 454 pyrosequencing to characterize and compare the bacterial communities of these ants. Our findings reveal that (i) the microbiota harbored by different ant species differs, (ii) that some of this variation is explained by the host's trophic level, and (iii) the microbial consortia are not influenced by nesting strategy. Our results support the hypothesis that herbivorous ants have a different bacterial consortium than omnivorous or predatory ants. This may be due to the herbivorous ant's need for additional sources of nitrogen that can be provided by microbes. While *Wolbachia* was a common taxon found in many of our samples, the abundance of sequences identified as Rhizobiales correlated with ant trophic level. These results suggest that while host species is the major driving force in microbial community composition, host diet is also significant, and that phylogenetically disparate ant species consuming similar diets have similarly structured microbial communities.

4.2 Introduction

Animals, including insects, rely on mutualistic bacteria for a variety of functions including, but not limited to, acquisition of nutrients. For example, pea aphids harbor the primary symbiont *Buchnera aphidicola*, one of the best described nutritional symbionts. *B. aphidicola* is directly involved in the production of essential amino acids for these phloem feeding insects that they cannot acquire from their diet [1, 2]. Similarly, *Sulcia muelleri* and *Baumannia cicadellinicola* are two bacterial endosymbionts in xylem feeding glassy-winged sharpshooters that produce amino acids, vitamins, and cofactors for their insect host [3–5]. Numerous insects are thought to require microbial symbionts to supplement nutrient poor food sources, including ants, in addition to other roles bacteria may serve in insects [6, 7].

Ants are dominant members of arboreal communities in the topics, yet much of their biology remains understudied [8]. Many ants were previously thought to be general carnivores, but the larger presence of ants in arboreal fogging samples than potential prey items suggests that ants are closer to the base of the trophic pyramid [9, 10]. Nitrogen isotope studies support this hypothesis, indicating that many ants are cryptic and functional herbivores, and perhaps complement their diet with animal protein when it is readily available [9, 10]. Herbivorous diets are often low in nitrogen and cannot account for the nitrogen needs of many insects [11]. Microbial symbionts have been suggested to provide this nutrient by either fixing or recycling nitrogen for their ant host.

Our understanding of the role of nutritional symbionts in ants is expanding, especially in regards to acquisition of nitrogen. The bacteria *Blochmannia floridanus* has been shown to recycle nitrogen, from urea to ammonia, for their ant host *Camponotus floridanus* [12]. Other ants in the tribe Camponotini also harbor *Blochmannia* that may serve the same purpose [13].

Nitrogen fixation has also been demonstrated in the fungus gardens of *Atta* and *Acromyrmex* ants, where the resulting usable nitrogen from *Pantoea* and *Klebsiella* was later detected in the ants [14]. Likewise, putative nitrogen fixers and recyclers have been found in other ants such as *Pseudomyrmex ferrugineus* and a number of different species of *Tetraponera* [15–17]. Additional research suggests that the need for nitrogen fixers and recyclers may also be tied to trophic level. In the guts of ants, a correlation has been found between herbivory and the presence of bacteria in the order Rhizobiales, many species of which are involved in nitrogen acquisition [18].

In addition to the function of specific bacteria in insects, the composition of bacterial communities is thought to be shaped by host phylogeny and diet. Native and invasive fire ants (*Solenopsis geminata* and *S. invicta*) have minimal differences in their bacterial communities, despite a different life history for the invasive *S. invicta* [19]. Host species was found to be more significant than location for the bacterial communities in the mycangia of ambrosia beetles, and phylogenetically related beetles did not necessarily have similar bacterial communities [20]. Similarly a study across the diversity of insects found that diet was a poor predictor of bacterial community composition, while more phylogenetically related hosts were more likely to have similar bacterial communities [21]. However, in this study, Jones *et al.* acknowledge that the effects of diet may be better resolved by focusing on more closely related hosts [21].

In this study we sought to address the influence of ant species, nesting strategy (obligate ant-plant or carton nester), and trophic level on the bacterial communities associated with arboreal ants. We characterized and compared the bacterial communities of seven species of arboreal ants at La Selva Biological Station in Costa Rica including *Azteca xanthochroa, Crematogaster longispina, Myrmelachista flavocotea, Nylanderia caeciliae, Pheidole bicornis,*

P. fiorii, and *Tapinoma ramulorum inrectum*. We hypothesized that hosts that are more phylogenetically related have similar bacterial communities, but that communities are also influenced by diet and life history.

4.3 Materials and methods

4.3.1 Colony collection

Five colonies each of *Crematogaster longispina, Myrmelachista flavocotea, Nylanderia caeciliae, Pheidole bicornis, Pheidole fiorii,* and *Tapinoma ramulorum inrectum* were collected from La Selva Biological Station in Costa Rica in July 2011 (Table 4.1). Five workers or five brood per colony were sterilely collected, pooled, and placed directly into 95% ethanol for transportation to the laboratory at the University of Wisconsin-Madison for DNA extraction.

4.3.2 DNA extraction, PCR, and 454 pyrosequencing

DNA extractions were performed as found in [22, 23]. Briefly, samples were removed from ethanol and ants were placed in 2 ml screw-top tubes with a single 3 mm steel bead and 500 μl of extraction buffer from an EpiCenter MasterPure Complete DNA and RNA Purification Kit (Illumina, Madison, WI). Samples were shaken in a Mini-beadbeater (Biospec Products, Bartlesville, OK) for 2.5 min. Following bead beating, DNA was extracted following the remainder of the DNA extraction kit instructions.

Preparation of 16S rRNA bacterial amplicons for 454 pyrosequencing were performed following the method of Hanshew *et al.* with the following parameters: forward primer 799F-mod6 at 250 nM final concentration, reverse primer 1392R at 250 nM final concentration, 20 ng of DNA for each sample, a total PCR volume of 25 μl, and performed with Herculase II DNA polymerase (Agilent Technologies, Santa Clara, CA) [22, 23]. Two-step PCR was performed, the first without primer modifications, and the second with primers with the 454 pyrosequencing A-

and B-adapters and 5 bp multiplex identifiers for each sample (Roche, Indianapolis, IN). 454 pyrosequencing with Lib-L Titanium chemistry was conducted on a Roche GS Junior following manufacturer's guidelines for amplicon sequencing with shotgun processing, using modifications described in [22, 23].

4.3.3 Data processing and statistical analysis

Sequence processing was performed using the bioinformatics program suite mothur (v 1.31.1) with standard amplicon processing pipeline with default parameters [24]. Worker and brood sequences from *A. xanthochroa* from La Selva Biological Station were included for comparison from previous work [22]. Alignment of processed sequences was done against the combined bacterial, archaeal, and eukaryotic Silva-derived database implemented in mothur, and chimeras were removed using the program UCHIME [25, 26]. Sequences identified as chloroplasts, eukaryotes, or unclassifiable at the kingdom level were removed after initial data assessment. Sequences were binned into operational taxonomic units (OTUs) at 95% similarity and any OTU with 2 or less reads in a sample was removed. Samples were standardized to 900 by randomly selecting sequences per sample prior to downstream analyses. Observed number of OTUs (S_{obs}), Good's coverage, Chao1, Simpson's Diversity, and Shannon's Evenness were computed in mothur. Clearcut, implemented in mothur, was used to create a relaxed neighbor joining tree of community structure, which was used for the creation of weighted and unweighted UniFrac dissimilarity matrices, also subsampled to 900 sequences [27, 28]. Differences between samples was assessed using non-metric multidimensional scaling (NMDS) analysis in PRIMER (v 6) using UniFrac dissimilarity matrices and a Bray-Curtis similarity matrix [27, 29]. Statistical differences in the overall community composition in workers was assessed using ant species, nesting strategy, and trophic level as factors using both the

Permutational multivariate analysis of variance (PERMANOVA) package in PRIMER and weighted UniFrac in mothur [30].

4.4 Results and Discussion

4.4.1 Analysis of 454 pyrosequencing data

We characterized the microbiota of seven species of arboreal ants in six genera and three subfamilies, totaling 73 samples (Table 4.1, Supplemental Table 4.1) from La Selva Biological Station in Costa Rica. 454 pyrosequencing provided a total of 232,256 sequences after all quality filtering steps and removal of nonbacterial sequences. The number of sequences per sample ranged from 994 to 18,234 (mean 3,181.6 ± 344.9 SE; Table 4.2; Supplemental Table 4.1) before the removal of rare reads. Sequences were on average 267.326 bp long. At the phylum level, samples had minimal diversity, with four phyla representing at least 84% of total sequences in each sample (Figure 4.1). While most of the sequences were identified as Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes, some ants also had a small portion of their total sequences that were classified as Acidobacteria, Planctomycetes, a bacterial group unclassifiable at the phylum level, and other low abundance phyla (Figure 4.1).

Alpha diversity varied between the ant species. Samples had on average 50.7 (± 5.7 SE) OTUs (Figure 4.2, Table 4.2, Supplemental Table 4.1). Good's coverage approached one for all samples indicating that they were sequenced to a sufficient depth (Table 4.2; Supplemental Table 4.1). Richness and evenness, measured by Chao1, and Shannon's and Simpson's indices, in samples also varied across ant species (Table 4.2; Supplemental Table 4.1). Some species, such as *P. bicornis*, were dominated by single OTUs, while others, such as *C. longispina*, were more diverse. The consistency of the microbiota of arboreal ants is possibly skewed here by our sampling technique. As we were interested in all bacteria associated with these ants, both

external and internal, whole insects were used. There is the potential that transient ectobionts are included in our data, and could influence the richness of the actual bacterial community members associated with these ants.

4.4.2 Comparisons across ant species and nesting strategy

Unweighted UniFrac, based solely on community membership, showed that the bacterial communities varied across the various ant species (Figure 4.3A). This is likely a product of the fact that 37% of OTUs after subsampling were only detected in single ant samples. However, when abundance was taken into account using weighted UniFrac, the separation between ant species became more apparent (Figure 4.3B). Similarly, when the communities were assessed using a Bray-Curtis similarity matrix, some ant species, *P. bicornis* and most samples of *T. inrectum,* became nearly indistinguishable, while others remained more diffuse (Figure 4.4). Nevertheless, the bacterial communities were significantly more similar within ant species than across species (Figure 4.4).

We used PERMANOVA to test the null hypothesis that no differences existed between the bacterial communities found in the workers of different ant species or nesting strategies. Results demonstrated that the bacterial communities are unique to individual ant species (*P*=0.001; Table 4.3). Pair-wise PERMANOVA showed significant differences in microbiota structures across only some of the ant species (Table 4.4, lower portion). This was further supported by weighted UniFrac (Table 4.4, upper portion). Surprisingly, samples within the same subfamily did not have similar bacterial communities. The two species in the subfamily Dolichoderinae, *A. xanthochroa* and *T. inrectum*, were significantly different from each other. This was also the case for the three species in the subfamily Formicinae, *M. flavocotea, P. bicornis*, and *P. fiorii*. This suggests that the driving force for microbial community composition

is not related to the phylogeny of the host. Similarly, PERMANOVA and weighted UniFrac

demonstrated that bacterial communities did not correlate with nesting strategy (Table 4.3)

4.4.3 The effect of trophic level on arboreal ant bacterial communities

Diet may also be a factor in determining the microbiota of these ants, which is suggested

to influence the composition of gut communities in other animals (Table 4.1) [18]. Amongst the

three obligate ant-plant systems studied here, only *M. flavocotea* occupies a plant, *Ocotea*, which

does not provide it with specialized food bodies or extrafloral nectar. These ants have relatively

high nitrogen signatures, suggesting that these ants might be predators and not rely on their host

tree for food, as found in many other ant-plant systems [10, 31]. The omnivore *P. bicornis*

consumes the eggs and early life stages of insects found on their host *Piper* tree in addition to

consuming pearl bodies produced by the plant [32]. *Cecropia*-obligate *Azteca* ants are thought to

be predominately herbivorous, consuming Müllerian bodies produced by their host *Cecropia*

tree, supplemented by honey dew produced by coccids in the domatia [33]. Less is known about

the dietary choices of the carton nesters. *C. longispina* and *P. fiorii* are thought to be

herbivorous, while almost nothing is known about the dietary choices of *N. caeciliae* and *T.*

inrectum (J. Longino, personal communication) [34, 35].

Previously it was shown that herbivorous ants are more likely to harbor Rhizobiales than

either omnivorous or predatory ants [18]. It has been suggested that these bacteria may be

integral in nitrogen acquisition for ants that consume nitrogen-poor substrates. The presence of

high levels of Rhizobiales in *A. xanthochroa* (12.8%), *C. longispina* (7.1%), *N. caeciliae* (8.4%),

and *P. fiorii* (9.1%), suggests that these ants are all herbivores (Figure 4.5). In contrast, the

microbial communities of the known predator and omnivore, *M. flavocotea* and *P. bicornis*,

contained very low levels of Rhizobiales, 0.7% and 0.6% of total sequences, respectively (Figure

4.5). *T. inrectum*, with 1.9% Rhizobiales, is unlikely to be an herbivore, and likely consumes substances with higher nitrogen content than that found in the extrafloral nectar they have been observed to consume [35].

Members of the family Enterobacteriaceae are also known to fix and recycle nitrogen in ants [12–14]. The presence of known insect-associated nitrogen fixers, such as *Pantoea* and *Klebsiella*, was inconsistent across our samples, and only found in abundances greater than 1% in five *A. xanthochroa* and two *T. inrectum* samples. However, there were two unclassified groups of Enterobacteriaceae associated with our samples, henceforth termed Enteric1 and Enteric2 (Figure 4.5). Enteric1 was present in one third of *A. xanthochroa* (5/15) and *T. inrectum* (3/10) samples. Enteric2 however was found in every sample and in high abundance in *M. flavocotea*. While *Blochmannia* was not identified in any of our ant samples using the Silva derived database, a blastN search with Enteric2 suggests this Gammaproteobacteria is closely related to *Baumannia* or *Blochmannia*, both bacteria associated with production of nutritive compounds in other insects [3, 13]. Adding the presence of Enteric2 with the high nitrogen signature of *M. flavocotea* suggests that these predators might consume the phloem-feeding Hemiptera that have been observed in their domatia [10, 31].

A single *Wolbachia* OTU was present in 85% of samples (62/73). The role of *Wolbachia* in ants remains unknown. While this bacterium is known to skew sex ratios in several insects, reproductive manipulation is unlikely in ants due their reproductive strategies [36, 37]. *P. bicornis* and most of the *T. inrectum* samples were almost completely dominated by *Wolbachia*, while *M. flavocotea* had more moderate levels (mean 21.5% ± 5.5). While this could be tied to the absence of Rhizobiales and perhaps tied to a non-herbivorous diet, *C. longispina*, a proposed herbivore, also had moderate levels of *Wolbachia*, but higher levels of Rhizobiales. Recent work

in *Acromyrmex octospinosus*, a leaf-cutting ant, suggests that *Wolbachia* could have a nutritional role as the bacteria are found predominately extracellularly in the gut [38]. We cannot exclude the possibility that the presence of *Wolbachia* may also be associated with trophic level.

4.5 Conclusion

The cross-Formicidae analyses presented here represent the broadest comparison of whole microbial communities in ants to date. The bacterial communities of these arboreal ants were host specific, but diet was also found to be a significant factor. Ants within the same trophic level were more likely to be similar than ants within the same subfamily, or even within the same genus. We also found trends in the abundance of certain bacterial groups that have been suggested to be associated with trophic level. These data support the hypotheses that bacterial communities are selected for by the environment of the host, but are also directly associated with dietary needs.

4.6 Acknowledgments

4.7 References

1.   Douglas AE, Prosser WA (1992) Synthesis of the essential amino acid tryptophan in the pea aphid (*Acyrthosiphon pisum*) symbiosis. Journal of Insect Physiology 38: 565–568

2.   Wilson ACC et al. (2010) Genomic insight into the amino acid relations of the pea aphid, *Acyrthosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. Insect Molecular Biology 19 Suppl 2: 249–258. doi:10.1111/j.1365-2583.2009.00942.x

3. Wu D et al. (2006) Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. PLoS Biology 4: e188. doi:10.1371/journal.pbio.0040188

4. Moran NA, Tran P, Gerardo NM (2005) Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum Bacteroidetes. Applied and Environmental Microbiology 71: 8802–8810. doi:10.1128/AEM.71.12.8802

5. Moran NA, Dale C, Dunbar H, Smith WA, Ochman H (2003) Intracellular symbionts of sharpshooters (Insecta: Hemiptera: Cicadellinae) form a distinct clade with a small genome. Environmental Microbiology 5: 116–126

6. Douglas AE (2009) The microbial dimension in insect nutritional ecology. Functional Ecology 23: 38–47

7. Douglas AE (2011) Lessons from studying insect symbioses. Cell Host & Microbe 10: 359–367. doi:10.1016/j.chom.2011.09.001

8. Davidson D (1997) The role of resource imbalances in the evolutionary ecology of tropical arboreal ants. Biological Journal of the Linnean Society 61: 153–181. doi:10.1006/bijl.1996.0128

9. Tobin JE (1991) A neotropical rainforest canopy, ant community: some ecological considerations. In: Huxley CR, Cutler DF (eds) Ant-Plant Interactions. Oxford University Press, Oxford, UK, pp 536–538

10. Davidson DW, Cook SC, Snelling RR, Chua TH (2003) Explaining the abundance of ants in lowland tropical rainforest canopies. Science 300: 969–972. doi:10.1126/science.1082074

11. Nardi JB, Mackie RI, Dawson JO (2002) Could microbial symbionts of arthropod guts contribute significantly to nitrogen fixation in terrestrial ecosystems? Journal of Insect Physiology 48: 751–763

12. Feldhaar H et al. (2007) Nutritional upgrading for omnivorous carpenter ants by the endosymbiont *Blochmannia*. BMC Biology 5: 48. doi:10.1186/1741-7007-5-48

13. Wernegreen JJ, Kauppinen SN, Brady SG, Ward PS (2009) One nutritional symbiosis begat another: Phylogenetic evidence that the ant tribe Camponotini acquired *Blochmannia* by tending sap-feeding insects. BMC Evolutionary Biology 9: 292. doi:10.1186/1471-2148-9-292

14. Pinto-Tomás AA et al. (2009) Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. Science 326: 1120–1123. doi:10.1126/science.1173036

15. Eilmus S, Heil M (2009) Bacterial associates of arboreal ants and their putative functions in an obligate ant-plant mutualism. Applied and Environmental Microbiology 75: 4324–4332. doi:10.1128/AEM.00455-09

16. Van Borm S, Buschinger A, Boomsma JJ, Billen J (2002) *Tetraponera* ants have gut symbionts related to nitrogen-fixing root-nodule bacteria. Proceedings of the Royal Society - Biological Sciences 269: 2023–2027. doi:10.1098/rspb.2002.2101

17. Stoll S, Gadau J, Gross R, Feldhaar H (2007) Bacterial microbiota associated with ants of the genus *Tetraponera*. Biological Journal of the Linnean Society 90: 399–412. doi:10.1111/j.1095-8312.2006.00730.x

18. Russell JA et al. (2009) Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. Proceedings of the National Academy of Sciences 106: 21236–21241. doi:10.1073/pnas.0907926106

19. Ishak HD et al. (2011) Bacterial diversity in *Solenopsis invicta* and *Solenopsis geminata* ant colonies characterized by 16S amplicon 454 pyrosequencing. Microbial Ecology 61: 821–831. doi:10.1007/s00248-010-9793-4

20. Hulcr J et al. (2012) Mycangia of ambrosia beetles host communities of bacteria. Microbial Ecology 64: 784–793. doi:10.1007/s00248-012-0055-5

21. Jones RT, Sanchez LG, Fierer N (2013) A cross-taxon analysis of insect-associated bacterial diversity. PLoS ONE 8: e61218. doi:10.1371/journal.pone.0061218

22. Hanshew AS, Currie CR ([date unknown]) The microbiota of *Cecropia*-obligate *Azteca* ants in Costa Rica. in preparation

23. Hanshew AS, Mason CJ, Raffa KF, Currie CR (2013) Minimization of chloroplast contamination in 16S rRNA gene pyrosequencing of insect herbivore bacterial communities. In review

24. Schloss PD, Gevers D, Westcott SL (2011) Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PLoS ONE 6: e27310. doi:10.1371/journal.pone.0027310

25. Pruesse E et al. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35: 7188–7196. doi:10.1093/nar/gkm864

26. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27: 2194–2200. doi:10.1093/bioinformatics/btr381

27. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R (2011) UniFrac: an effective distance metric for microbial community comparison. The ISME Journal 5: 169–172. doi:10.1038/ismej.2010.133

28. Evans J, Sheneman L, Foster J (2006) Relaxed neighbor joining: a fast distance-based phylogenetic tree construction method. Journal of Molecular Evolution 62: 785–792. doi:10.1007/s00239-005-0176-2

29. Clarke K, Gorley R (2006) PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth, UK.

30. Anderson MJ, Gorley RN, Clarke KR (2008) Permanova+ for PRIMER. PRIMER-E, Plymouth, UK.

31. McNett K et al. (2010) Stable isotope investigation of a cryptic ant-plant association: *Myrmelachista flavocotea* (Hymenoptera, Formicidae) and *Ocotea* spp. (Lauraceae). Insectes Sociaux 57: 67–72. doi:10.1007/s00040-009-0051-z

32. Dyer LA, Palmer ADN (2004) *Piper*: A Model Genus for Studies of Phytochemistry, Ecology, and Evolution. Kluwer Academic/Plenum Publishers, New York.

33. Longino JT (1991) *Azteca* ants in *Cecropia* trees: taxonomy, colony structure, and behaviour. In: Huxley CR, Cutler DF (eds) Ant-Plant Interactions. Oxford University Press, Oxford, pp 271–288

34. Kleinfeldt SE (1978) Ant-Gardens: The interaction of *Codonanthe crassifolia* (Gesneriaceae) and *Crematogaster Longispina* (Formicidae). Ecological Society of America 59: 449–456

35. Black II RW (1987) The Biology of Leaf Nesting Ants in a Tropical Wet Forest. Biotropica 19: 319–325

36.   Russell JA (2012) The ants (Hymenoptera: Formicidae) are unique and enigmatic hosts of prevalent *Wolbachia* (Alphaproteobacteria) symbionts. Myrmecological News 16: 7–23

37.   Wenseleers T, Sundström L, Billen J (2002) Deleterious *Wolbachia* in the ant *Formica truncorum*. Proceedings of the Royal Society - Biological Sciences 269: 623–629. doi:10.1098/rspb.2001.1927

38.   Andersen SB, Boye M, Nash DR, Boomsma JJ (2012) Dynamic *Wolbachia* prevalence in *Acromyrmex* leaf-cutting ants: potential for a nutritional symbiosis. Journal of Evolutionary Biology 25: 1340–1350. doi:10.1111/j.1420-9101.2012.02521.x

Table 4.1. **List of ant species analyzed in this paper and basic natural history information.**

Ant	Subfamily	Nesting site	Trophic Level	Diet
Azteca xanthochroa	Dolichoderinae	*Cecropia* trees	Herbivore	Muellerian bodies, Hemipteran honeydew
Crematogaster longispina	Myrmicinae	Carton nests	Herbivore	Fruits, and pulp and seed arils, extrafloral nectar, floral nectar
Myrmelachista flavocotea	Formicinae	*Ocotea* trees	Predator	Carnivores
Nylanderia caeciliae	Pseudomyrmecinae	Carton nests	Unknown[a]	No published information[a]
Pheidole bicornis	Formicinae	*Piper* trees	Omnivore	Pearl bodies, insect eggs and young insects
Pheidole fiorii	Formicinae	Carton nests	Herbivore	Seeds
Tapinoma ramulorum inrectum	Dolichoderinae	Carton nests	Unknown[a]	Extrafloral nectar?[a]

[a] The current assessment of the diets of *N. caeciliae* and *T. inrectum* are based on personal observations (J. Longino, personal communication).

Table 4.2. Average number of sequences, number of observed OTUs, sequence coverage, richness and evenness of bacterial communities in each ant species.

	Average No. Sequences	S_{obs}	Good's coverage	Chao	Shannon	Simpson
A. xanthochroa	5895.4	60.36	0.98	73.32	2.6	0.22
C. longispina	3361.8	85.8	0.97	113.47	2.28	0.37
M. flavocotea	2488.2	17.1	1.00	19.07	0.97	0.3
N. caeciliae	2255.9	75.33	1.00	85.15	2.61	0.55
P. bicornis	2040.4	5.33	1.00	5.43	0.09	0.97
P. fiorii	2020	74.89	0.99	80.2	2.71	0.25
T. inrectum	3009.7	32.9	0.99	39.95	1.02	0.67

Table 4.3. PERMANOVA results of ant species and nest strategy using Bray-Curtis similarity matrix for workers.

Factor	d.f.	SS	MS	Pseudo-F	P
Ant species	6	67745	11291	5.3634	0.001
Nest strategy	1	6640.9	6640.9	1.9126	0.07

d.f, degrees of freedom; SS, sum of squares; MS, mean squares.

Table 4.4. **Comparison of bacterial communities in workers using Pairwise PERMANOVA and weighted UniFrac.** PERMANOVA values appear in the lower half; UniFrac in the upper half.

	A. xanthochroa	C. longispina	M. flavocotea	N. caeciliae	P. bicornis	P. fiorii	T. inrectum
A. xanthochroa	-	0.83692	0.934771	0.805992	0.873447	0.87256	0.82608
C. longispina	0.002	-	0.895907	0.928359	0.375733	0.829703	0.37555
M. flavocotea	0.001	0.005	-	0.914955	0.911413	0.771276	0.890949
N. caeciliae	0.003	0.007	0.008	-	0.95236	0.862628	0.95236
P. bicornis	0.003	0.069	0.018	0.011	-	0.861839	0.221996
P. fiorii	0.003	0.031	0.162	0.008	0.026	-	0.86154
T. inrectum	0.001	0.19	0.019	0.013	0.491	0.024	-
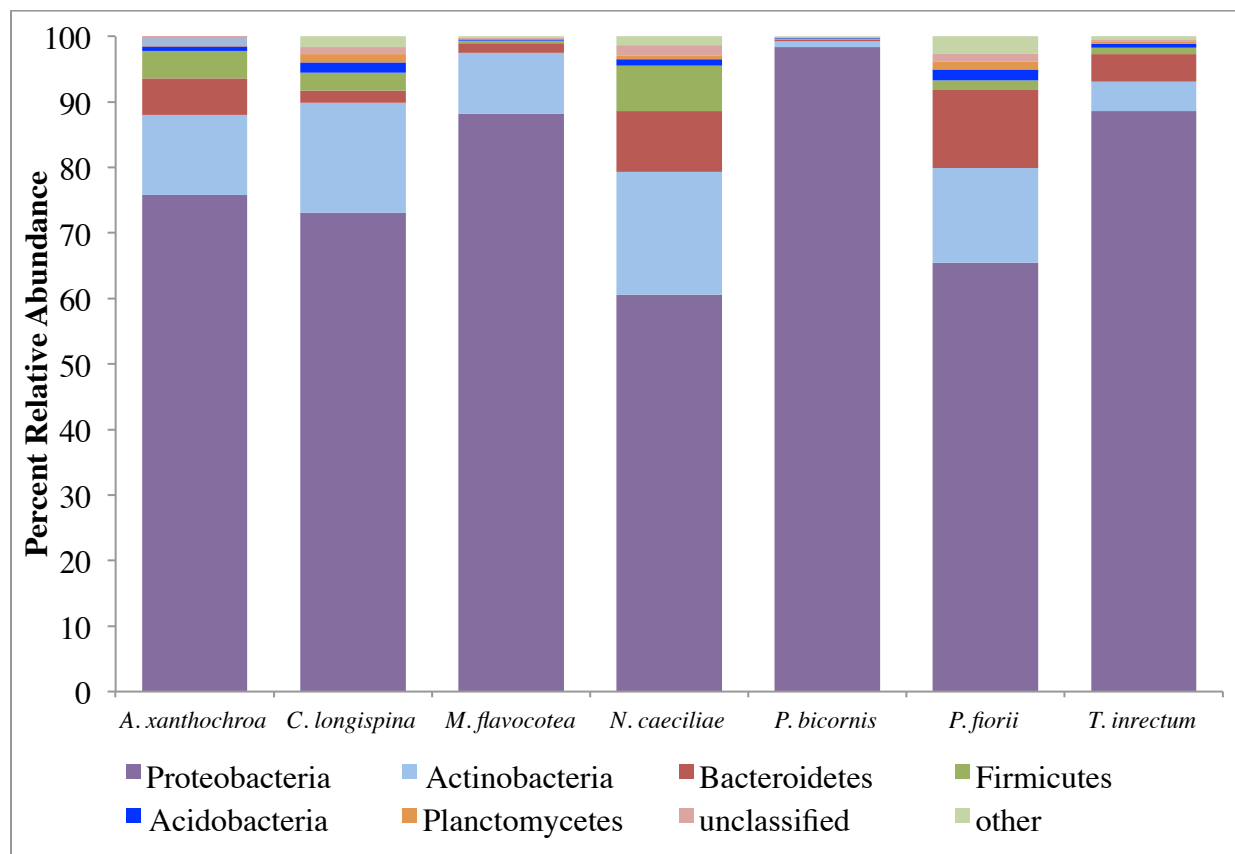
Figure 4.1. Classification of microbiota community members for each ant system. Values are averaged across brood and workers within each ant species.
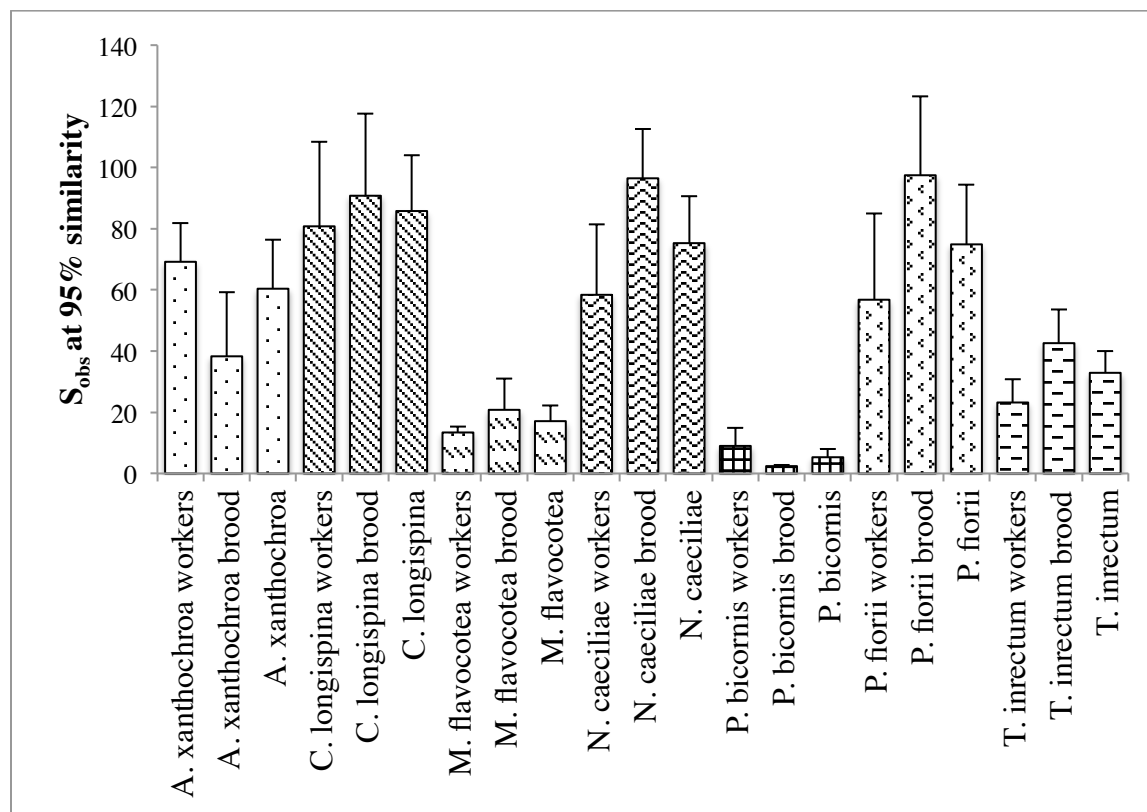
Figure 4.2. Average number of observed OTUs at 95% similarity after subsampling to 900 sequences. Error bars represent standard error.

A.

2D Stress: 0.13

B.

2D Stress: 0.19

C.

▼ *A. xanthochroa* ✛ *M. flavocotea* ✖ *P. bicornis* △ *T. inrectum*

◆ *C. longispina* ● *N. caeciliae* ▽ *P. fiorii*
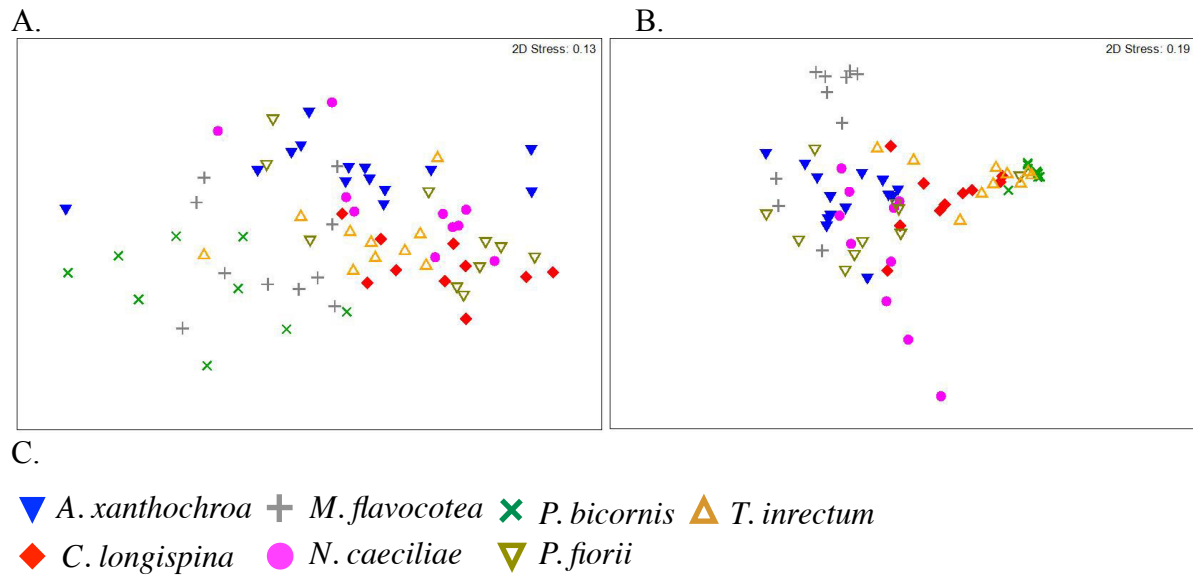
Figure 4.3. NMDS plots using unweighted (A) and weighted (B) UniFrac. (C) Key for figures 4.3 and 4.4.

2D Stress: 0.16
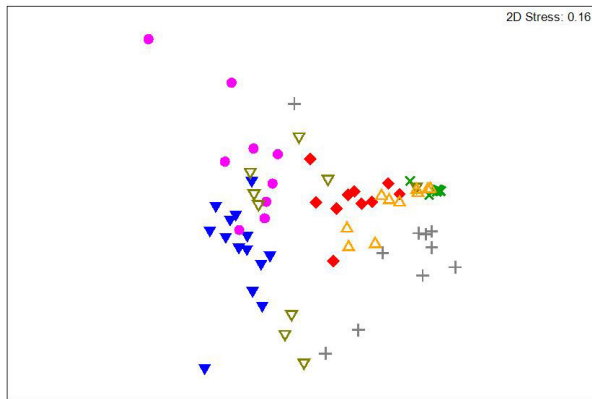
Figure 4.4. NMDS plot using a Bray-Curtis distance matrix.
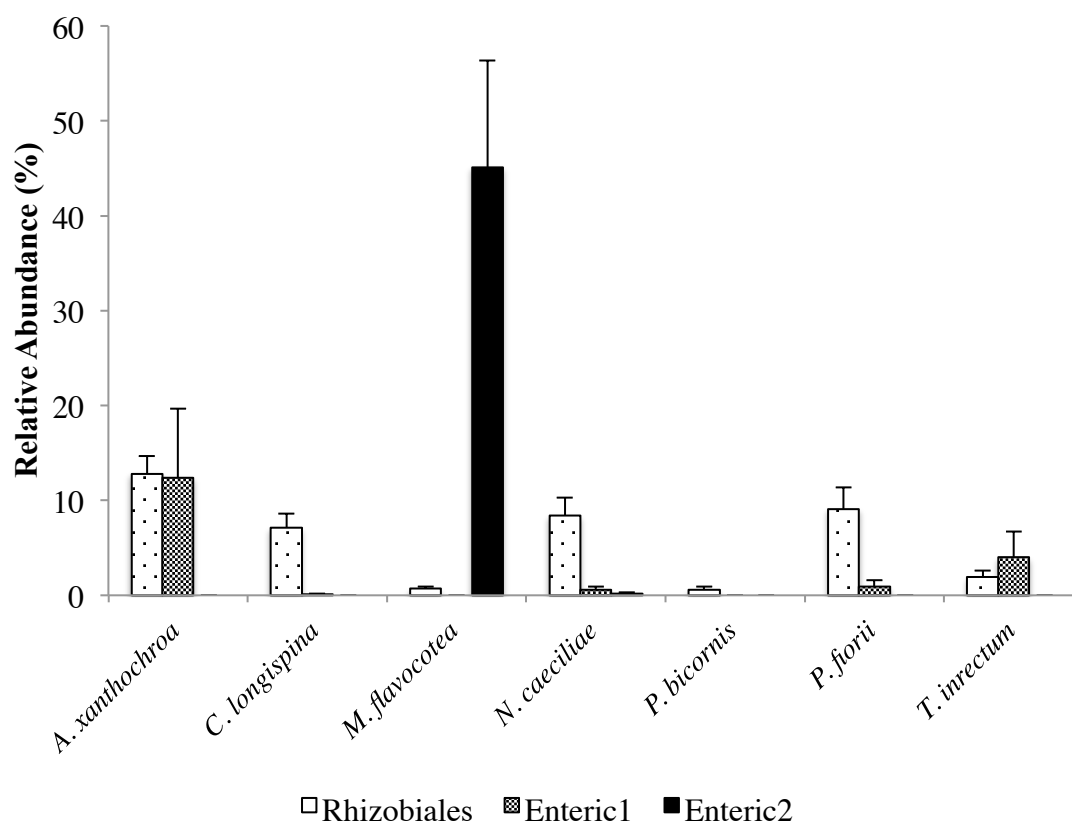
Figure 4.5. Average relative abundance of sequences identified as Rhizobiales, Enteric1, and Enteric2. Error bars represent standard error.

Supplemental Table 4.1. Number of sequences, observed OTUs, sequence coverage, richness and evenness of bacterial communities in each sample.

Sample Code	Ant species	Life Stage	Bacterial Sequences	S_{obs}	Good's coverage	Chao	Shannon	Simpson
Ax-W32	A. xanthochroa	workers	1263	22	1.00	25.00	1.43	0.44
Ax-W33	A. xanthochroa	workers	1438	24	1.00	24.00	2.16	0.18
Ax-L33	A. xanthochroa	brood	1385	1	1.00	1.00	0	1.00
Ax-W34	A. xanthochroa	workers	6934	72	0.98	81.00	2.86	0.12
Ax-W37	A. xanthochroa	workers	1604	39	1.00	39.75	2.74	0.11
Ax-W38	A. xanthochroa	workers	11234	61	0.98	71.91	2.89	0.11
Ax-W40	A. xanthochroa	workers	10713	76	0.98	95.09	3.38	0.05
Ax-W42	A. xanthochroa	workers	2584	82	0.98	97.00	3.08	0.12
Ax-W43	A. xanthochroa	workers	11190	143	0.94	183.83	4.14	0.03
Ax-W47	A. xanthochroa	workers	18234	123	0.95	166.24	3.70	0.05
Ax-P47	A. xanthochroa	brood	6335	98	0.97	131.21	3.49	0.08
Ax-W49	A. xanthochroa	workers	5297	50	0.99	56.43	2.53	0.19
Ax-L49	A. xanthochroa	brood	2247	24	1.00	24.00	1.94	0.29
Ax-P49	A. xanthochroa	brood	2078	30	1.00	30.00	2.08	0.27
Cl-W1	C. longispina	workers	2647	27	0.98	40.00	0.49	0.84
Cl-B1	C. longispina	brood	1474	74	1.00	74.55	3.40	0.07
Cl-W2	C. longispina	workers	4242	74	0.97	87.80	1.77	0.46
Cl-B2	C. longispina	brood	3442	186	0.93	235.88	4.31	0.04
Cl-W3	C. longispina	workers	9478	176	0.90	297.42	3.08	0.24
Cl-B3	C. longispina	brood	4180	109	0.95	160.25	3.15	0.14
Cl-W4	C. longispina	workers	2325	100	0.97	118.91	2.77	0.24
Cl-B4	C. longispina	brood	2010	49	0.99	51.50	1.24	0.60
Cl-W5	C. longispina	workers	1929	27	0.99	31.00	0.67	0.79
Cl-B5	C. longispina	brood	1891	36	0.99	37.36	1.92	0.27
Mf-W1	M. flavocotea	workers	994	13	1.00	13.00	1.19	0.38
Mf-B1	M. flavocotea	brood	1846	19	0.99	20.67	0.87	0.11
Mf-W2	M. flavocotea	workers	1900	11	1.00	11.25	0.84	0.97
Mf-B2	M. flavocotea	brood	1787	2	1.00	2.00	0.41	0.63
Mf-W3	M. flavocotea	workers	1850	12	1.00	12.50	0.95	0.07
Mf-B3	M. flavocotea	brood	1497	6	1.00	6.00	0.60	0.13
Mf-W4	M. flavocotea	workers	5541	10	1.00	10.00	0.93	0.04
Mf-B4	M. flavocotea	brood	3415	17	0.99	28.25	0.87	0.07
Mf-W5	M. flavocotea	workers	2928	21	1.00	22.20	1.45	0.15
Mf-B5	M. flavocotea	brood	3124	60	0.98	64.79	1.57	0.48
Nc-W1	N. caeciliae	workers	1976	48	0.99	51.11	1.88	0.62
Nc-B1	N. caeciliae	brood	1714	58	1.00	58.17	3.08	0.50
Nc-W2	N. caeciliae	workers	2182	5	1.00	5.00	0.09	0.75

Nc-B2	*N. caeciliae*	brood	1061	na	na	na	na	na
Nc-W3	*N. caeciliae*	workers	1021	23	1.0	23.0	1.0	0.5
Nc-B3	*N. caeciliae*	brood	1618	83	1.0	83.5	3.6	0.6
Nc-W4	*N. caeciliae*	workers	3339	81	1.0	93.2	3.0	0.6
Nc-B4	*N. caeciliae*	brood	2890	130	1.0	144.0	4.1	0.5
Nc-W5	*N. caeciliae*	workers	3766	135	1.0	163.7	3.8	0.3
Nc-B5	*N. caeciliae*	brood	2992	115	1.0	144.7	3.0	0.5
Pb-B1	*P. bicornis*	brood	3661	3	1.0	3.0	0.1	1.0
Pb-W2	*P. bicornis*	workers	1294	27	1.0	27.9	0.6	0.8
Pb-B2	*P. bicornis*	brood	1016	1	1.00	1.00	0	1
Pb-W3	*P. bicornis*	workers	1415	2	1.00	2.00	0.01	1.00
Pc-B3	*P. bicornis*	brood	1005	3	1.00	3.00	0.03	0.99
Pb-W4	*P. bicornis*	workers	1820	3	1.00	3.00	0.02	0.99
Pb-B4	*P. bicornis*	brood	1601	3	1.00	3.00	0.03	0.99
Pb-W5	*P. bicornis*	workers	4073	4	1.00	4.00	0.04	0.99
Pb-B5	*P. bicornis*	brood	2479	2	1.00	2.00	0.02	1.00
Pf-W1	*P. fiorii*	workers	1326	18	1.00	18.25	1.72	0.29
Pf-B1	*P. fiorii*	brood	1064	na	na	na	na	na
Pf-W2	*P. fiorii*	workers	2203	19	1.00	19.00	0.36	0.89
Pf-B2	*P. fiorii*	brood	1457	42	0.99	43.88	1.86	0.29
Pf-W3	*P. fiorii*	workers	3273	10	1.00	10.00	0.92	0.51
Pf-B3	*P. fiorii*	brood	2207	118	0.98	122.94	4.00	0.04
Pf-W4	*P. fiorii*	workers	1489	78	1.00	78.08	3.14	0.14
Pf-B4	*P. fiorii*	brood	1265	71	1.00	71.00	3.60	0.05
Pf-W5	*P. fiorii*	workers	2589	159	0.96	179.09	4.54	0.02
Pf-B5	*P. fiorii*	brood	3327	159	0.95	179.57	4.26	0.03
Ti-W1	*T. ramulorum inrectum*	workers	1658	18	0.99	20.50	0.33	0.90
Ti-B1	*T. ramulorum inrectum*	brood	3139	47	0.98	68.00	1.02	0.69
Ti-W2	*T. ramulorum inrectum*	workers	1057	51	1.00	51.00	2.74	0.14
Ti-B2	*T. ramulorum inrectum*	brood	2154	82	0.98	96.00	2.50	0.25
Ti-W3	*T. ramulorum inrectum*	workers	1432	13	1.00	16.00	0.39	0.86
Ti-B3	*T. ramulorum inrectum*	brood	2371	32	1.00	32.38	0.79	0.75
Ti-W4	*T. ramulorum inrectum*	workers	2843	26	0.99	32.43	0.57	0.81
Ti-B4	*T. ramulorum inrectum*	brood	5033	36	0.98	49.00	1.55	0.37
Ti-W5	*T. ramulorum inrectum*	workers	2752	8	1.00	11.00	0.10	0.97
Ti-B5	*T. ramulorum inrectum*	brood	7658	16	0.99	23.20	0.19	0.95
Total			232,256	1556				

na: not available. These samples had less than 900 sequences after removal of rare reads, and were removed from further analyses.

Chapter 5. Summary and Future Directions

In this dissertation I explored the microbial ecology of tropical arboreal ants. In Chapter 2 I presented data on the presence and abundance of culturable Actinobacteria in three ant-plant-fungi mutualisms. *Pseudomyrmex penetrator*/*Tachigali* sp. from French Guiana, *Petalomyrmex phylax*/*Leonardoxa africana* and *Crematogaster margaritae*/*Keetia hispida*, both from Cameroon, all contained culturable Actinobacteria that spans the phylogeny of the phylum. Isolates from all three systems had antifungal properties, with the *Streptomyces* isolates producing larger zones of inhibition than the *Nocardiodes* isolates. *Streptomyces* isolates from *P. phylax*/*L. africana* and *C. margaritae*/*K. hispida* were capable of degrading cellulose *in vitro*. Together, this suggests that these ant-plant-fungi systems are niches for Actinobacteria, and that these bacteria could be playing protective or nutritional roles. In Chapter 3, using 454 pyrosequencing I described for the first time the bacteria communities associated with Costa Rican *Cecropia*-obligate *Azteca* ants. Actinomycetales, Burkholderiales, Enterobacteriales, Pseudomonadales, Rhizobiales, and Xanthomonadales were common bacterial orders in these samples regardless of host ant or tree species. There was high variability between bacterial communities, but this variation does not correlate with *Azteca* species, *Cecropia* species, nor geographic location. The variability of the bacterial communities could be associated with life stage, as queens were more similar to each other than any other life stage, or it might correlate with the primary food source colonies are capable of acquiring. In Chapter 4 I explored the impact of trophic level, nesting strategy, and phylogeny on the bacterial communities in seven arboreal ant species from La Selva Biological Station. Using 454 pyrosequencing I showed that hosts from the same genera, or even subfamily, did not necessarily have similar bacterial

communities. Likewise, nesting strategy in the form of ant-plant mutualisms versus carton nesters did not explain the variation we saw in community structure. Diet is likely a major factor in deterring bacterial community structure and can partially be explained by the nitrogen needs of functionally herbivorous ants.

There are numerous experiments that I can envision would add greatly to our knowledge about the roles of bacteria in arboreal ants, and the factors that explain the presence and absence of certain bacteria. To expand on the data I presented in Chapter 2, I think it would be interesting to better explore the antifungal properties of the strains I isolated. Testing these bacteria against the known fungal symbiont (Chaetothyriales) would provide the first clue on whether these Actinobacteria are indeed mutualists and not just transients or potential pathogens in these systems. Likewise, testing against more applicable pathogens from these systems would serve better to indicate if these bacteria do indeed serve as protective symbionts.

The use of 454 pyrosequencing, especially in describing the microbial communities of arthropods, is still in its infancy. With a better handle now on what we can do with and get from next-gen sequencing, I think it is now becoming important to ensure proper sampling techniques, and to start to add in additional support for the conclusions many draw from their data. While I made a modest effort to collect sufficient sample sizes of the ant species that were subjected to 454 pyrosequencing here, I do think repeat sequencing of *Azteca alfari, A. coerulepennis,* and *A. constructor* would be informative. Recent work suggests that was closely related species are reproductively separate due in large part to the difference in their microbial consortia. A similar scenario could be at play in *Cecropia*-obligate *Azteca* ants, as some of these ant species are so similar, they are nearly impossible to tell apart by workers. Perhaps the differences in microbial communities could be keeping the species reproductively isolated.

Additionally, collecting samples from the same colonies for both N isotope studies and sequencing at the same time would be more indicative of the link between trophic level and microbial community composition. Many ants, including a few species used here, are facultative predators. Likely some of the variation we observed in community structure is due to this variation in diet, which likely also varies over time as the diet of the colony as a whole shifts based on season and availability of potential prey items. Studying multiple indicators of diet at the same time would be more informative, and provide better evidence to the idea that host communities are intimately linked to life style in insects and not just a random assortment of bacteria that they happen to encounter during their life cycle.

Appendix 1. Minimization of chloroplast contamination in 16S rRNA gene pyrosequencing of insect herbivore bacterial communities

Coauthors contributed in the following ways. *These authors contributed equally to this work. ASH and CJM designed the experiments, performed all the experiments, and wrote the manuscript. CRC and KFR provided lab material and support.

A1.1 Abstract

Chloroplast sequence contamination in 16S ribosomal RNA gene (16S) analyses can be particularly problematic when sampling microbial communities in plants and folivorous arthropods. We previously encountered high levels of plastid contamination in herbivorous insect samples when we used the predominant 454 pyrosequencing 16S methodologies described in the literature. 799F, a primer previously found to exclude chloroplast sequences, was modified to enhance its efficacy, and we describe, in detail, our methodology throughout amplicon pyrosequencing. Thirteen versions of 799F were assessed for the exclusion of chloroplast sequences from our samples. We found that a shift in the mismatch between 799F and chloroplast 16S resulted in significant reduction of chloroplast reads. Our results also indicate that amplifying sequences from environmental samples in a two-step PCR process, with the addition of the multiplex identifiers and 454 adapters in a second round of PCR, further improved primer specificity. Primers that included 3' phosphorothioate bonds, which were designed to block primer degradation, did not amplify consistently across samples. The different forward primers do not appear to bias the bacterial communities detected. We provide a methodological framework for reducing chloroplast reads in high-throughput sequencing data sets that can be applied to a number of environmental samples and sequencing techniques.

A1.2 Introduction

The advent of next-generation sequencing technologies has revolutionized methods for studying microbial ecology and symbiosis. Barcoded amplicon pyrosequencing has enabled researchers to multiplex samples and generate a tremendous amount of data in a variety of systems (Hamady et al., 2008; Weinstock, 2012). Microbial communities associated with arthropods and plants have received increased attention during this time. Studies on insects such as those describing establishment of a core microbiome (Moran et al., 2012), microbial transmission (Sudakaran et al., 2012), community dynamics through development (Wong et al., 2011), phylogenies (Hulcr et al., 2012), and across populations (Jones et al., 2011) have been made possible from these technological advancements. Additionally, these advances have improved our understanding of the mechanisms and ecological consequences of bacterial community acquisition in plants (Bengtsson et al., 2012; Gottel et al., 2011; Koopman et al., 2010; Redford et al., 2010).

While these studies utilize a diversity of model insects and plants, their investigations lack information on bacterial communities of folivorous insects. There are major methodological obstacles in addressing questions pertaining to foliage-feeding herbivores as several of the published primer pairs used in bacterial 16S ribosomal RNA gene (henceforth abbreviated 16S) pyrosequencing are not feasible for projects studying these systems. The homology between bacterial 16S, chloroplast 16S, plant nuclear and mitochondrial 18S rRNA genes (18S), and arthropod 18S leads to challenges in choosing the appropriate primer pairs. For example, the commonly used universal bacterial primers 926F and 1392R are sufficiently similar to arthropod 18S, resulting in the amplification of these non-bacterial sequences (Dams et al., 1988). The universal 16S primers targeting hypervariable regions V1-V3 (27F, 338R, 519R), V3-V6 (534F,

926F, 1114R), and V6-V8 (926F, 1392R), used in other insect systems (Andreotti et al., 2011; Fagen et al., 2012; Hail et al., 2012, 2011; Hulcr et al., 2012; Jones et al., 2013, 2010; Lalzar et al., 2012; Moran et al., 2012; Osei-Poku et al., 2012; Palavesam et al., 2012; Reid et al., 2011; Wang et al., 2011; Wong et al., 2011) are homologous to chloroplast 16S (Dams et al., 1988; Lane, 1991; Rastogi et al., 2010). Many of the phytophagous insects covered in the pyrosequencing literature consume substrates other than leaves, such as phloem, wood, xylem, and seeds, that contain low abundances of chloroplasts (Fagen et al., 2012; Hail et al., 2012, 2011; Köhler et al., 2012; Reid et al., 2011; Salem et al., 2013; Sudakaran et al., 2012). However, in several pyrosequencing studies focusing on insects, authors have reported unintended chloroplast contamination of varying degrees (Hulcr et al., 2012; Kelley and Dobler, 2011; Moran et al., 2012; Reid et al., 2011; Russell et al., 2013).

For the study of maize roots, Chelius and Triplett (2001) designed 799F, a primer intended to amplify bacterial 16S sequences while avoiding chloroplast 16S sequences, by using a two base pair mismatch on the 3' end of the primer (Chelius and Triplett, 2001). This primer, and other versions designed from it (799F2, 783R, 783Rabc), have been sufficient for some plant systems (Edwards et al., 2007; Rastogi et al., 2010; Sakai et al., 2004; Sun et al., 2008), but it has not been entirely effective in eliminating amplification of chloroplast 16S sequences in all samples (Bodenhausen et al., 2013; Bulgarelli et al., 2012; Leveau and Tech, 2011; Sagaram et al., 2009; Shade et al., 2013). Others have made no direct comments on the presence or absence of chloroplast sequences in their data sets (Jones et al., 2013; Lundberg et al., 2012; Rastogi et al., 2012; Redford and Fierer, 2009; Redford et al., 2010). To our knowledge, this primer has not been applied in arthropod systems.

Chloroplasts are evolutionarily descended from bacteria, so it is not surprising that the 16S genes are nearly homologous between the two. One of the only regions appropriate for primer design that allows broad bacterial 16S amplification while potentially blocking chloroplast 16S exists between positions 783-799 of the 16S gene (*E. coli* numbering system). Chloroplast 16S genes have two base pair mismatches at positions 798 and 799 that Chelius and Triplett took advantage of in designing 799F and two additional mismatches at positions 783 and 784 (Chelius and Triplett, 2001). Mismatches between the 3' end of the primer and the targeted sequence are commonly thought to block amplification (Klindworth et al., 2013; Kumar et al., 2011; Lane, 1991; Nossa et al., 2010; Rastogi et al., 2010; Sipos et al., 2007; Wang and Qian, 2009). As noted by Chelius and Triplett, these minor differences can be exploited in an attempt to avoid chloroplast 16S sequences (Chelius and Triplett, 2001; Sakai et al., 2004). Primer design may also have a major influence on data analysis and interpretation of bacterial community data sets. There is an extensive literature debating which of the various primer combinations are best suited for 16S pyrosequencing projects, but much of this work has been done using marine ecosystems (Klindworth et al., 2013) or the Human Microbiome Project (Kumar et al., 2011; Nossa et al., 2010) as models.

Additional complications in primer design include length of the primer, and potential exonuclease activity from error correcting polymerases. Primers that are too short will anneal to non-targeted sequences while primers that are too long will be overly specific which may incorporate additional biases in pyrosequencing (Sergeant et al., 2012). Moreover, exonuclease activities observed in error correcting polymerases can degrade primers, causing unintended amplification of undesired targets (Ahn et al., 2012). Modifications on the 3' ends of primers,

such as phosphorothioate bonds, create highly stable primers that are not degraded by polymerase exonuclease activities and may be used to circumvent this polymerase issue.

We found that following the current methodology in the published insect bacterial 16S literature was inadequate for our samples, resulting in significant loss of sequencing reads to chloroplast or arthropod sequences, rendering our data useless. Here we test modifications that improve upon the previously published primer, 799F, and show how these modifications perform in 454 pyrosequencing of herbivorous insect samples (Chelius and Triplett, 2001). We tested these primers in two distantly related arthropods that consume plant substrates with high chloroplast abundances, gypsy moth (*Lymantria dispar*) and the herbivorous ant *Azteca constructor*, along with a mock community consisting of 50% chloroplast 16S and 50% *Streptomyces* 16S. We designed 13 modifications to 799F for use in pyrosequencing, with the objective of complete elimination of chloroplast 16S reads in our arthropod systems. Our results indicate that 799F does reduce chloroplast abundance relative to other published primers, but primer length and various 3' modifications appear to be more effective at further minimizing plastid contamination. These primer modifications are appropriate for use in folivorous arthropod bacterial community analysis and in other samples that may contain chloroplasts, such as plants and algae.

A1.3 Materials & Methods

A1.3.1 Sample collection, preparation, and DNA extraction

Two herbivorous insects and a mock mixture of chloroplast and bacterial 16S were used for the analysis. Five *Azteca constructor* workers were collected sterilely from a *Cecropia insignis* tree at Carara Biological Reserve, Costa Rica, May 2011, pooled, stored in 99% ethanol, and transported to the laboratory in Madison, WI, for DNA extraction. Gypsy moth (*Lymantria*

dispar) larvae were reared in petri dishes from egg masses obtained from a wild population in Temperance, MI. Larvae were fed white birch (*Betula papyrifera*) obtained from WI-DNR (Wilson Nursery, Boscobel, WI), with diet being replaced daily. At fifth instar, ten larvae were starved for 24 hours, and then anesthetized by placing at -20ºC for 15 min. Larvae were surface sterilized in 70% ethanol for 30 s, air-dried for 1 min, and midguts were dissected with flame sterilized instruments. Midguts were pooled and stored at -80ºC until DNA extraction.

DNA was extracted from both insect tissues with the Epicenter Master Pure Complete DNA and RNA Purification Kit (Illumina, Madison, WI) with modifications. Tissues were homogenized in 2.0 mL screw-cap vials with one 3 mm diameter steel bead in 500 µL T&C buffer, samples were centrifuged at 500 x g for 3 min, supernatant was collected, and the remaining manufacturer's directions were followed. DNA was resuspended in TE and stored at -20ºC until use.

A1.3.2 Construction of mock mixture

To test the effectiveness of the newly designed primers, a mock mixture was constructed to contain half chloroplast 16S and half *Streptomyces* 16S. The chloroplast 16S was cloned from an *Azteca alfari* larvae sample found to contain 99% chloroplast reads from a previous 454 pyrosequencing run (data not shown). A near-full length sequence of the 16S was PCR amplified in 50 µL total volume, containing 100 ng template DNA, 0.5 µL Herculase II DNA polymerase (Agilent Technologies, Santa Clara, CA), 1.0 nM dNTPs, 1.25 µL DMSO, 10 µL buffer, 250 nM each of the primers 27F (5'AGAGTTTGATCNTGGCTCAG) and 1492R (5' TACGGYTACCTTGTTACG). Reaction conditions were as follows: 95ºC for 2 min, 32 cycles of 95ºC for 20 s, 55ºC for 20 s, 72ºC for 1:30 min, and a final elongation of 72ºC for 3 min. PCR products were cleaned using Promega Wizard SV gel and PCR clean-up system as per

manufacturer's directions (Promega, Madison, WI). One μL of cleaned PCR product was used in Novagen Perfectly Blunt Cloning kit with the pT7 Blue Blunt vector, performed as per manufacturer's directions (EMD Millipore, Billerica, MA). Colonies were selected based on blue/white screening and colony PCRs were performed, with a total volume of 20 μL containing 4 μL of boiled and centrifuged colony supernatant, 0.4 μL Herculase II DNA polymerase, 1.0 nM dNTPs, 0.5 μL DMSO, 4 μL buffer, and 100 nM of each of the primers M13F (5'GTTTTCCCAGTCACGAC) and M13R (5'CAGGAAACAGCTATGAC). Reaction conditions were as follows: 95°C for 2 min, 35 cycles at 95°C for 20 s, 55°C for 20 s, 72°C for 2 min, and a final elongation of 72°C for 3 min. Three colonies were positive and selected for sequencing using Big Dye chemistry. A 10 μL total volume PCR contained 1 μL Big Dye, 1.5 μL Big Dye buffer, 100 nM primer (27F or 1492R), 0.5 μL DMSO, and 0.5 μL PCR template. Reaction conditions were as follows: 96°C for 2 min, 36 cycles of 96°C for 10 s, 52°C for 15 s, 60°C for 3 min, and a final elongation of 72°C for 2 min. Products were cleaned with Agencourt CleanSeq beads (Beckman Coulter, Brea, CA), as per manufacturer's directions, and sequenced at the University of Wisconsin-Madison Biotechnology Center. Sequences were edited and aligned in Sequencher v4.5 (Gene Codes Corporation, Ann Arbor, MI), and confirmed via NCBI blastN to be chloroplast (Altschul et al., 1990).

A laboratory strain of *Streptomyces* spp. was used as the bacterial 16S, and amplified as described above using 27F and 1492R. The PCR products from both chloroplast and *Streptomyces* were quantified by Invitrogen Qubit Fluorometer (Life Sciences, Grand Island, NY) and mixed in equal concentrations to create the 50:50 mock mixture.

A1.3.3 Cross-phyla small subunit ribosomal DNA alignment

A framework by Dams *et al* (1988) was used for the alignment between bacterial 16S, chloroplast 16S, arthropod 18S, plant nuclear 18S, and plant mitochondrial 18S consensus sequences (Dams et al., 1988). The 16S consensus sequence for bacteria from Baker *et al* (2003) was used here with no modifications (Baker et al., 2003). A chloroplast 16S consensus sequence was created from the chloroplast sequenced above, the top 15 full length Blast matches to that sequence representing different genera within the rosids clade (DQ226511, JN884817, JQ041763, AP012207, HQ336405, HQ664605, JF317356, HQ244500, HQ664560, HQ664552, FJ895895, HQ664600, EF207453, HQ664619, EU431223), and 5 partial *Betula* chloroplast 16S sequences (GQ284846-50). Sequences were aligned in MEGA 5.2.1 using default ClustalW parameters, and the chloroplast consensus sequence was manually made (Tamura et al., 2011) (Chloroplast consensus sequence in Supplemental Text A1.1). *Azteca ovaticeps* 18S (EF012842) and *L. dispar* 18S (DQ186972.1) were used as representatives of arthropod 18S. A plant mitochondrial 18S consensus sequence was created, as described for the chloroplast 16S consensus, using two near full length rosid sequences, *Oenothera berteriana* (X61277) and *Arabidopsis thaliana* (Y08501). A plant nuclear 18S consensus sequence was created, as described for the chloroplast 16S consensus, using *Arabidopsis thaliana* (X16077), *Betula pendula* (GU476453), and three sequences from the same family as *Cecropia* (Urticaceae), *Pilea cadierei* (JF317373.1), *Debregeasia saeneb* (JF317363), and *Boehmeria nivea* (AF206870). All sequences were assessed for the presence of universal bacterial primers, including the forward primers 27F, 338F, 534F, 799F, 926F, and 1114F, in addition to the reverse primers 338R, 519R, 1114R, 1392R, and 1492R (Table A1.1).

A1.3.4 Primer design and assessment

The primers used in this study were developed to minimize chloroplast contamination and amplify the V5-V8 region of the 16S. Chloroplast and bacterial 16S are, as expected, highly conserved, severely limiting the number of potential primer mismatches (Table A1.1, Supplemental Table A1.1). Besides the four mismatches around 799F, no other conserved region of the bacterial 16S appropriate for primer design contains sufficient mismatches to chloroplast 16S. Consensus sequences of chloroplast and bacterial 16S were aligned along with the previously published 799F (Chelius and Triplett, 2001). 799F was modified on both the 5' and 3' ends, resulting in 9 different versions of the primer (Table A1.2). 799F-mod2, -mod4, and -mod7 were also synthesized with phosphorothioate bonds on the 3' end, resulting in 13 total versions of 799F (Table A1.2). Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

All versions of 799F, including 799F-tags which includes the 454 B adapter, were assessed for their ability to amplify chloroplast 16S, bacterial 16S, the 50:50 mock mixture, and environmental samples, in addition to their predicted coverage rate using probe match in the Ribosomal Database Project (RDP) (Cole et al., 2009). 19.2 ng of chloroplast 16S, bacterial 16S and 50:50 mock mixture was used as template, while 50 ng of environmental DNA was used. A 50 μL total volume PCR contained 0.5 μL Herculase II DNA polymerase, 1.0 nM dNTPs, 1 μL DMSO, 10 μL buffer, 300 nM 1392R (5' ACGGGCGGTGTGTRC) and 300 nM forward primer. Reaction conditions were as follows: 95ºC for 3 min, 30 cycles of 95ºC for 20 s, 45ºC for 30 s, 72ºC for 30 s, and a final elongation of 72ºC for 3 min. PCR products were visualized and assessed on a 2% agarose gel. Primers not capable of amplifying the *Streptomyces* 16S (799F-mod5, -mod9, and -mod4thio) were excluded from further testing. Likewise, primers unable to

amplify either environmental sample (799F-mod4, -mod8 and -mod2thio) were removed. In addition to 799F and 799F-tags, the modified versions 799F-mod2, -mod3, -mod6, -mod7, -thio and -mod7thio were included in further assessment.

A1.3.5 PCR for 454 Pyrosequencing

With two exceptions, PCRs were carried out using a two-step PCR procedure, utilizing a gel extraction of the 16S band between the first and second steps (Berry et al., 2011). The first PCR was done with primers that lacked the 454 adapters or multiplex identifiers (MIDs), while the second added the required 454 A- and B-adapters along with a 5 bp MID for each respective sample. PCRs with the primer pairs 799F-tags/1392R and 27F/519R were tagged in the first round of PCR, and were not done in a two-step process. All tested versions of the forward primer 799F (799F, 799F-tags, 799F-mod2, 799F-mod3, 799F-mod6, 799F-mod7, 799F-thio, and 799F-mod7thio) were paired with the reverse primer 1392R.

PCRs were done in triplicates containing 30-50 ng template DNA, 0.5 μL Herculase II DNA polymerase, 1.0 nM dNTPs, 1.0 μL DMSO, 10 μL buffer, 300 nM of forward and reverse primers, and water amounting to a final volume of 50 μL. Reaction conditions were as follows: 95ºC for 2 min, 30 cycles of 95ºC for 20 s, 48-50ºC for 30 s, 72ºC for 30 s, and a final elongation of 72ºC for 3 min. The optimal annealing temperature differed between the two environmental samples, 50ºC for *A. constructor*, and 48ºC for *L. dispar*. Triplicate reactions were pooled and an aliquot was used for gel extraction. The ~600 bp band expected from the 16S amplicon was extracted from a low-melt agarose gel using a Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA) by visualizing on a blue light transilluminator (Clare Chemical Research, Dolores, CO). A second PCR was done on all primers pairs, except 799F-tags/1392R and 27F/519R, using 2 μL of the extraction product using primers that included the A- and B-

adaptors along with the MIDs. All conditions for the second PCR step were identical except that thermocycling was done for 10 cycles instead of 30. Five μL of the second PCR product was loaded on a 2.0% agarose gel to verify the presence of a ~700 bp fragment while lacking one at ~600 bp. PCR products were cleaned with three rounds of cleanup using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA) as per manufacturer's directions. PCR products were quantified with a Qubit, and, according to Roche 454 pyrosequencing protocols, diluted and pooled at 10^{-6} DNA molecules/μl.

A1.3.6 454 pyrosequencing and long read modifications

The expected fragment size from 799F and 1392R, including 454 adapters and MIDs (~700 bp), is longer than recommended for 454 pyrosequencing (Roche, Indianapolis, IN) on a GS Junior with FLX Titanium chemistry. Therefore, emPCR was modified to accommodate the longer fragment. The modifications were based on suggestions in technical bulletin TCB-11001 "Amplicon Sequencing on GS FLX System with Various emPCR Conditions" and from Roche technical support (personal communication). Modifications included increased Amp mix to 297 μL, Amp Primer to 104 μL, and decreased water to 359 μL. Thermocycler conditions were changed to: 94°C for 4 min, 50 cycles of 94°C for 30 sec, 60°C for 10 min, and storage at 10°C. No other modifications were made to the manufacturer's protocols. The 50:50 mock mixture and environmental sample amplified with 27F/519R were run separately, but with the identical protocols.

A1.3.7 Data Analysis

Raw data were processed using mothur (v. 1.23.1) (Schloss et al., 2009). Sequences were analyzed, allowing for no differences in the primers or MIDs, and with a minimum length of 200 bp. The data set was simplified using unique.seqs and aligned to the Silva-derived reference

database (v. 102 as implemented for mothur) (Pruesse et al., 2007). Chimeras were removed using UCHIME (Edgar et al., 2011). For the first data analysis to evaluate how many chloroplast reads were present, all processed reads were kept. After the command classify.seqs to a nogap version of Silva containing bacterial, archaea, and eukaryotic sequences, each sample was assessed for the percent of chloroplast. For the second data analysis, to compare what bacteria were detected for each primer pair for the two arthropod samples, all eukaryotic reads were removed, as were OTUs with less than 2 reads. Weighted and unweighted Unifrac distance matrices were constructed in mothur and analyzed with non-metric multidimensional scaling (MDS) plots in PRIMER (v6) (Clarke and Gorley, 2006; Lozupone et al., 2011).

A1.4 Results

All but three primers readily amplified bacterial 16S and produced a low-intensity band after PCR with pure chloroplast 16S. The additional modifications to 799F not used in the pyrosequencing analysis failed these initial quality controls. All primers successfully amplified the *L. dispar* sample, but the 3' phosphorothioate modifications, 799F-thio and 799F-mod7thio, failed to amplify the *A. constructor* sample. The 50:50 mock mixture yielded a clean, single band with all primers, but the environmental samples had variable numbers of bands present, including the ~900 bp fragment from amplification of plant mitochondria 18S (Chelius and Triplett, 2001; Dams et al., 1988). Within a sample the multiple bands detected did not differ between the various forward primers.

The 454 GS Junior runs resulted in 143,260 raw reads. After removal of short reads and chimeras, 136,270 reads remained. 1364 non-chloroplast Eukaryote reads were removed from one sample, resulting in 134,906 high quality reads (Table A1.3). Across all primer combinations, both environmental samples had on average less than 1.0% chloroplast reads with

the 50:50 mock mixture having 2.5% chloroplasts or less with any of the various versions of 799F. The primer combination 799F-tags, including the 454 B adapter, along with 1392R with the 454 A adapter and 5 bp MIDs, had the highest percentage of chloroplasts in all three cases with 2.47% for the mock mixture, 1.95% for the *A. constructor* sample, and 6.87% for the *L. dispar* sample. 799F-mod3, -mod6, and -mod7 resulted in no chloroplast reads in both environmental samples. In the *L. dispar* sample, 799F-thio also had no chloroplast reads. A previous 454 run with 27F/519R using the *A. constructor* and *L. dispar* environmental samples yielded 67% and 39.4% chloroplasts, respectively, while the 50:50 mock mixture resulted in 55% chloroplast reads.

Relative abundances of taxa at the order taxonomic level were minimally impacted by the different forward primers (Figure A1.1, Supplemental Table A1.2). A MDS of weighted Unifrac also showed minimal differences in the various forward primers (Figure A1.2). The unweighted Unifrac MDS was most affected by the various forward primers, which was most likely due to rare reads present in the analysis (Figure A1.2).

A1.5 Discussion

Chloroplast and other plastids of bacterial origin present problems unique to certain environments in 16S pyrosequencing (Bulgarelli et al., 2012; Chelius and Triplett, 2001; Lundberg et al., 2012; Rastogi et al., 2010; Sakai et al., 2004). Although this issue is potentially widespread in insects and plant samples, it has been understudied in next-generation sequencing. We tested a series of primers to eliminate plastid contamination in herbivorous insects, and found that our primers, specifically 799F–mod 3, –mod6, and –mod7, paired with the universal primer 1392R, drastically reduced these interfering sequences. Subsequent independent experiments using these modifications reduced chloroplast contamination from as much as

99.9% in insect and plant samples to between 0-10% (CJ Mason, unpublished data; AS Hanshew, unpublished data).

799F has been used extensively in the literature to minimize chloroplast contamination in plant samples (Bulgarelli et al., 2012; Chelius and Triplett, 2001; Redford and Fierer, 2009; Redford et al., 2010). While this primer has been successful in some cases (Sun et al., 2008), it has performed poorly in others (Sagaram et al., 2009). Our results show that 799F is capable of reducing chloroplast sequence contamination in our insect samples, but three of our modified versions of 799F appear to be more effective. The differences between 799F and 799F-mod3, -mod6, and -mod7 are the positions of the 3' mismatch between bacterial and chloroplast 16S, in addition to mismatches at the 5' end. Shifting the position of these mismatches 1 or 2 base pairs in the primer may reduce the likelihood of chloroplast sequence amplification greater than in 799F.

Bias is a well-documented problem with all primers used in 16S PCR (Wang and Qian, 2009). 799F-mod3, -mod6, and –mod7 all maintain the ability to detect many of the bacterial phyla, but potentially bias against Chloroflexi and Verrucomicrobia (Supplemental Table A1.1). These phyla tend to be in minimal abundances in many animal microbial communities (Colman et al., 2012; Jones et al., 2013; Lozupone et al., 2012). In our study, we were able to detect these phyla, but at very low abundance, and they have been detected in subsequent analyses using these primers (N Davis, E Houck, K Dill-McFarland, personal communications). Cyanobacteria have a shared evolutionary history with Chloroplast that makes it challenging to have confidence in separating these two DNA sources in culture independent analyses in known plastid-abundant samples. While a biasing of community membership may occur with our primers, this is a long-

standing issue that exists for all 16S primers and the reduction of chloroplast sequences in samples similar to ours greatly outweighs this shortcoming.

To our knowledge, this is the first study using these primers in insect samples. Similar to 799F use with plant samples, we found our samples produced multiple banding patterns (Bulgarelli et al., 2012; Chelius and Triplett, 2001). Unlike previous studies, banding patterns differed from plant samples in that *A. constructor* had 3-4 bands while *L. dispar* had 7-9 bands. Changes in annealing temperature and other aspects of the PCR protocol did not reduce these patterns. Thus, utilizing gel extraction between the two-step PCR was necessary and effective in targeting our band of interest. As universal bacterial primers are capable of amplifying non-bacterial sequences, it is not surprising that these complex samples resulted in multiple bands from PCR. These other bands may represent plant mitochondrial 18S, which also has homology to 799F and 1392R, but produces a band roughly 1.5 times as long as that for 16S. It is unlikely this primer amplifies arthropod 18S or plant nuclear 18S, but there may be other non-target sequences amplified by these primers. Indeed, the 1364 eukaryotic reads present in one sample were likely a result from including an additional band during gel extraction by mistake. The two-step PCR separated by a gel extraction also circumvents the concern of primer length causing non-specific amplification and biases (Berry et al., 2011). In our results, the primer pairs including the 454 adapters and MIDs were less efficacious as opposed to a two-step PCR method. The two-step PCR method produced results with fewer unwanted sequences, as well as increased consistency.

Throughout our experiments, we tested a number of polymerases with different exonuclease activities, many of which are used extensively in the literature (data not shown). With few exceptions, many were unable to produce amplicons from our samples. Our samples

contain a myriad of sources of DNA, including at a minimum bacterial, insect, and plant DNA. Therefore, we think that the complexity of our samples may have reduced the effectiveness of amplification. In order to minimize primer degradation by polymerase exonuclease activities, we incorporated 3' phosphorothioate modifications into four versions of our primers to attempt to preserve the mismatches to chloroplast 16S on the 3' end of the primers. We found that the phosphorothioate primers performed inconsistently between samples. These modifications worked as well as the unmodified versions in the *L. dispar* sample, but failed to amplify in the *A. constructor* sample. Therefore, this primer modification should be used on a case-by-case basis.

Recently, 16S amplicon sequencing on high-throughput platforms has had increased use across a number of environmental samples, including insects and plants. Investigating bacterial communities in these systems with standard protocols can present challenges that previous studies have not yet encountered. The methods outlined in this study provide a much-needed methodological framework for addressing issues pertaining to unwanted sequence contamination. Our methods enable comparison of microbial communities in systems that were previously intractable to bacterial community analyses that can be modified to other PCR-based platforms. Many systems, including herbivorous hosts, can be chloroplast-laden and our methods establish a way of contending with this issue.

A1.6 Acknowledgements

to CRC. Funding sources had no involvement in conducting this research or preparing this

publication.

A1.7 References

Ahn, J.-H., Kim, B.-Y., Song, J., Weon, H.-Y., 2012. Effects of PCR cycle number and DNA polymerase type on the 16S rRNA gene pyrosequencing analysis of bacterial communities. Journal of Microbiology 50, 1071–1074.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic Local Alignment Search Tool. Journal of Molecular Biology 215, 403–410.

Andreotti, R., Perez de Leon, A.A., Dowd, S.E., Guerrero, F.D., Bendele, K.G., Scoles, G.A., 2011. Assessment of bacterial diversity in the cattle tick Rhipicephalus (Boophilus) microplus through tag-encoded pyrosequencing. BMC Microbiology 11, 6.

Baker, G.C., Smith, J.J., Cowan, D.A., 2003. Review and re-analysis of domain-specific 16S primers. Journal of Microbiological Methods 55, 541–555.

Bengtsson, M.M., Sjøtun, K., Lanzén, A., Ovreås, L., 2012. Bacterial diversity in relation to secondary production and succession on surfaces of the kelp Laminaria hyperborea. The ISME Journal 6, 2188–2198.

Berry, D., Mahfoudh, K. Ben, Wagner, M., Loy, A., 2011. Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. Applied and Environmental Microbiology 77, 7846–7849.

Bodenhausen, N., Horton, M.W., Bergelson, J., 2013. Bacterial communities associated with the leaves and the roots of Arabidopsis thaliana. PLoS ONE 8, e56329.

Bulgarelli, D., Rott, M., Schlaeppi, K., van Themaat, E.V.L., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F.O., Amann, R., Eickhorst, T., Schulze-Lefert, P., 2012. Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature 488, 91–95.

Chelius, M.K., Triplett, E.W., 2001. The diversity of archaea and bacteria in association with the roots of Zea mays L. Microbial Ecology 41, 252–263.

Clarke, K., Gorley, R., 2006. PRIMER v6: User Manual/Tutorial. PRIMER-E, Plymouth, UK.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research 37, D141–145.

Colman, D.R., Toolson, E.C., Takacs-Vesbach, C.D., 2012. Do diet and taxonomy influence insect gut bacterial communities? Molecular ecology 5124–5137.

Dams, E., Hendriks, L., Peer, Y. Van De, Neefs, J.-M., Smits, G., Vandenbempt, I., Wachter, R. De, 1988. Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Research 16, r87–r173.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.

Edwards, J.E., Huws, S.A., Kim, E.J., Kingston-Smith, A.H., 2007. Characterization of the dynamics of initial bacterial colonization of nonconserved forage in the bovine rumen. FEMS Microbiology Ecology 62, 323–335.

Fagen, J.R., Giongo, A., Brown, C.T., Davis-Richardson, A.G., Gano, K.A., Triplett, E.W., 2012. Characterization of the relative abundance of the citrus pathogen Ca. Liberibacter asiaticus in the microbiome of its insect vector, Diaphorina citri, using high throughput 16S rRNA sequencing. Open Microbiology Journal 6, 29–33.

Gottel, N.R., Castro, H.F., Kerley, M., Yang, Z., Pelletier, D.A., Podar, M., Karpinets, T., Uberbacher, E., Tuskan, G.A., Vilgalys, R., Doktycz, M.J., Schadt, C.W., 2011. Distinct microbial communities within the endosphere and rhizosphere of Populus deltoides roots across contrasting soil types. Applied and Environmental Microbiology 77, 5934–5944.

Hail, D., Dowd, S.E., Bextine, B., 2012. Identification and location of symbionts associated with potato psyllid (Bactericera cockerelli) lifestages. Environmental Entomology 41, 98–107.

Hail, D., Lauzi, I., Dowd, S.E., Bextine, B., Lauzìere, I., 2011. Culture independent survey of the microbiota of the glassy-winged sharpshooter (Homalodisca vitripennis) using 454 pyrosequencing. Environmental Entomology 40, 23–29.

Hamady, M., Walker, J.J., Harris, J.K., Gold, N.J., Knight, R., 2008. Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. Nature Methods 5, 235–237.

Hulcr, J., Rountree, N.R., Diamond, S.E., Stelinski, L.L., Fierer, N., Dunn, R.R., 2012. Mycangia of ambrosia beetles host communities of bacteria. Microbial Ecology 64, 784–793.

Jones, R.T., Bressan, A., Greenwell, A.M., Fierer, N., 2011. Bacterial communities of two parthenogenetic aphid species cocolonizing two host plants across the Hawaiian Islands. Applied and Environmental Microbiology 77, 8345–8349.

Jones, R.T., Knight, R., Martin, A.P., 2010. Bacterial communities of disease vectors sampled across time, space, and species. The ISME Journal 4, 223–231.

Jones, R.T., Sanchez, L.G., Fierer, N., 2013. A cross-taxon analysis of insect-associated bacterial diversity. PLoS ONE 8, e61218.

Kelley, S.T., Dobler, S., 2011. Comparative analysis of microbial diversity in Longitarsus flea beetles (Coleoptera: Chrysomelidae). Genetica 139, 541–50.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research 41, e1.

Köhler, T., Dietrich, C., Scheffrahn, R.H., Brune, A., 2012. High-resolution analysis of gut environment and bacterial microbiota reveals functional compartmentation in the gut of wood-feeding higher termites (Nasutitermes spp.). Applied and Environmental Microbiology 78, 4691–4701.

Koopman, M.M., Fuselier, D.M., Hird, S., Carstens, B.C., 2010. The carnivorous pale pitcher plant harbors diverse, distinct, and time-dependent bacterial communities. Applied and Environmental Microbiology 76, 1851–1860.

Kumar, P.S., Brooker, M.R., Dowd, S.E., Camerlengo, T., 2011. Target region selection is a critical determinant of community fingerprints generated by 16S pyrosequencing. PLoS ONE 6, e20956.

Lalzar, I., Harrus, S., Mumcuoglu, K.Y., Gottlieb, Y., 2012. Composition and seasonal variation of Rhipicephalus turanicus and Rhipicephalus sanguineus bacterial communities. Applied and Environmental Microbiology 78, 4110–4116.

Lane, D.J., 1991. 16S/23S rRNA Sequencing, in: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, Ltd, New York, pp. 115–175.

Leveau, J.H.J., Tech, J.J., 2011. Grapevine microbiomics: bacterial diversity on grape leaves and berries revealed by high-throughput sequence analysis of 16S rRNA amplicons. Acta Horticulturae 905, 31–42.

Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J., Knight, R., 2011. UniFrac: an effective distance metric for microbial community comparison. The ISME Journal 5, 169–172.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., Knight, R., 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489, 220–230.

Lundberg, D.S., Lebeis, S.L., Paredes, S.H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., Rio, T.G. Del, Edgar, R.C., Eickhorst, T., Ley, R.E., Hugenholtz, P., Tringe, S.G., Dangl, J.L., 2012. Defining the core Arabidopsis thaliana root microbiome. Nature 488, 86–90.

Moran, N.A., Hansen, A.K., Powell, J.E., Sabree, Z.L., 2012. Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. PLoS ONE 7, e36393.

Nossa, C.W., Oberdorf, W.E., Yang, L., Aas, J.A., Paster, B.J., DeSantis, T.Z., Brodie, E.L., Malamud, D., Poles, M.A., Pei, Z., 2010. Design of 16S rRNA gene primers for 454 pyrosequencing of the human foregut microbiome. World Journal of Gastroenterology 16, 4135–4144.

Osei-Poku, J., Mbogo, C.M., Palmer, W.J., Jiggins, F.M., 2012. Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya. Molecular Ecology 21, 5138–5150.

Palavesam, A., Guerrero, F.D., Heekin, A.M., Wang, J., Dowd, S.E., Sun, Y., Foil, L.D., Pérez de León, A.A., 2012. Pyrosequencing-based analysis of the microbiome associated with the horn fly, Haematobia irritans. PLoS ONE 7, e44390.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., Glöckner, F.O., 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35, 7188–7196.

Rastogi, G., Sbodio, A., Tech, J.J., Suslow, T. V, Coaker, G.L., Leveau, J.H.J., 2012. Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. The ISME Journal 6, 1812–1822.

Rastogi, G., Tech, J.J., Coaker, G.L., Leveau, J.H.J., 2010. A PCR-based toolbox for the culture-independent quantification of total bacterial abundances in plant environments. Journal of Microbiological Methods 83, 127–132.

Redford, A.J., Bowers, R.M., Knight, R., Linhart, Y., Fierer, N., 2010. The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. Environmental Microbiology 12, 2885–2893.

Redford, A.J., Fierer, N., 2009. Bacterial succession on the leaf surface: a novel system for studying successional dynamics. Microbial Ecology 58, 189–198.

Reid, N.M., Addison, S.L., Macdonald, L.J., Lloyd-Jones, G., 2011. Biodiversity of active and inactive bacteria in the gut flora of wood-feeding huhu beetle larvae (Prionoplus reticularis). Applied and Environmental Microbiology 77, 7000–7006.

Russell, J.A., Weldon, S., Smith, A.H., Kim, K.L., Hu, Y., Lukasik, P., Doll, S., Anastopoulos, I., Novin, M., Oliver, K.M., 2013. Uncovering symbiont-driven genetic diversity across North American pea aphids. Molecular Ecology 22, 2045–2059.

Sagaram, U.S., DeAngelis, K.M., Trivedi, P., Andersen, G.L., Lu, S.-E., Wang, N., 2009. Bacterial diversity analysis of Huanglongbing pathogen-infected citrus, using PhyloChip arrays and 16S rRNA gene clone library sequencing. Applied and Environmental Microbiology 75, 1566–1574.

Sakai, M., Matsuka, A., Komura, T., Kanazawa, S., 2004. Application of a new PCR primer for terminal restriction fragment length polymorphism analysis of the bacterial communities in plant roots. Journal of Microbiological Methods 59, 81–89.

Salem, H., Kreutzer, E., Sudakaran, S., Kaltenpoth, M., 2013. Actinobacteria as essential symbionts in firebugs and cotton stainers (Hemiptera, Pyrrhocoridae). Environmental Microbiology 15, 1956–1968.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology 75, 7537–7541.

Sergeant, M.J., Constantinidou, C., Cogan, T., Penn, C.W., Pallen, M.J., 2012. High-throughput sequencing of 16S rRNA gene amplicons: effects of extraction procedure, primer length and annealing temperature. PLoS ONE 7, e38094.

Shade, A., Mcmanus, P.S., Handelsman, J., 2013. Unexpected diversity during community succession in the apple flower microbiome. mBio 4, e00602–12.

Sipos, R., Székely, A.J., Palatinszky, M., Révész, S., Márialigeti, K., Nikolausz, M., 2007. Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis. FEMS Microbiology Ecology 60, 341–350.

Sudakaran, S., Salem, H., Kost, C., Kaltenpoth, M., 2012. Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, Pyrrhocoris apterus (Hemiptera, Pyrrhocoridae). Molecular Ecology 21, 6134–6151.

Sun, L., Qiu, F., Zhang, X., Dai, X., Dong, X., Song, W., 2008. Endophytic bacterial diversity in rice (Oryza sativa L.) roots estimated by 16S rDNA sequence analysis. Microbial Ecology 55, 415–424.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28, 2731–2739.

Wang, Y., Gilbreath, T.M., Kukutla, P., Yan, G., Xu, J., 2011. Dynamic gut microbiome across life history of the malaria mosquito Anopheles gambiae in Kenya. PLoS ONE 6, e24767.

Wang, Y., Qian, P.-Y., 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. PLoS ONE 4, e7401.

Weinstock, G.M., 2012. Genomic approaches to studying the human microbiota. Nature 489, 250–256.

Wong, C.N.A., Ng, P., Douglas, A.E., 2011. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environmental Microbiology 13, 1889–1900.

Table A1.1 Cross phyla alignment of the universal bacterial primers with 16S- and 18S-rRNA genes from bacteria and eukaryotes. Primer sequences written 5' → 3'. Shaded bases differ from the corresponding nucleotides in the primer. Numbers ending in F are forward primers and numbers ending in R are reverse primers.

Sequences[a]	27F	338F	338R	534F	519R
Primer	AGAGTTTGATCNTGGCTCAG	ACTCCTACGGGAGGCAGCAGT	CATGCTGCCTCCCGTAGGAGT	GTGTGCCAGCTGCCGCGGTAA	TTACCGCGGCDGCTGGC
Bacteria	AGAGTTTGATCATGGCTCAG	ACTCCTACGGGACGCAGCAGT	CATGCTGCCTCCCGTAGGAGT	CCGTGCCAGCAGCCGCGGTAA	TTACCGCGGCAGCTGGC
Chloroplast	AGAGTTCGATCCTGGCTCAG	ACTCMTACGGGAGGCAGCAGT	CATGCTGCCTCCCGTAKGAGT	CTGTGCCAGCAGCCGCGGTAA	TTACCGCGGCAGCTGGC
A. ovaticeps	nd[b]	ACATCAAGGAAGGCAGCAGG	CCTGCTGCCTTCCTTGGATGT	TGTGCCAGCTGCCGCGGTAA	TTACCGCGGCAGCTGGC
L. dispar	nd	ACATCAAGGAAGGCAGCAGG	CCTGCTGCCTTCCTTGGATGT	TGTGCCAGCTGCCGCGGTAA	TTACCGCGGCAGCTGGC
Plant mito.	AGAGTTTGATCCTGGCTCAG	ACTCCACGGGGGCAGCAGT	CATGCTGCCCCCGTGGGAGT	CCGTGCCAGCAGCCGCGGTAA	TTACCGCGGCAGCTGGC
Plant nuclear	nd	ACATCAAGGAAGGCAGCAGG	CCTGCTGCCTTCCTTGGATGT	TGTGCCAGCAGCCGCGGTAA	TTACCGCGGCAGCTGGC

Sequences[a]	799F	926F	1114F	1114R	1392R	1492R
Primer	AACMGGATTAGATACCCKG	AAACTYAAAKGAATTGACGG	GCAACGAGCGCAACCC	GGGTTGCGCTCGTTGC	ACGGGCGGTGTGTRC	GGNTACCTTGTTACGACTT
Bacteria	GCAAACAGGATTAGATACCCTGGT	AAACTCAAATGAATTGACGG	GCAACGAGCGCAACCC	GGGTTGCGCTCGTTGC	ACGGGCGGTGTGTAC	GGATACCTTGTTACGACTT
Chloroplast	GCRAATCGGATTAGATACCCCAGT	AAACTCAAAGGAATTGACGG	GCAACGAGCGCAACCC	GGGTTGCGCTCGTTGC	ACGGGCGGTGTGTAC	GGGTACCTTGTTACGACTT
A. ovaticeps	TCGAAGCCGATCAGATACCGCCCT	AAACTTAAAGGAATTGACGG	ATAACGAACGAGACTC	GAGTCTCGTTCGTTAT	ACGGGCGGTGTGTAC	nd
L. dispar	TCGAAGCCGATTAGATACCGCCCT	AAACTTAAAGGAATTGACGG	GTAACGAACGAGACTC	GAGTCTCGTTCGTTAC	ACGGGCGGTGTGTAC	nd
Plant mito.	GCGAACCGGATTAGATACCCTGGT	AAACTCAAAGGAATTGACGG	WTAACGAGCGAAACCC	GGGTTTCGCTCGTTAW	ACGGGCGGTGTGTAC	GGCTACCTTGTTACGACTT
Plant nuclear	TCGAAGACGATCAGATACCGTCCT	AAACTTAAAGGAATTGACGG	nd	nd	ACGGGCGGTGTGTAC	nd

a. Primer: Universal primers, Bactera: Bacterial consensus 16S, Chloroplast: Chloroplast consensus 16S, A ovaticeps: Azteca ovaticeps 18S, L. dispar: Lymantria dispar 18S, Plant mito: Plant mitochondrial 18S, Plant nuclear: Plant nuclear 18S

b. nd = Sequence not detected

Table A1.2 Sequence and coverage rate of forward primers.

Primer Name	Primer Sequence (5' → 3')	Primer Length (bp)	% RDP predicted coverage[b]		
			M=0	M=1	M=2
799F	AACMGGATTAGATACCCKG	19	81.8	88.1	95.9
799F-mod1	AACMGGATTAGATACCCKGG	20	81.6	88.1	95.8
799F-mod2	AACMGGATTAGATACCCKGGT	21	81.4	88.0	95.7
799F-mod3	CMGGATTAGATACCCKGG	18	82.1	88.2	95.9
799F-mod4	GGATTAGATACCCKGG	16	82.4	95.5	99.3
799F-mod5	ATTAGATACCCKGG	14	82.7	95.7	99.5
799F-mod6	CMGGATTAGATACCCKGGT	19	81.9	88.1	95.9
799F-mod7	GGATTAGATACCCKGGT	17	82.2	95.4	99.3
799F-mod8	ATTAGATACCCKGGT	15	82.5	95.6	99.4
799F-mod9	TAGATACCCKGGT	13	85.8	95.8	99.6
799F-thio	AACMGGATTAGATACCCKG	19	81.8	88.1	95.9
799F-mod2thio	AACMGGATTAGATACCCKGGT	21	81.4	88.0	95.7
799F-mod4thio	GGATTAGATACCCKGG	16	82.4	95.5	99.3
799F-mod7thio	GGATTAGATACCCKGGT	17	82.2	95.4	99.3

Underlined bases are bonded with phosphorothioate bonds. The number of mismatches (M), zero, one, or two, allowed between the primer and target region in a RDP search.

Table A1.3 Total number of sequence reads and percent aligning to chloroplasts with the various forward primers.

Primer combination	50:50 Mock mixture		A. constructor		L. dispar	
	# reads	%chloroplast	# reads	%chloroplast	# reads	%chloroplast
27F/519R	661	55.4	1839	67	374	39.4
799F/1392R	7966	1.66	10759	0.37	2208	0.36
799F-tags/1392R	9764	2.47	3176	1.95	1675	6.87
799F-mod2/1392R	4390	1.09	8586	0.02	2798	0.11
799F-mod3/1392R	8657	0.88	8772	0	2307	0
799F-mod6/1392R	6717	0.76	8548	0	2115	0
799F-mod7/1392R	6370	0.63	6638	0	1795	0
799F-thio/1392R	10133	2.34	N/A	N/A	254	0
799F-mod7thio/1392R	5196	2.1	N/A	N/A	865	0
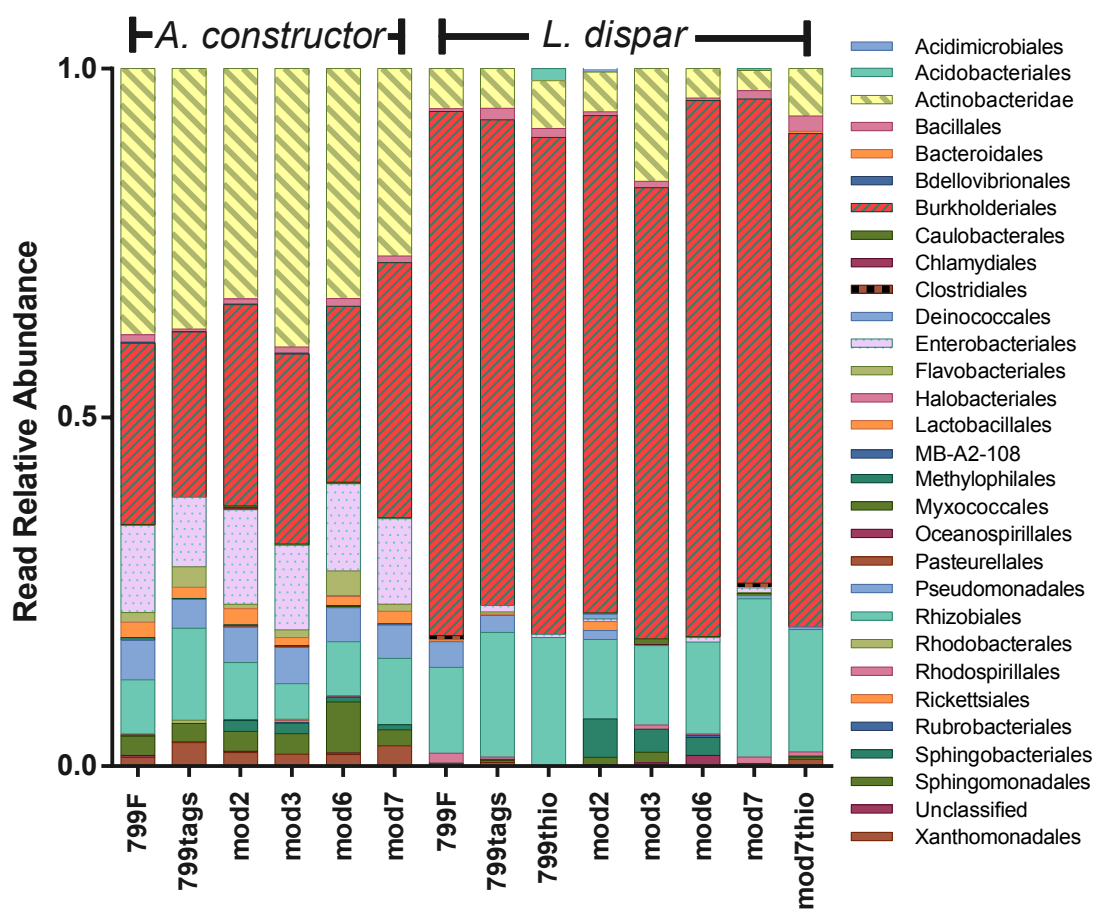
Figure A1.1 Relative abundances of sequences at the bacterial order taxonomic classification sequenced with the tested forward primers.
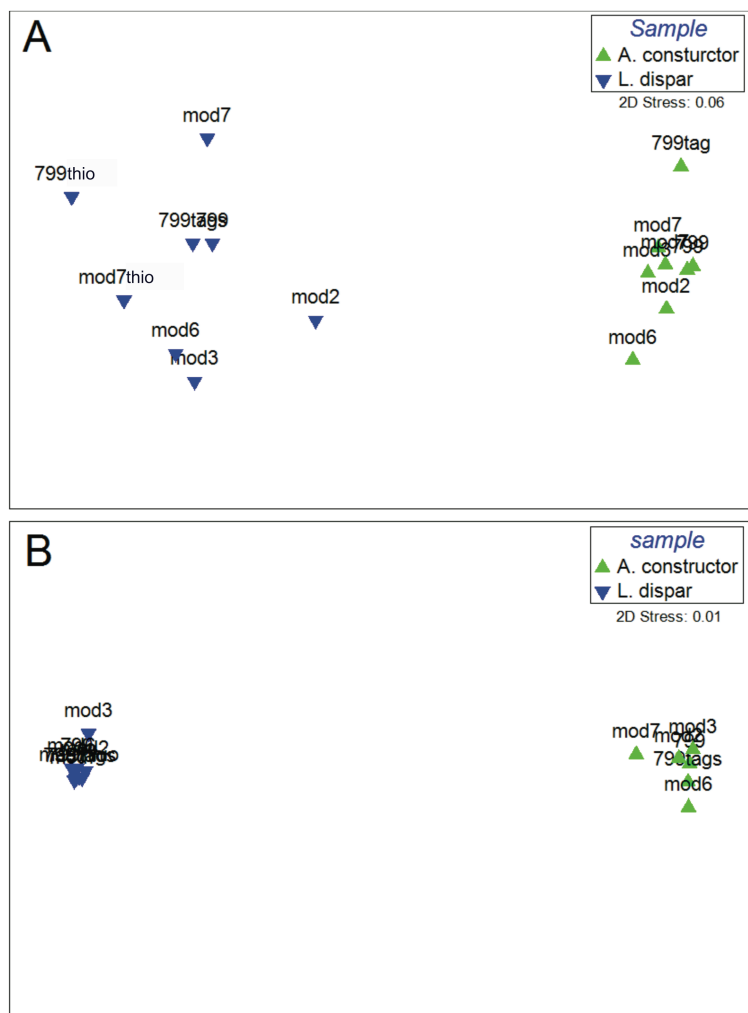
Figure A1.2 MDS plot illustrating the differences between the bacterial communities in two arthropod samples sequenced with 8 primer pairs. Pairwise community distances determined using the unweighted Unifrac algorithm (A) and the weighted Unifrac algorithm (B). These two panels show that while there may be more bacterial taxa detected with different primers (A), when abundance is taken into account, the communities in each respective insect are nearly indistinguishable (B).

Supplemental Text A1.1 Chloroplast 16S consensus sequence

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TCTCATGGAGAGTTCGATCCTGGCTCAGGATGAACGCTGGCGGCATGCTTAACACAT
GCAAGTCGGACGGGAAGTGGTGTTTCCAGTGGCGGACGGGTGAGTAACGCGTAAGA
ACCTGCCCTTGGGAGGGGAACAACAGCTGGAAACGGCWGCTAATACCCCGTAGGCT
GAGGAGCAAAAGGAGGAATCCGCCYGAGGAGGGGCTCGCGTCTGATTAGCTAGTTG
GTGAGGCAATAGCTTACCAAGGCGATGATCAGTAGCTGGTCCGAGAGGATGATCAG
CCACACTGGGACTGAGACACGGCCCAGACTCMTACGGGAGGCAGCAGTGGGGAAT
TTTNCCGCAATGGGCGAAAGCCTGACGGAGCAATGCCGCGTGGAGGTAGAAGGCCY
ACGGGTCRTGAACTTCTTTTCCCGGAGAAGAARCAATGACGGTATCYGGGGAATAA
GCATCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGATGCAAGCGTTATC
CGGAATGATTGGGCGTAAAGCGTCTGTAGGTGGCTTTTTAAGTCCGCCGTCAAATCC
CAGGGCTCAACCCYGGACAGGCGGTGGAAACTACCAAGCTGGAGTACGGTAGGGG
CAGAGGGAATTTCCGGTGGAGCGGTGAAATGCGTAGAGATCGGAAAGAACACCAA
CGGCGAAAGCACTCTGCTGGGCCGACACTGACACTGAGAGACGAAAGCTAGGGGA
GCRAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGC
TGTGCGTATCGACCCGTGCAGTGCTGTAGCTAACGCGTTAARTATCCCGCCTGGGGA
GTACGTTCGCAAGAATGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGATGCAAAGCGAAGAACCTTACCAGGGCTTGACATGCCG
CGAATCCTCTTGAAAGAGAGGGGTGCCTTCGGGAACGCGGACACAGGTGGTGCATG
GCTGTCGTCAGCTCGTGCCGTAAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TCGTGTTTAGTTGCCACCGTTGAGTTTGGAACCCTGARCAGACTGCCGGTGATAAGC
CGGAGGAAGGTGAGGATGACGTCAAGTCATCATGCCCCTTATGCCCTGGGCGACAC
ACGTGCTACAATGGMCGGGACAAAGGGTCGCGATCCCGCGAGGGTGAGCTAACYC
CAAAAACCCGTCCTCAGTTCGGATTGYAGGCTGCAACTCGCCTRCATGAAGCCGGA
ATCGCTAGTAATCGCCGGTCAGCCATACGGCGGTGAATTCGTTCCCGGGCCTTGTAC
ACACCGCCCGTCACACTATGGGAGCTGGCCATGCCCGAAGTCGTTACCTTAACCGCA
AGGRGGGGGATGCCGAAGGCMGGGCTAGTGACTGGAGTGAAGTCGTAACAAGGTA
GCCGTACTGGAAGGTGCGGCTGGATCACCTCCTTT
```

Supplemental Table A1.2 Relative abundances of all bacterial phyla sequenced with the tested forward primers.

| | A. constructor | | | | | | L. dispar | | | | | | | |
	799F/1392R	799F-tags/1392R	799F-mod2/1392R	799F-mod3/1392R	799F-mod6/1392R	799F-mod7/1392R	799F/1392R	799F-tags/1392R	799F-thio/1392R	799F-mod2/1392R	799F-mod3/1392R	799F-mod6/1392R	799F-mod7/1392R	799F-mod7thio/1392R
Acidobacteria	0	0	0	0	0.1	0	0	0	1.6	0	0	0	0.3	0
Actinobacteria	38.1	36.4	33.1	39.9	32.7	27.0	6.0	5.4	6.7	6.4	16.1	4.6	3.1	6.9
Bacteroidetes	1.6	2.8	2.2	2.8	4.3	1.9	0.3	0.5	0	5.4	3.4	2.6	0.2	0.6
Chlamydiae	0	0	0.2	0	0	0	0	0	0	0	0	0	0	0
Chloroflexi	0	0	0.01	0	0.01	0	0	0	0	0	0	0	0	0
Cyanobacteria	0.4	2.0	0.02	0	0	0	0.4	6.9	0	0.1	0	0	0	0
Deinococcus-Thermus	0	0	0	0	0	0	0	0	0	0.6	0	0	0	0
Firmicutes	3.3	2.0	3.1	2.1	2.6	2.9	1.2	1.7	1.6	1.9	1.0	0.6	2.2	2.8
Planctomycetes	0	0.1	0.02	0	0.01	0	0	0	0	0	0	0	0	0
Proteobacteria	56.4	56.6	61.2	55.3	60.0	68.1	92.1	85.6	89.8	85.6	78.9	90.5	94.2	89.6
Verrucomicrobia	0.01	0	0	0	0	0.06	0	0	0	0	0	0	0	0
unclassified	0.3	0	0.2	0	0.2	0	0	0	0	0	0.5	1.5	0	0

Appendix 2. The epiphytic microbiota of the globally widespread macroalga
***Cladophora glomerata* (Chlorophyta, Cladophorales)**

ASH contributed in the following ways: Assisted in design and setup of 454 pyrosequencing and data analysis. Contributed to writing the manuscript.

A2.1 Abstract

- *Premise of the study*: The filamentous chlorophyte *Cladophora* Kütz. produces abundant nearshore populations in marine and freshwaters worldwide, often dominating periphyton communities and producing nuisance growths under eutrophic conditions. High surface area and environmental persistence foster such high functional and taxonomic diversity of epiphytic microfauna and microalgae that *Cladophora* has been labeled an ecological engineer. We tested the hypotheses that: 1) *Cladophora* supports a structurally and functionally diverse epiphytic prokaryotic microbiota that influences materials cycling and 2) mutualistic host-microbial interactions occur. Because previous molecular sequencing-based analyses of the microbiota of *C. glomerata* found as western Lake Michigan beach drift had identified pathogenic associates such as *Escherichia coli*, we also asked if actively growing lentic *C. glomerata* harbors known pathogens.

- *Methods*: We used 16S rRNA gene amplicon pyrosequencing to examine the microbiota of *C. glomerata* of Lake Mendota, Dane, Co., WI during the growing season of 2011, at the genus- or species-level to infer functional phenotypes. We used correlative scanning electron and fluorescence microscopy to describe major prokaryotic morphotypes.

- *Key results*: We found microscopic evidence for diverse bacterial morphotypes, and molecular evidence for about 100 distinct sequence types classifiable to genus at the 80% confidence level or species at the 96-97% level within 9 bacterial phyla, but not *E. coli* or related human pathogens.

- *Conclusions*: We inferred that lentic *C. glomerata* bacterial epiphytes display diverse materials cycling functions, including traits that indicate the occurrence of mutualistic interactions with the algal host.

A2.2 Introduction

The green algal genus *Cladophora* Kützing (1843) (Ulvophyceae) produces conspicuous and persistent growths on hard substrata along shorelines of pristine to nutrient-rich marine, estuarine, brackish, and lentic and lotic freshwaters of temperate and tropical, high and low-altitude biomes worldwide (reviewed by Whitton, 1970; Dodds and Gudder, 1992; Higgins et al., 2008; Graham et al., 2009; Zulkifly, 2012). While many of the factors contributing to *Cladophora*'s abundance and persistence are understood and summarized next, associated communities of bacteria–the microbiota–and their functional effects are not well known.

Attributes contributing to *Cladophora*'s success include rapid growth rate. Auer and Canale (1982) showed that at optimum irradiance and temperature, Lake Huron *C. glomerata* achieved a net growth rate of 0.6 day^{-1} when P was not limiting, i.e. could increase its mass by about 60% per day. *Cladophora* can use dissolved carbon dioxide or bicarbonate as an inorganic carbon source (Choo et al., 2002), and is able to rapidly acclimate to changes in irradiance (Ensminger et al., 2000a,b; Bautista and Necchi-Junior 2008). Laboratory experiments indicate that optimum irradiances for freshwater *C. glomerata* are 300-600 μmol photons m^{-2} sec^{-1} for freshwater *C. glomerata* (Graham et al., 1982), consistent with field observations that this alga

commonly occupies littoral areas ranging from 0 to 10 or more meters depth, depending on water clarity. In the Laurentian Great Lakes, invasive planktivorous dreissenid mussels enhance the growth of *Cladophora*-dominated periphyton by clarifying the water column, thereby increasing benthic irradiance (reviewed by Auer et al., 2010).

Resistance to herbivory also contributes to ecological abundance and persistence (Canale and Auer, 1982), possibly due to complex branching that deters herbivores, and/or chemical constituents that render *C. glomerata* distasteful (Patrick et al., 1983). Environmental abundance of *Cladophora* is aided by continuous production of asexual zoospores in filament tips (Graham et al., 2009), resistance to hydrodynamic drag (Dodds and Gudder, 1992; Bergey et al., 1995), and high body tensile strength (9-35 MPa) (Johnson et al., 1996), as well as holdfast attachment features and the cellulosic cell wall, whose highly crystalline structure (reviewed by Mihranyan, 2011) resists microbial attack (Paalme et al., 2002; Birch et al., 1983). Once *Cladophora* has been able to colonize a solid substratum, a population may continue in that location for years, overwintering in the form of robust basal parts that regenerate multicellular bodies when growth conditions allow and provide a consistent inoculum and substratum for epiphytes.

Global abundance arising from the above-described features, together with distinctive structure, fosters high functional and taxonomic diversity of associated microfauna (Kraufvelin and Salovius, 2004). Consequently, Ward and Riccardi (2010) characterized *Cladophora* as an autogenic ecological engineer, an organism that creates, modifies, and maintains habitat by means of its own physical structure (Jones et al., 1994). *Cladophora* likewise provides habitat for numerous microalgal species, primarily diatoms; Stevenson and Stoermer (1982), found 245 diatom taxa on *Cladophora* collected from Lake Huron near Harbor Beach, MI, and Mpawenayo and Mathooko (2005) found 60 diatom species associated with *Cladophora* in a highland stream

(the River Njoro) in Kenya. The role of *Cladophora* as habitat for microfauna and microalgae arises from an extraordinarily high surface area; Jansson (1967) estimated that a one-month-aged, 0.78 g dry weight biomass of *C. glomerata* occupying 1 dm^2 of substratum had a surface area of 50 dm^2.

Such high surface area, together with abundant secretion of metabolizable organic compounds such as glycolate (Cheney and Hough 1983), offers the potential for *Cladophora* to also support a large and diverse bacterial community (Lowe et al., 1982) that may display materials cycling functions and the capacity to foster host growth. For example, microscopic evidence indicates that bacteria associated with Lake Michigan *C. glomerata* express alkaline phosphatases that may foster the growth of *Cladophora* (and microalgal epiphytes) in P-limiting conditions (Young et al. 2010). Further, *Cladophora glomerata* has been shown to require vitamin B$_{12}$ (Gerloff and Fitzgerald, 1976, Hoffmann and Graham, 1984; Hoffmann, 1990), and algae can gain this nutrient from associated bacteria (Croft et al., 2005, 2006). Despite the essential functional roles bacteria likely play in materials cycling in periphytic communities and in fostering algal growth, a detailed analysis of the microbiota associated with actively growing *C. glomerata* has not been reported.

Recent studies of decomposing *Cladophora glomerata* in beach drift along western Lake Michigan shorelines have revealed the presence of potentially pathogenic bacteria such as *Escherichia coli*, *Salmonella*, *Shigella*, and *Campylobacter* (Byappanahalli, et al. 2003; Whitman et al., 2003; Ishii et al., 2006; Olapade et al., 2006; Englebert et al., 2008a,b; Badgley et al., 2011). For this reason it is important to determine if actively growing *Cladophora* likewise functions as a reservoir for pathogenic bacteria that would present a human health issue.

We sampled *C. glomerata* from Lake Mendota, Dane Co., WI to address the following

key questions: 1) Can genotypes within the *C. glomerata* bacterial epiflora be related to key materials cycling processes such as nitrogen fixation, methane oxidation, or cellulose degradation? 2) Does evidence exist for mutualistic algal-bacterial interactions? 3) Does actively growing *C. glomerata* host *E. coli* or other enteric mammalian pathogens?

To answer these questions, we conducted a 16S rRNA gene amplicon pyrosequencing analysis of the prokaryotic genera or species associated with *C. glomerata* sampled from Lake Mendota during the growth season of 2011. Such sequence data are widely assumed to reflect the taxonomic composition of the prokaryotic communities sampled, and generic- and species-level identification facilitates inference of functional phenotype. We also performed correlative fluorescence microscopy and scanning electron microscopy to characterize the major bacterial morphotypes associated with *Cladophora*.

A2.3 Materials and Methods

A2.3.1 Study site

Lake Mendota, Dane Co., WI was chosen as a study site because this hardwater hypereutrophic lake supports robust populations of *Cladophora*, has been the subject of many classic studies (summarized by Brock, 1985), and is part of the North Temperate Lakes Long Term Ecological Research site. *Cladophora*-dominated periphyton occurs throughout the mostly rocky 35.2 km long shoreline. Lake Mendota shoreline is mostly rocky because the lake occupies a glacial basin that is a product of the final glaciation event in southern Wisconsin; thus, most of the shoreline displays Quaternary glacial deposits (Brock, 1985). To the south Lake Mendota is connected to a chain of lakes by the Yahara River, which discharges to the Rock River, a tributary of the Mississippi River. Lake water level is controlled by the county to reduce the risk of flooding, so Lake Mendota *Cladophora* is not subject to large fluctuations in water level

during the growth season.

The specific study site, 43°4.5'N, 89°35.5'W, locally known as Picnic Point, was chosen because periphyton growths were readily accessible, yet were relatively isolated from disturbances resulting from activities at boat docks or swimming areas that occur elsewhere along the shore. The Picnic Point site was unshaded by overhanging vegetation, which can decrease the abundance of *Cladophora*-dominated periphyton (Whitton, 1970).

A2.3.2 Periphyton sampling, collecting, and cleaning

Triplicate samples of *Cladophora*-dominated periphyton were collected from emergent and submerged rocks located within 10 feet of shore and 0-0.5 m depth during the months of June, July, August, and September of 2011. Samples were put into Nasco Whirl-Packs (Fort Atkinson, Wisconsin) filled with lake water.

Immediately after sampling, periphyton samples were transported to the lab, transferred to sterile plastic dishes, and washed with sterile SD11 medium to remove loosely associated materials such as silt, zooplankton, bacterioplankton, and phytoplankton. SD11 medium is a modification (Arancibia-Avila et al., 2000) of D11, a medium optimized for growth of *Cladophora* (Gerloff and Fitzgerald, 1976) that maintains the osmotic strength of natural Lake Mendota water. Portions of the *Cladophora* body nearest holdfasts were selected for microbial community analysis because these are the oldest regions and thus had accumulated denser microbial communities. Personnel handling *C. glomerata* collections consistently wore latex gloves as a protection from potential pathogens and to prevent contamination of the samples with bacterial DNA from human skin.

A2.3.3 DNA extraction, amplification, and preparation for pyrosequencing

DNA extraction was accomplished using the MoBio Ultra Clean Soil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA), modified by adding 100 µl of lysozyme (10mg/ml) in the initial step. Lysozyme attacks the peptidoglycan walls of bacteria, so this modification allowed us to avoid grinding to break bacterial cell walls, a process that would also have increased breakage of host and attached microalgal cell walls. We avoided damaging host and microalgal walls to prevent swamping bacterial community DNA with eukaryotic DNA. 16S rRNA gene amplicons were used to assess prokaryotic community composition because the 16S marker has become a standard. This sequence region is present in all cellular organisms, has a low rate of horizontal gene transfer and recombination, has sufficient genetic variation to distinguish among closely related species, and is represented by many reference sequences in public databases (Clarridge, 2004).

We utilized Roche 454 GS Junior technology to perform amplicon sequencing by synthesis: polymerase-catalyzed extension of a primed template that adds single nucleotides in each cycle, thereby releasing inorganic phosphate used in formation of ATP, which powers light emission by luciferin (Metzker, 2010). Extracted DNA was PCR amplified using universal bacterial 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 909 R(5'-CCC CGY CAA TTC MTT TRA GT-3') primers to amplify 16S rRNA gene regions V1-3 (Baker, 2003; Wang and Qian, 2009), according to my.jgi.doe.gov/general/protocols/SOP_16S18S_rRNA_PCR_Library_Creation.pdf. Primers were designed to avoid amplifying host 18S rRNA genes and to generate sequences 200-600 base pairs in length, to determine generic or species level bacterial identifications for high-resolution inference of bacterial functional properties.

For each environmental sample PCR was performed using Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland), with 100ng/μL of DNA template, and 1μL of forward and 1μL of reverse primer. The thermal cycling program was based on a modified version of the recommended JGI protocol (94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, 50°C for 45 seconds, 68°C for 90 seconds, and a final extention of 68°C for 10 minutes). Forward primers were fused with Roche 454-Titanium adapter A and five base barcodes (Multiplex Identifiers) and reverse primers were fused with only the Roche 454-Titanium adapter B. (Barcodes were used to differentiate the 3 replicates for each of 4 sampling dates for epiphytic bacterial community dynamics analyses at family and higher taxonomic levels that are not described in this article, which instead focuses on genera and species present at any time during the growth period.) Amplicon samples were then subjected to three rounds of purification using Agencourt AMPure XP beads and Agencourt SPRI magnetic plate (Beckman Coulter, Massachusetts). Individual samples were diluted to 1 X 10^7 molecules/μl, titrated to contain 0.25, 0.5, 1 and 1.5 copies of DNA per bead, pooled and sequenced using GS FLX Titanium Sequencing Kit XLR79 on a Roche 454 GS Junior located in the Rennebohm instructional laboratory in the Department of Bacteriology, UW-Madison.

A2.3.4 Sequence data processing and archiving

The entire set of fasta files for all reads contained 34,570 sequences. This set of sequences represents a pool of replicate samples taken monthly at four times during the growth season. According to Roche Technical Bulletin No. T2011-001TTT, the number of sequences we obtained was likely somewhat lower than if we had utilized primers giving shorter amplicon lengths, but we elected to pursue longer reads in view of evidence that pyrosequencing read length matters in assessing microbial communities (Wommack et al. 2008).

The use of pyrosequencing in 16S rRNA gene analyses of bacterial communities has the potential to introduce biases (misrepresentation of abundances of microbial populations) and errors (mistakes in actual sequence), and chimeric sequences can form when incomplete PCR products function as primers and amplify related sequences, all of which can affect perceived biodiversity (Schloss et al., 2011). Therefore, the entire set of fasta files were first quality-filtered. The set of sequences having Qual scores above 25 with correct assigned barcodes was 19,215.

The RDP (Ribosomal Database Project) SeqMatch command line utility program and RNA Classifier version 2.3 (Wang et al., 2007) were used to classify a subset of quality-filtered 11,670 sequences that were 200-600 bp length using a non-redundant curated reference dataset of 16S rRNA bacterial sequences. A custom perl script was used to parse the dataset of sequence classifications generated by RDP SeqMatch for unique genera having confidence values $\geq 80\%$. In this process, a random subset of "words" (short sequence regions) for each sequence is used in a probability calculation repeated in 100 bootstrap trials. The number of times a genus is most likely generates a confidence estimate for that assignment. Sequences classifiable to genus at the 80% level of confidence would not be expected to include chimeras that impact classification. All hits to Enterobacteriaceae (the family into which *E. coli* and other coliform pathogens are classified) and several other sequences of interest were linked to species by using BLASTN against the NCBI 16S Bacteria and Archaea database. To infer structural and functional phenotypes, we employed one or more recent literature article per genus or species to focus on phenotypic characters consistent within that taxon.

In accordance with database policy, following publication of this article, raw and processed sequences and other information related to the pyrosequencing run will become

publically accessible from the NSF-supported Long Term Ecological Research North Temperate Lakes Microbial Observatory (LTER NTL MO) database, which contains additional Lake Mendota microbial sequencing data.

A2.3.5 Bacterial morphotype characterization

For morphological characterization samples of *C. glomerata* collected on July 8, 2011 were fixed with a 0.5% solution of glutaraldehyde (EM grade, Polysciences Inc., Warrington, PA) just prior to treatment with a 50 μg/ml solution of the DNA-specific fluorochrome DAPI (4',6-diamidino-2-phenylindole). At this point in the growing season, an extensive community of microalgal epiphytes had not yet developed on *C. glomerata*, which allowed us to more easily image bacterial elements of the epiphyton. A Zeiss Axioplan fluorescence microscope equipped for UV excitation (G365 FT395 LP420) and digital image capture (Nikon DM300, Camera Control Pro software) was used to image bacterial morphotypes. No image modifications were made except for the addition of labels. We also used DAPI staining to check that our washing technique (described above) had effectively removed loosely attached material while leaving intact bacterial epiphytes for molecular analyses. Whitton (2002) was used as a taxonomic reference to determine the cyanobacterial species *Chamaesiphon incrustans* based on characteristics visible with light microscopy.

Samples of *Cladophora glomerata* collected on the same day were fixed in 2% glutaraldehyde (EM grade, Polysciences Inc., Warrington, PA) in 0.05 M phosphate buffer, dehydrated in an ethanol series, then transported to the University of Wisconsin-Milwaukee Electron Microscopy Laboratory, where samples were critical-point dried, then metal-coated for scanning electron microscopy, using a Hitachi S-4800 Ultra High Resolution Cold Cathode Field Emission Scanning Electron Microscope (FE-SEM) operated at 5 kV.

A2.4 Results

Our analysis of bacterial epiphytes on *Cladophora glomerata* revealed the occurrence of diverse genotypes and several distinctive morphotypes. We employed 11,670 quality-filtered sequences of 200-600 bp length to infer bacterial genus- or species-level identifications for 99 unique bacterial genera (at the 0.80 confidence level) or species (at the 0.96 or 0.97 confidence level). We then used the results of an extensive literature search to infer specific phenotypes for each taxon (Table A2.1), thereby providing a substantial base of community function information.

A2.4.1 Prokaryotic genotypes

The majority of the 99 unique bacterial genera or species whose presence on surfaces of *C. glomerata* we inferred from sequence data were classified into Class Betaproteobacteria (21), Class Alphaproteobacteria (17), Class Gammaproteobacteria (12), or Class Deltaproteobacteria (9) in Phylum Proteobacteria; or Class Sphingobacteria (16), Class Flavobacteria (7), or Class Bacteroidia (1) in Phylum Bacteriodetes; Phylum Planctomycetes (4), or Phylum Verrucomicrobiae (4, including 1 member of class Opitutae). Other prokaryotic genera that could be identified by sequence database comparisons fell into Phylum Deinococcus-Thermus (3), Phylum Lentisphaerae (1), Phylum Fusobacteria (1) or Phylum Nitrospirae (1) (Figure 1). Six unique sequences were classified as Phylum Cyanobacteria and into Groups I, IIa, IV, VI, XI, and XIII, but could not be further classified into genera or species using the RNA Classifier, possibly due to limited database information for these taxa. Eukaryotic chloroplast sequences linked to Chlorophyta (green algae) and Bacillariophyta (diatoms) were also detected. In addition, at least one proteobacterial sequence that could not be identified to genus occurred in >100 reads, and may be new to science if the presence of artifact can be discounted.

We found no sequence evidence for the occurrence of Phylum Proteobacteria, Class Epsilonproteobacteria, into which the pathogenic genus *Campylobacter* is classified, and only a few reads were classifiable to Enterobacteriaceae (of Class Gammaproteobacteria). We found no sequence evidence for the presence of *Escherichia* or *Salmonella* (Enterobacteriaceae), though 4 reads of a sequence that was 97% identical to a NCBI 16S reference sequence for the putative pathogen *Plesiomonas shigelloides* were observed.

A2.4.2 Bacterial morphotypes

In a snapshot sample collected at the beginning of our study on July 8, 2011, and imaged with epifluorescence microscopy and SEM we distinguished several prokaryotic morphotypes among the microbial epiphytes consistently present on randomly surveyed areas of the surface of the host *Cladophora glomerata*. First, DAPI staining showed relatively common occurrence of the exospore-producing cyanobacterium *Chamaesiphon incrustans* appearing as pink clubs (color caused by combination of red chlorophyll autofluorescence with the blue-white DAPI-DNA complex) (Fig. A2.2A). In SEM view, *C. incrustans* was distinctively greater in width (about 4 μm) than co-occurring bacterial cells (Figure A2.2B). Segmented, unbranched bacterial filaments of intermediate width were likely cyanobacteria of uncertain identity (see Fig. A2.2B).

A second common prokaryotic morphotype was long, narrow filaments that stood erect from the host surface or were flattened against it (Fig. A2.3A). These long filaments did not appear to be segmented in either fluorescence or SEM (Fig. A2.3B) views, were < 1 μm in diameter, and did not exhibit chlorophyll autofluorescence, indicating that they were not cyanobacteria. Short rods that did not display chlorophyll autofluorescence occurred commonly on the surface of *Cladophora* (see Figs. A2.2A and A2.3A).

Bushy colonies of tiny cocci or very short rods were consistently present (Fig. A2.3A). In

SEM view, the lobed nature of these bushy colonies was distinctive and the diameter of the cells could be seen to be much < 1 μm (Fig. A2.3B). Somewhat less common was a distinctive morphotype consisting of fairly long cylindrical axes with short branchlike appendages (Figs. A2.4A,B).

A2.5 Discussion

This is the first detailed molecular sequence survey of the prokaryotic epiphytes occupying the surface of actively growing samples of the periphytic green alga *Cladophora glomerata* in samples taken over a growth season, together with correlative fluorescence and scanning electron microscopic observations made near the beginning of that season. Our inference from molecular sequence data that at least 99 bacterial genera (at the 80% confidence level) or species (at the 96-97% level) were present at some time during the sampling period was consistent with our SEM and fluorescence microscopic results indicating the occurrence of diverse microbial morphotypes. More than half of the genera inferred from sequence information were previously known to occur in aquatic habitats (see Table A2.1 and Supplementary Table A2.1), an observation consistent with the study habitat, a hypereutrophic lake.

Our compilation of the extent of genetic, functional, and structural diversity of the microbial community (in Table A2.1, Supplementary Table A2.1) enables us to address specific questions regarding host-epiphyte relationships: 1) Can genotypes within the *C. glomerata* bacterial epiflora be related to key functional processes such as nitrogen fixation, methane oxidation, or cellulose degradation? 2) Does evidence exist for mutualistic algal-bacterial interactions? 3) Does actively growing *C. glomerata* host *E. coli* or other enteric mammalian pathogens? In addition the data allow us to compare the *C. glomerata* microbiota with bacterial communities observed on other green algae and to putatively link prokaryotic morphotypes

observed with microscopy with particular genotypes.

A2.5.1 Sequence data indicate that the *Cladophora glomerata* prokaryotic epiflora displays diverse ecological function

Complexly structured *Cladophora*-dominated periphyton communities–which often include several other filamentous algal genera, hundreds of microalgal species, diverse microfauna, and a large number of prokaryotic taxa–are microscopic forests providing many ecological niches. It is therefore not surprising that pyrosequencing indicated diverse prokaryotic ecological functions. Our detailed functional analysis of *C. glomerata* bacterial epiflora, based on recent literature bearing on the phenotypic traits of genera or species (see Supplementary Table A2.1), provides information important in understanding the ecology of periphyton communities. The results also aid the application of *Cladophora* communities to wastewater remediation and the potential for industrial production of renewable biofuel feedstocks (Hoover et al., 2011).

Inferred autotrophic functions included anoxygenic photoautotrophy–indicated by the presence of sequences related to *Rhodoferax*, *Erythromicrobium*, *Sandarakinorhabdus*, and *Blastomonas*–and chemolithoautotrophy, indicated by the presence of sequence related to *Thiobacter*. Diverse capacities for mineral redox reactions were inferred from sequences related to specific taxa: nitrogen fixation (*Dechloromonas*, *Blastobacter*, *Devosia*), Fe(III) reduction (*Ferribacterium, Geobacter*), U(IV) reduction (*Geobacter*), sulfate reduction (*Desulfobulbus*), Fe(II) oxidation (*Leptothrix*), nitrite oxidation (*Nitrospira*), and H-oxidation (*Hydrogenophaga*). Presence of the sulfur-cycle chemolithoheterotroph *Limnobacter* was also inferred. Sequence data also suggested the presence of taxa having diverse chemoheterotrophic capacities including degradation of methane (*Methylotenera*, *Methylibium*, *Methylobacter*) and cellulose (*Sorangium*,

Byssovorax, *Opitutus, Cellvibrio*, as well as alkanes (*Alkanindiges*) and other organic pollutants (*Anaeromyxobacter*, *Dechloromonas*, *Sphingomonas*, *Ideonella*). *Bdellovibrio*, a genus that consumes other bacteria, could be inferred from sequence analysis (see Table A2.1). Nitrogen-fixation, methanotrophy, and cellulose degradation are addressed next in more detail.

A2.5.2 The inferred *C. glomerata* microbiota includes nitrogen fixers, methanotrophs, and chitin and cellulose degraders

Nitrogen fixers. Evidence for the occurrence of heterotrophic nitrogen-fixing bacteria associated with *Cladophora* comes from sequences inferred to represent *Dechloromonas*, *Blastobacter*, and *Devosia*. Diazotrophic capacity of *Dechloromonas* is putative and based on the presence in the *D. aromatica* genome of genes known to be associated with N-fixation in other bacterial species (Salinero et al., 2009). *Blastobacter denitrificans*, closely related to *Bradyrhizobium*, is a confirmed diazotroph that occurs in root nodules of the flood tolerant legume *Aeschynomene indica* (van Berkum and Eardly, 2002). We were not able to link *Blastobacter*-like sequences (which were >500 bp in length) to database species sequences, though *Cladophora*-associated *Devosia* sequences were 96% similar to *D. insulae*. *Devosia neptuniae* (closely related to *Rhizobium*) is a diazotrophic symbiont in root nodules of the aquatic legume *Neptunia natans* (Rivas et al., 2002), but the nitrogen-fixing capacity of *D. insulae* is uncertain. Hypereutrophic Lake Mendota often displays large populations of nitrogen-fixing planktonic cyanobacteria, so that *Cladophora*-dominated periphyton is likely not often N-limited.

Though we obtained sequence evidence for presence of six types of cyanobacteria, many of which have nitrogen fixation capability, we did not find microscopic evidence for the common presence of heterocyte- (heterocyst)-bearing cyanobacteria on *C. glomerata*. In a related study

focused on microalgae present in Lake Mendota *Cladophora*-dominated periphyton (Zulkifly, 2012), we did not find diatom taxa (*Epithemia* or *Rhopalodia* species) known to harbor N-fixing cyanobacterial endobionts. This is consistent with observations that *Epithemia* species bearing N-fixing endobionts are more abundantly associated with *Cladophora* in N-limited habitats (Bergey et al., 1995).

Methanotrophs. Lakes are "hot spots" of methane production generated by a benthic community of methanogens, and waterbodies such as Lake Mendota that are >1 km^2 in area are particularly important in global methane cycling (Bastviken et al., 2004, 2011). We observed the occurrence of several *Cladophora*-associated sequences that are related to known methanotrophs (*Methylotenera*, *Methylibium,* and *Methylobacter*), which use oxygen to metabolize methane. We speculate that if methanotrophic (and respiratory) consumption of oxygen produced by the algal host occurs at a high enough level, photorespiration and consequent host glycolate excretion might decrease. Reduction in glycolate excretion could foster algal growth by preventing loss of photosynthate. By harboring methanotrophs and producing copious amounts of the O_2 they need to combust methane, globally abundant *Cladophora* might function like *Sphagnum* moss and other aquatic plants having methanotrophic symbionts that reduce the amount of methane emitted from aquatic systems that would otherwise enter Earth's atmosphere. Such function is regarded as an important ecological service because, on a per molecule basis, over the past 100 years atmospheric methane has had 21 times the climate warming impact of CO_2 (Intergovernmental Panel on Climate Change, 2012)

Cellulose degraders. *Cladophora* is known for its cellulose-rich cell walls that have diverse technological applications (Hoover, et al., 2011; Mihranyan, 2011). Therefore, we expected to find sequence evidence for cellulose degraders associated with *Cladophora*-

dominated periphyton. Cellulose-degrading bacteria and the cellulase enzyme complexes they produce are not only ecologically significant, but also have potential technological applications in processing cellulosic feedstocks for the renewable biofuels industry (Voget et al., 2006). We observed sequences inferred to represent two bacterial genera frequently associated with cellulose degradation, *Byssovorax* and *Sorangium* and several other genera for which genome sequences (completed for some species) indicate that cellulases may be produced. The *Cellvibrio japonicus* genome contains 21 cellulase genes, the *Sorangium cellulosum* genome 17 and the *Opitutus terrae* genome 14 (Medie et al., 2012). Genome sequences for certain species in a few other genera whose presence we inferred from sequence data display fewer cellulase genes: *Leadbetterella byssophila* (3) *Legionella longbeachae* (2), *Rhodoferax ferrireducens* (2), *Planctomyces brasiliensis* (2), *Deinococcus geothermalis* (2), *Rhodopirellula baltica* (1), *Truepera radiovictrix* (1), and multiple strains of *Pseudomonas* (1-2) (Medie et al., 2012). We did not detect some bacterial genera or species known to occur in freshwaters and also degrade cellulose, such as *Clostridium cellulolyticum* (Guedon et al., 1999), *Bradybacterium* (Zhang et al., 2007), or *Fibrobacter* (McDonald et al., 2009; Suen et al., 2011). Some other known cellulose degraders are classified in Phylum Actinobacteria (DeMenzes et al., 2008), which were not observed among Lake Mendota *Cladophora*-associated sequences at the 80% confidence level.

A2.5.3 Sequence evidence suggests the occurrence of mutualistic algal-bacterial nutritional interactions

Marine bacterial epiphytes have been shown to provide vitamin B_{12} to microalgal partners (Croft et al., 2005), and it has also been demonstrated that growth and reproductive capacities of freshwater *C. glomerata* depend upon exogenous B_{12} (Gerloff and Fitzgerald, 1976;

Hoffmann and Graham, 1984; Hoffmann 1990). Using a factorial experimental design, Hoffmann and Graham (1984) found that vitamins B_1 and B_{12}, temperature, irradiance, and photoperiod were all statistically significant factors in the production of zoospores and dry weight by a laboratory culture of *C. glomerata* isolated from freshwater Lake Mendota, WI and that zoosporogenesis was more sensitive than dry weight production to vitamin limitation and photoperiod. These authors also noted that reduction in vitamin B levels significantly decreased zoosporogenesis. Using radiolabeled vitamin B_{12}, Hoffmann (1990) demonstrated the dependence of *C. glomerata* photosynthesis and growth on this nutrient, inferring that the critical concentration range was 0.3-0.5 ng per mg dry weight and that luxury storage occurs. This author suggested that vitamin B_{12} likely limits zoosporogenesis by constraining essential energetic resources that would arise from photosynthesis in vitamin-replete cells. Hoffmann (1990) states that despite intensive efforts, he was unable to obtain completely axenic cultures of *C. glomerata* from Lake Mendota collections for his studies of B_{12} requirements, suggesting some degree of host dependency on one or more bacterial associates. The present study provides evidence that some of the bacteria present in the *Cladophora*-dominated periphyton community may provide the host with B-vitamins.

Cetobacterium (Fusobacteria) is known to produce vitamin B_{12} (Sugita et al., 1994), as does *Plesiomonas* (Sugita et al. 1991). Planktonic *Flavobacterium* (phylum Bacteriodetes) isolated from eutrophic Lake Jeziorak (Poland) also produced several types of B vitamins (Donderski, 1991). Our finding of sequences related to *Flavobacterium*, *Cetobacterum,* and *Plesiomonas shigelloides* associated with L. Mendota *Cladophora* suggests that such bacteria may supply B_{12} to *Cladophora* and its microalgal associates. Under eutrophic conditions in freshwater and marine systems, *Cladophora* can form nuisance level blooms bearing coverings

of silica-rich diatoms so dense that they impact lake silica cycling (Malkin et al., 2009). Bacterial vitamin production may foster both host and microalgal epiphyte proliferations. It is known that most harmful phytoplankton bloom species are dependent on an exogenous supply of vitamins B_1 and B_{12} (Tang et al., 2010).)

In a mutualistic interaction *Cladophora* (and its microalgal epiphytes) may supply epiphytic heterotrophic vitamin producers with organic exudates such as glycolate, which can be used by heterotrophic bacteria as a substrate (Paver and Kent, 2010). Freshwater *Cladophora fracta*, for example, has been observed to excrete as much as 90% of daily fixed carbon, primarily at the time of greatest growth (not during die-off periods) (Cheney and Hough 1983). It is possible that *Cladophora* exudates support not only vitamin producers, but also heterotrophic nitrogen fixers and bacteria that efficiently harvest phosphate, thereby making additional nutrients more available to the host and associated microalgae.

Microscopic evidence for abundant bacterial alkaline phosphatase activity in *Cladophora*-epiphyte communities from Lake Michigan suggests that bacterial phosphorus acquisition might aid *Cladophora* and algal epiphytes within the community, particularly in chronically P-limiting conditions that occur in some lakes (Young et al. 2010). Our sequence survey revealed evidence for *Gemmatimonas*, a polyphosphate-accumulator (Zhang et al., 2003), suggesting that in P-rich conditions, *Cladophora* and its microalgal and bacterial epiphytes may all be important in sequestering phosphate from the water column, a property potentially useful in wastewater effluent remediation applications.

A2.5.4 Actively-growing *Cladophora glomerata* does not seem to be a natural host for *E. coli* or other enteric mammalian pathogens

Previous sequencing studies had suggested that *C. glomerata* collected from beach drift

hosts enteric human pathogens. *E. coli* and other enteric bacteria were detected in 97% of *Cladophora* samples taken from 10 beach sites on Lake Michigan shorelines; when rehydrated these bacteria rapidly grew after more than 6 months in the sun-dried condition followed by storage at 4 °C (Whitman et al., 2003). Olapade et al. (2006) collected stranded mats of *Cladophora glomerata* from three beach areas of Lake Michigan, used the DNA-localizing fluorochrome dye DAPI to examine bacteria that were detached by sonication then filtered onto micropore membranes, and generated 16S rRNA clone libraries. Though 40% of the Olapade et al. (2006) sequences fell into the Cytophaga-Flavobacterium-Bacteroides cluster–previously found to occur widely in aquatic biofilms, including the surfaces of freshwater green algae (Fisher et al., 1998)–the Firmicutes *Clostridium carboxidivorans*, certain Actinobacteria, and several types of Proteobacteria (including Enterobacteriaceae) were also observed. Because human populations and agricultural operations strongly influence southern Lake Michigan, and animal hosts are likely the primary habitat for various enteric bacteria, Ishii et al. (2006) considered that beach drift *Cladophora* might be a secondary habitat for pathogenic bacteria, a conclusion consistent with the presence of *E. coli* in beach sand in some shoreline areas of Lake Michigan (Beversdorf, et al. 2007; Zehms et al., 2008).

Our results suggest that mammalian pathogens are probably not a general feature of the microbiota of actively-growing lentic *Cladophora*, since we did not find sequence evidence for the presence of *E. coli*, *Salmonella*, or other common enteric genera known to be mammalian pathogens and sequence evidence for the presence of Enterobacteriaceae was sparse. We did find sequence evidence for the presence of *Plesiomonas shigelloides* (Enterobacteriaceae)*,* a gram-negative bacillus occurring in freshwater and soil that is regarded as a commensal in diverse animals; *P. shigelloides* can cause human illness, hence antibiotic sensitivities have been

determined (Stock and Wiedemann, 2001). Although our sequence data indicated the presence of *Legionella* and *Pseudomonas*, some species of which are human pathogens, representatives of these genera are known to commonly occur in water samples. These data indicate that the prospective technological application of *Cladophora*-dominated periphyton to improve water quality and at the same time generate renewable biofuel feedstocks (Hoover, et al., 2011) does not necessarily pose a threat of enteric bacterial infection to workers.

A2.5.5 The microbiota of *C. glomerata* shares some components with microbiotas of other green algae, but has distinctive character

In an earlier molecular study of the bacterial epiphytes on periphytic charophycean algae in low pH humic lakes (Fisher et al., 1998), cloned 16S rRNA gene sequences were reported to group with the genera *Sphingobacterium, Cytophaga*, *Flexibacter*, *Methylobacterium*, *Brachyomonas*, *Comamonas*, *Variovorax*, *Hydrogenoluteola*, *Acinetobacter* and *Flavobacterium*; of these, only *Flexibacter* and *Flavobacterium* were also found in the present study of Lake Mendota *C. glomerata*. This difference suggests host-specific and/or environment-specific differences in bacterial floras that should be further investigated by the application of modern sequencing methods to the periphytic algae of humic lakes.

Sheath and Morison (1982) microscopically surveyed the microalgal epiphytes of Great Lakes *Cladophora,* finding that the cyanobacterial species *Phormidium diguetti*, *Leibleinia epiphytica*, and *Chamaesiphon incrustans* comprised 53-90% of the epiphytic cell density. Filamentous cyanobacteria (*Fischerella* sp.) have also been noted microscopically on *Cladophora* from nearshore Lake Michigan (Young et al. 2010). We observed large populations of exospore-producing (budding) *Chamaesiphon incrustans* on *C. glomerata* from Lake Mendota by using microscopic methods and had thought that it might be possible to consider this species a

positive control for sequencing-based surveys. However, too little sequence was available in databases to allow molecular detection of the species *C. incrustans*.

A recent study of the epiphytic bacterial community of the marine green alga *Ulva australis* based on 16S rRNA gene libraries indicated: 1) the dominance of Alphaproteobacteria and Bacteroidetes, particularly the Bacteroidetes families Rhodobacteriaceae, Sphingomonadaceae, Flavobacteriaceae and Saprospiraceae; 2) that epiphytic communities could be distinguished from those of surrounding seawater; and 3) that variability between libraries was high (Burke et al., 2011). We likewise found dominance by Bacteroidetes and Alphaproteobacteria. As more 16S rRNA gene based analyses of marine macroalgal bacterial endophytes and epiphytes are accomplished (e.g. Hollants et al., 2011; Lachnit et al., 2011) it may become possible to better parse the effects of host and environment on epiphytic community composition.

Studies of marine ulvophycean algae have revealed that one or more bacterial epiphytes related to the Cytophaga-Flavobacterium-Bacteroides group secrete chemical compounds that influence algal development and body structure (Provasoli and Pintner, 1980; Tatewaki et al., 1983; Nakanishi et al, 1996; Matsuo et al., 2005). Although the precise source taxa remain uncertain, researchers have explored the morphological effects on ulvaleans of adding bacterial isolates cultured from ulvalean algae (Marshall et al., 2006) or used molecular methods to characterize the bacterial communities present on ulvalean algae (Tujula et al., 2010). While we found evidence within the *Cladophora glomerata* microbiota for about 20 bacterial genera known to occur in marine habitats (see Table A2.1), of these only *Sphingopyxis*, *Sphingomonas*, and *Pseudomonas* overlapped with lists of bacterial genera found in recent analysis of marine ulvaleans (Marshall et al., 2006; Tujula et al., 2010). The *C. glomerata* microbiota that we

investigated also did not include the Flavobacteriaceae *Costertonia aggregata*, which occurs

in marine biofilms (Kwon et al., 2006). Because the vast majority of cladophoralean algae are

marine, *Cladophora* is thought to have secondarily invaded freshwaters (Graham, 1982),

possibly bringing along essential bacterial symbionts. Another explanation for the possible

occurrence of marine-adapted bacteria in the periphyton of Lake Mendota is that this lake

receives a certain amount of ion-rich runoff as a consequence of road-salting during the winter.

Future transcriptomic or metagenomic approaches could be used to test hypotheses of

microbiota function inferred in this study. Pyrosequences generated in this study will be used in

future work to compare the Lake Mendota *C. glomerata* microbiota to that of other aquatic

systems and to bacterioplankton communities of the same aquatic systems.

A2.5.6 Common bacterial morphotypes observed on the surface of *Cladophora* can

tentatively be linked to particular genotypes

We conjecture that long filaments (see Fig. A2.3A,B) that we observed microscopically

as epiphytes on *C. glomerata* might represent *Leptothrix*, which has 1-2 wide μm sheaths that

appear solid in SEM views (Fleming et al., 2011), the similarly sheathed *Haliscomenobacter*

(Daligault et al., 2011), or *Flexibacter*. Other candidates for the relatively long rod-shaped

bacterial morphotypes (see Fig. A2.3A,B) that commonly and consistently occurred on the

surface of *Cladophora* include *Malikia granulosa*, *Anaeromyxobacter*, *Flavobacterium*, and

Runella. We suggest that bushy aggregations of prokaryotic cells (see Fig. A2.4A,B) might be

myxobacterial fruiting bodies of such genera as *Byssovorax* or *Sorangium*. We hypothesize that

the branched prokaryotic filaments we observed (see Fig. A2.5A,B) might be members of the

actinobacteria, which are known for production of branched filaments. However, we did not

observe actinobacterial sequences that passed our 80% reliability criterion for generic

identification. Fluorescence in situ hybridization (FISH) could be used to test these hypotheses.

In summary, our sequencing and microscopy analyses of the microbiota epiphytic on surfaces of Lake Mendota *C. glomerata* display previously unrecognized bacterial genetic and morphological diversity and imply diverse functional attributes important in materials cycling and fostering host growth. The inferred *C. glomerata* microbiota is also noteworthy for presence of bacteria known for unusual environmental tolerances (*Porphyrobacter, Altererythrobacter, Sphingopyxis, Thiobacter*, *Deinococcus*, *Meiothermus*, and *Truepera)*. The latter three genera might seem surprising because they are known for resistance to ionizing radiation, not likely to be a selective influence in the littoral of Lake Mendota. However, Ragon et al. (2011) found that biofilm communities growing in sunlight-exposed habitats rich in UV that are periodically desiccated (such as a lake littoral zone), are able to cope with increased mutation rates and thus are preadapted to cope with ionizing radiation. This observation helps to explain our observation of deinococci in *Cladophora*-dominated periphyton communities of the exposed littoral zone. Lake Mendota periphyton also seems to include several bacterial genera that display cellular compartmentation (*Verrucomicrobium*, *Prosthecobacter*, *Gemmata*, *Rhodopirellula*, and *Pirellulla*) (Lee et al., 2009a,b), and thus are of intense evolutionary interest. *Cladophora glomerata* is not just a nuisance alga, but also represents a microscopic forest that provides many ecological niches, fostering a diverse microbiota with important materials-cycling functionalities such as autotrophy and mineral redox and organic degradation reactions.

A2.6 Literature Cited

Arancibia-Avila, P., J. R. Coleman, W. A. Russin, L. W. Wilcox, J. M. Graham, and L. E. Graham. 2000. Effects of pH on cell morphology and carbonic anhydrase activity and localization in bloom-forming *Mougeotia* (Chlorophyta, Charophyceae). *Canadian Journal of Botany*. 78: 1206-1214.

Auer, M. T. and R. P. Canale. 1982. Ecological studies and mathematical modeling of *Cladophora* in Lake Huron: 3. The dependence of growth rates on internal phosphorus pool size. *Journal of Great Lakes Res*earch 8: 93-99.

Auer, M. T., L. M. Tomlinson, S. N. Higgins, S. Y. Malkin, E. T. Howell, and H. A. Bootsma. 2010. Great Lakes *Cladophora* in the 21[st] century: same algae–different ecosystem. *Journal of Great Lakes Research* 36 :248-255.

Badgley, B. D., J. Ferguson, A. Vanden Heuvel, G. T. Kleinheinz, C. M. McDermott, T. R. Sandrin, J. Kinzelman, et al. 2011. Multi-scale temporal and spatial variation in genotypic composition of *Cladophora*-borne *Escherichia coli* populations in Lake Michigan. *Water Research* 45: 721-731.

Baker G. C., J. J. Smith, and D. A. Cowan. 2003. Review and re-analysis of domain-specific 16S primers. *Journal of Microbiological Methods* 55: 541-555.

Bastviken, D., J. Cole, and M. Pace, and L. Tranvik. 2004. Methane emission from lakes: Dependence of lake characteristics, two regional assessments, and a global estimate. *Global Biogeochemical Cycles* 18: GB409, doi: 10.1029/2004GB002238,2004

Bastviken, D., L. J. Tranvik, J. A. Downing, P. M Crill, and A. Enrich-Prast. 2011. Freshwater methane emissions offset the continental carbon sink. *Science* 331:50.

Bautista, A. I. N., and O. Necchi-Júnior. 2008. Photoacclimation in a tropical population of *Cladophora glomerata* (L.) Kützing 1843 (Chlorophyta) from southeastern Brazil. *Brazilian Journal of Biology* 68: 129-136.

Bergey E. A., C. A. Boettiger, and V. H. Resh. 1995. Effects of water velocity on the architecture and epiphytes of *Cladophora glomerata* (Chlorophyta). *Journal of Phycol*ogy 31: 264-271.

Beversdorf L., S. Bornstein-Forst, and S. L. McLellan. 2007. The potential for beach sand to serve as a reservoir for *Escherichia coli* and the physical influences on cell die-off. *Journal of Applied Microbiology* 102: 1372-1381.

Birch, PB, JO Gabrielson, and KS Hamel. 1983. Decomposition of *Cladophora*. I. Field studies in the Peel-Harvey Estuarine System, Western Australia. *Botanica Marina* 26: 165-171.

Brock, T. D. 1985. *A Eutrophic Lake, Lake Mendota, Wisconsin*. Springer-Verlag, New York.

Burke, C., P. Steinberg, D. Rusch, S. Kjelleberg, and T. Thomas. 2011. Bacterial community assembly based on functional genes rather than species. *Proceedings of the National Academy of Sciences, USA* 34: 14288-14293.

Byappanahalli, M. N., D. A. Shively, M. B. Nevers, M. J. Sadowsky, and R. L. Whitman. 2003. Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga *Cladophora* (Chlorophyta). *Federation of European Microbiological Societies Microbial Ecol*ogy 46:203-11.

Canale, R. P., and M. T. Auer. 1982. Ecological studies and mathematical modeling of *Cladophora* in Lake Huron: 5. Model development and calibration. *Journal of Great Lakes Res*earch 8: 112-125.

Cheney, C. and R. A. Hough. 1983. Factors controlling photosynthetic productivity in a population of *Cladophora fracta* (Chlorophyta). *Ecology* 64: 68-77.

Choo, K.-S., P. Snoeijs, and M. Pedersén. 2002. Uptake of inorganic carbon by *Cladophora glomerata* (Chlorophyta) from the Baltic Sea. *Journal of Phycology* 38: 493-502.

Clarridge J. E. 2004. Impact of 16S rRNA gene sequence analysis for identification of Bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Review* 17(4): 840-862.

Croft, M. T., A. D. Lawrence, E. Raux-Deery, M. J. Warren, and A. G. Smith. 2005. Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria. *Nature* 438: 90-93.

Croft, M. T., M. J. Warren, and A. G. Smith. 2006. Algae need their vitamins. *Eukaryotic Cell* 5: 1175-1183.

Daligault, H., A. Lapida, A. Zeytun, M. Nolan, S. Lucas, T. G. Del Rio, H. Tice, et al. 2011. Complete genome sequence of *Haliscomenobacter hydrossis* type strain (O[T]). *Standards in Genomic Sciences* 4: 352-360.

De Menezes, A. B., R. J. Lockhard, M. J. Cox, H. E. Allison, and A. J. McCarthy. 2008. Cellulose degradation by micromonosporas recovered from freshwater lakes and classification of the actinomycetes by DNA gyrase B gene sequencing. *Applied and Environmental Microbiology* 74: 7080-7084.

Dodds, W. K., and D. A. Gudder. 1992. The ecology of *Cladophora*. *Journal of Phycol*ogy 28: 415-427.

Donderski, W. 1991. Production of B-vitamins by heterotrophic planktonic bacteria isolated from pelagic zone of the Lake Jeziorak. *Journal of the Islamic Academy of Science* 4:54-57.

Englebert, E. T., C. McDermott, and G. T. Kleinheinz. 2008a. Impact of the alga *Cladophora* on the survival of *E. coli*, *Salmonella*, and *Shigella* in laboratory microcosm. *Journal of Great Lakes Research* 34: 377-382.

Englebert, E. T., C. McDermott, and G. T. Kleinheinz. 2008b. Effects of the nuisance algae, *Cladophora*, on *Escherichia coli* at recreational beaches in Wisconsin. *Science of the Total Environment* 404: 10-17.

Ensminger, I., C. Hagen, and W. Braune. 2000a. Strategies providing success in a variable habitat: I. Relationships of environmental factors and dominance of *Cladophora glomerata*. *Plant, Cell and Environment* 23: 1119-1128.

Ensminger, I., C. Hagen, and W. Braune. 2000b. Strategies providing success in a variable habitat: Ecophysiology of photosynthesis of *Cladophora glomerata*. *Plant, Cell and Environment* 23: 1129-1136.

Fisher, M. W., Wilcox, L. W. & Graham, L. E. 1998. Molecular characterization of epiphytic bacterial communities on charophycean green algae. *Applied and Environmental Microbiology* 64: 4384-4389.

Gerloff, G. C., and G. P. Fitzgerald. 1976. The nutrition of Great Lakes *Cladophora*. EPA-600/3-79-044, US Environmental Protection Agency, Office of Research and Development, Environmental Research Laboratory, Duluth, MN 55804.

Graham, J. M,. M. T. Auer, R. P. Canale, and J. P. Hoffmann. 1982. Ecological studies and mathematical modeling of *Cladophora* in Lake Huron: 4. Photosynthesis and respirations as functions of light and temperature. *Journal of Great Lakes Research* 8: 100-111.

Graham, L. E. 1982. Cytology, ultrastructure, taxonomy, and phylogenetic relationships of Great Lakes filamentous algae. *Journal of Great Lakes Research* 8: 3-9.

Graham, L. E., J. M. Graham, and L. W. Wilcox. 2009. *Algae*. Pearson, San Francisco.

Guedon, E., S. Payot, M. Desvaux, and H. Petitmange. 1999. Carbon and electron flow in *Clostridium cellulolyticum* grown in chemostat culture on synthetic medium. *Journal of Bacterio*logy 181: 3262-9.

Higgins, S. N., S. Y. Malkin, E. T. Howell, S. J. Guildford, L. Campbell, V. Hirart-Baer, and R. E. Hecky. 2008. An ecological review of *Cladophora glomerata* (Chlorophyta) in the Laurentian Great Lakes. *Journal of Phycol*ogy 44: 1-16.

Hoffmann, J. P. 1990. Dependence of photosynthesis and vitamin B_{12} uptake on cellular viamin B_{12} concentration in the multicellular alga *Cladophora glomerata* (Chlorophyta). *Limnology and Oceanography* 35: 100-108.

Hoffmann, J. P., and L. E. Graham. 1984. Effects of selected physicochemical factors on growth and zoosporogenesis of *Cladophora glomerata* (Chlorophyta). *Journal of Phycol*ogy 20: 1-7.

Hollants, J., O. Leroux, F. Leliaert, H. Decleyre, O. De Clerck, and A. Willems. 2011. Who is in there? Exploration of endophytic bacteria within the siphonous green seaweed *Bryopsis* (Bryopsidales, Chlorophyta). *Public Library of Science One* 6: e26458.

Hoover, S. W., W. D. Marner II, A. K. Brownson, R. M. Lennen, T. M. Wittkopp, J. Yoshitani, S. Zulkifly, et al. 2011. Bacterial production of free fatty acids from freshwater macroalgal cellulose. *Applied Microbiology and Biotechnology* 91: 435-446.

Intergovernmental Panel on Climate Change. 2012. 2.10.2 Direct global warming potentials http://www.ipcc.ch/publications_and_data/ar4/wg1/en/ch2s2-10-2.html

Ishii, S., T. Yan, D.A. Shively, M. N., Byappanahalli, R. L. Whitman and M. J. Sadowsky. 2006. *Cladophora* (Chlorophyta) spp. Harbor human bacterial pathogens in nearshore water of Lake Michigan. *Applied and Environmental Microbiology* 72: 4545-4553.

Jansson, A.-M., 1967. The food-web of the *Cladophora*-belt fauna. *Helgoland Marine Research* 15: 574-588.

Johnson, M, S Shivkumar, and L. Berlowitz-Tarrant. 1996. Structure and properties of filamentous green algae. *Materials Science and Engineering* B38: 103-108.

Jones, CG, JH Lawton, and M Shachak. 1994. Organisms as ecological engineers. *Oikos* 69: 373-386.

Kraufvelin, P., and S. Salovius. 2004. Animal diversity in Baltic rocky shore macroalgae: Can *Cladophora glomerata* compensate for lost *Fucus vesiculosis*? *Estuarine, Coastal and Shelf Science* 61: 369-378.

Kwon, D. D., Y. K. Lee, and H. K. Lee. 2006. *Costertonia aggregata* gen. nov., sp. nov., a mesophilic marine bacterium of the family Flavobacteriaceae, isolated from a mature biofilm. *International Journal of Systematic and Evolutionary Microbiology* 56: 1349-1353.

Lachnit, T., D. Meske, M. Wahl, T. Harder, and R. Schmitz. 2011. Epibacterial community patterns on marine macroalgae are host-specific but temporally variable. *Environmental Microbiology* 13: 655-665.

Lee, K.-C., R. I. Webb, and J. A. Fuerst. 2009a. The cell cycle of the planctomycete *Gemmata obscuriglobus* with respect to cell compartmentalization. *BMC Cell Biology* 10: 4.

Lee, K.-C., R. I. Webb, P. H. Janssen, P. Sangwan, T. Romeo, J. T. Staley, and J. A. Fuerst. 2009b. Phylum Verrucomicrobia representatives share a compartmented cell plan with members of bacterial phylum Planctomycetes. *BMC Microbiology* 9:5 doi:10.1186/1471-2180-9-5

Lowe, R. L., B. H. Rosen, and J. C. Kingston. 1982. A comparison of epiphytes on *Bangia atropurpurea* (Rhodophyta) and *Cladophora glomerata* (Chlorophyta) from northern Lake Michigan. *Journal of Great Lakes Res*earch 8: 164-168.

McDonald, J. E., A. B. de Menezes, H. E. Allison, and A. J. McCarthy. 2009. Molecular biological detection and quantification of novel *Fibrobacter* populations in freshwater lakes. *Applied and Environmental Microbiol.* 75: 5148-5152.

Malkin, S. Y., R. J. Sorichetti, J. A. Wiklund., And R. E. Hecky. 2009. Seasonal abundance, community composition, and silica content of diatoms epiphytic on *Cladophora glomerata*. *Journal of Great Lakes Research* 35: 199-205.

Marshall, K., I. Joint, M. E. Callow, and J. A. Callow. 2006. Effect of marine bacterial isolates on the growth and morphology of axenic plantlets of the green alga *Ulva linza*. *Microbial Ecology* 52: 302-310.

Matsuo, Y., H. Imagawa, M. Nishizawa, and Y. Shizuri. Isolation of an algal morphogenesis inducer from a marine bacterium. *Science*. 307: 1598.

Medie, F. M., G. J. Davies, M. Drancourt, and B. Henrissat. 2012. Genome analyses highlight the different biological roles of cellulases. *Nature Reviews Microbiology* 10: 227-234.

Metzker, M. L. 2010. Sequencing technologies – the next generation. *Nature Reviews Genetics* 11:31-46.

Mihranyan, A. 2011. Cellulose from cladophorales green algae: From environmental problem to high-tech composite materials. *Journal of Applied Polymer Science* 119: 2449-2460.

Mpawenayo, B and JM Mathooko. 2005. The structure of diatom assemblages associated with *Cladophora* and sediments in a highland stream in Kenya. *Hydrobiologia* 544: 55-67.

Nakanishi, K., M. Nishijima, M. Nishimura, K. Kuwano, and N. Saga. 1996. Bacteria that induce morphogenesis in *Ulva pertusa* (Chlorophyta) grown under axenic conditions. *Journal of Phycology* 32: 479-482.

Olapade, O. A., M. M. Depas, E. T. Jensen, and S. L. McLellan. 2006. Microbial communities and fecal indicator bacteria associated with *Cladophora* mats on beach sites along Lake Michigan shores. *Applied and Environmental Microbiology* 72: 1932-1938.

Paalme, T, H Kukk, J Kotta, and H Orav. 2002. 'In vivo' and 'in situ' decomposition of nuisance macroalgae *Cladophora glomerata* and *Pilayella littoralis*. *Hydrobiologia* 475/476: 469-476.

Patrick, R., C. F. Rhyne, R. W. Richardson III, R. A. Larson, T. T. Bott, and K. Rogenmuser. 1983. The potential for biological controls of *Cladophora glomerata*. USEPA, EPA 600/3-83-065, 153 pp.

Paver, S. F, and A. D. Kent. 2010. Temporal patterns in glycolate-utilizing bacterial community composition correlate with phytoplankton population dynamics in humic lakes. *Microbial Ecol*ogy 60: 406-418.

Provasoli, L., and I. J. Pintner. 1980. Bacteria induced polymorphism in an axenic laboratory strain of *Ulva lactuca* (Chlorophyceae). *Journal of Phycology* 16:196-201.

Ragon, M., G. Restaux, D. Moreira, A. P. Møller, and P. López-Garcia. 2011. Sunlight-exposed biofilm microbial communities are naturally resistant to Chernobyl ionizing-radiation levels. *Public Library of Science One* 6:e21764.

Rivas, R., E. Velázquez, A. Willems, N. Viscaíno, N. S. Subba-Rao, P. F. Mateos, M. Gillis, et al. 2002. A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (L.f.) Druce. *Applied and Environmental Microbiology* 68: 5217-5222.

Salinero, K. K., K. Keller, W. S. Feil, H. Feil, S. Trong, G. DiBartolo, and A. Lapidus. 2009. Metabolic analysis of the soil microbe *Dechloromonas aromatica* str. RCB: indications of a surprisingly complex life-style and cryptic anaerobic pathways for aromatic degradation. *BMC Genomics* 10: 351.

Schloss, P. D., D. Gevers, and S. L. Westcott. 2011. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. Public Library of Science One 6: e273710.

Sheath, R. G., and M. O, Morison. 1982. Epiphytes on *Cladophora glomerata* in the Great Lakes and St. Lawrence seaway with particular reference to the red alga *Chroodactylon ramosum* (=*Asterocytis smargdina*). *Journal of Phycol*ogy 18: 385-391.

Stevenson, R. J., and E. F. Stoermer. 1982. Abundance patterns of diatoms on *Cladophora* in Lake Huron with respect to a point source of wastewater treatment plant effluent. *Journal of Great Lakes Research* 8: 184-195.

Stock, I., and Wiedemann, B. 2001. Natural antimicrobial susceptibilities of *Plesiomonas shigelloides* strains. *Journal of Antimicrobial Chemotherapy* 48: 803-811.

Suen, G., P. J. Weimer, D. M. Stevenson, F. O. Aylward, J. Boyum, J. Deneke, C. Drinkwater, et al. 2011. The complete genome sequence of *Fibrobacter succinogenes* S85 reveals a cellulolytic and metabolic specialist. Public Library of Science ONE 6(4): e18814. doi:10.1371/journal.pone.0018814

Sugita, H, C. Miyajima, and Y. Deguchi. 1991. The vitamin B_{12}-producing ability of the intestinal microflora of freshwater fish. *Aquaculture* 92: 267-276.

Sugita, H., A. Kirima, C. Hirato, T. Ohkoshi, R. Okada and Y. Deguchi. 1994. The vitamin-B_{12}-producing bacteria in the water and sediment of a carp culture pond. *Aquaculture* 119: 425-431.

Tang, Y. Z., F. Koch, and C. J. Gobler. 2010. Most harmful algal bloom species are vitamin B_1 and B_{12} auxotrophs. *Proceedings of the National Academy of Sciences, USA* 107: 20756-20761.

Tatewaki, M., L. Provasoli, and I. J. Pintner. 1983. Morphogenesis of *Monostroma oxyspermum* (Kütz.) Doty (Chlorophyceae) in axenic culture, especially in bialgal culture. *Journal of Phycology* 19: 409-416.

Tujula, N. A., G. R. Crocetti, C. Burke, T. Thomas, and C. Holmström. 2010. Variability and abundance of the epiphytic bacterial community associated with a green marine Ulvacean alga. *The ISME Journal* 4: 301-311.

Van Berkum, P., and B. D. Eardly. 2002. The aquatic budding bacterium *Blastobacter denitrificans* is a nitrogen-fixing symbiont of *Aeschynomene indica*. *Applied and Environmental Microbiology* 68: 1132-1136.

Voget, S., H. L. Steele, and W. R. Streit. 2006. Characterization of a metagenome-derived halotolerant cellulase. *Journal of Biotechnology* 126: 26-36.

Wang, Q, G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* 73: 5261-5267.

Wang, Y., and P. Y. Qian. 2009. Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies. Public Library of Science ONE 4: e7401.

Ward, J. M., and A. Ricciardi. 2010. Community-level effects of co-occurring native and exotic ecosystem engineers. *Freshwater Biology* 55: 1803-1817.

Whitman, R. L., D. A. Shively, H. Pawlik, M. B. Nevers, and M. N. Byappanahalli. 2003. Occurrence of *Escherichia coli* and enterococci in *Cladophora* (Chlorophyta) in nearshore water and beach sand of Lake Michigan. *Applied and Environmental Microbiology* 69: 4714-4719.

Whitton, B. A. 1970. Biology of *Cladophora* in freshwaters. *Water Research* 4: 457-476.

Whitton, B. A. 2002. Phylum Cyanophyta (Cyanobacteria). *In* D. M. John, B. A. Whitton, and A. J. Brook [eds.], The Freshwater Algal Flora of the British Isles: An Identification Guide to Freshwater and Terrestrial Algae, 25-122. Cambridge University Press, Cambridge, UK.

Wommack, K. E., J. Bhavsar, and J. Ravel. 2008. Metagenomics: Read length matters. *Applied and Environmental Microbiology* 74:1543-1463.

Yoon, J.-H., S.-J. Kang, S. Park, and T.-K. Oh. 2007a. *Devosia insulae* sp. nov., isolated from soil, and emended description of the genus *Devosia*. *International Journal of Systematic and Evolutionary Microbiology* 57: 1310-1314.

Young, E. B., R. C. Tucker, and L. Pansch. 2010. Alkaline phosphatase in freshwater *Cladophora*-epiphyte assemblages: regulation in response to phosphorus supply and localization. *Journal of Phycology* 46: 93-101.

Zehms, T. T., C. McDermott, and G. T. Kleinheinz. 2008. Microbial concentrations in sand and their effect on beach water in Door County, Wisconsin. *Journal of Great Lakes Research* 34: 524-534.

Zhang, G., G. Zeng, X. Cai, S. Deng, H. Luo, and G. Sun. 2007. *Brachybacterium zhongshanense* sp. nov., a cellulose-decomposing bacterium from sediment along the Qijiang River, Zhongshan City, China. *International Journal of Systematic and Evolutionary Microbiology* 57: 2519-2524.

Zhang, H., Y. Sekiguchi, S. Hanada, P. Hugenholtz, H. Kim, Y. Kamagata, and K. Nakamura. 2003. *Gemmatimonas aurantiaca* gen. nov., sp. nov., a Gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. nov. *International Journal of Systematic and Evolutionary Microbiology* 53: 1155-1163.

Zulkifly, S. 2012. Seasonal dynamics of chemical and microbial components of *Cladophora*-dominated freshwater periphyton communities. A thesis submitted in partial fulfillment of the requirements for the Ph.D. degree at the University of Wisconsin, Madison, WI.

Table. A2.1. Bacterial genera inferred from 16S rRNA gene sequences associated with Cladophora glomerata in replicate samples taken from Lake Mendota, WI, through the growth season of 2011. See Supplementary Table 1 for functionalities and literature references.

Domain Bacteria	Genus
Phylum Proteobacteria	
Class Alphaproteobacteria	*Erythromicrobium*
	Hyphomicrobium
	Porphyrobacter
	Sandarakinorhabdus
	Croceicoccus
	Devosia insulae
	Blastobacter
	Pseudorhodobacter
	Altererythrobacter
	Sphingopyxis
	Roseomonas
	Sphingomonas
	Novosphingobium
	Rhodobacter
	Brevundimonas
	Blastomonas
	Enhydrobacter
Class Betaproteobacteria	*Leptothrix*
	Hydrogenophaga
	Inhella
	Methylotenera
	Pseudorhodoferax
	Rhodoferax
	Ferribacterium
	Chitinbacter
	Methylibium
	Dechloromonas
	Macromonas
	Vogesella
	Thiobacter
	Malikia
	Mitsuaria
	Curvibacter
	Ideonella
	Massilia
	Undibacterium
	Limnobacter
	Aquabacterium
Class Deltaproteobacteria	*Byssovorax*

	Enhygromyxa
	Geobacter
	Anaeromyxobacter
	Kofleria
	Sorangium
	Bdellovibrio
	Nannocystis
	Desulfobulbus
Class Gammaproteobacteria	*Haliea*
	Pseudomonas
	Silanimonas
	Lysobacter
	Alishewanella
	Legionella
	Luteimonas
	Alkanindiges
	Rheinheimera
	Plesiomonas
	Methylobacter
	Cellvibrio

Phylum Bacteriodetes

| Class Sphingobacteria | *Sediminibacterium* |
	Terrimonas
	Aquiflexum
	Emticicia
	Flavisolibacter
	Lactibacter
	Leadbetterella
	Arcicella
	Filimonas
	Ferruginibacter
	Haliscomenobacter
	Niabella
	Segetibacter
	Runella
	Flectobacillus
	Flexibacter
Class Flavobacteria	*Crocinitomix*
	Flavobacterium
	Wautersiella
	Formosa
	Fluviicola
	Mariniflexile
	Cloacibacterium

Class Bacteroidia *Porphyromonas*
Phylum Planctomycetes

Planctomyces
Gemmata
Rhodopirellula
Pirellula
Zavarzinella

Phylum Verrucomicrobiae

Luteolibacter
Verrucomicrobium
Prosthecobacter
Opitutus

Phylum Deinococcus-Thermus

Meiothermus
Truepera
Deinococcus

Phylum Lentisphaerae

Lentisphaera

Phylum Nitrospirae

Nitrospira

Phylum Fusobacteria

*Cetobacterium*

Phylum Gemmatimonadetes *Gemmatimonas*

Figure A2.1. Phylum (and major class-level) classification of bacterial genera or species (listed in Table A2.1) that could be identified from quality-filtered pyrosequences 200-600 base pairs in length, amplified from the Lake Mendota *Cladophora glomerata* epiphyte community sampled in replicate monthly from June through September, 2011.
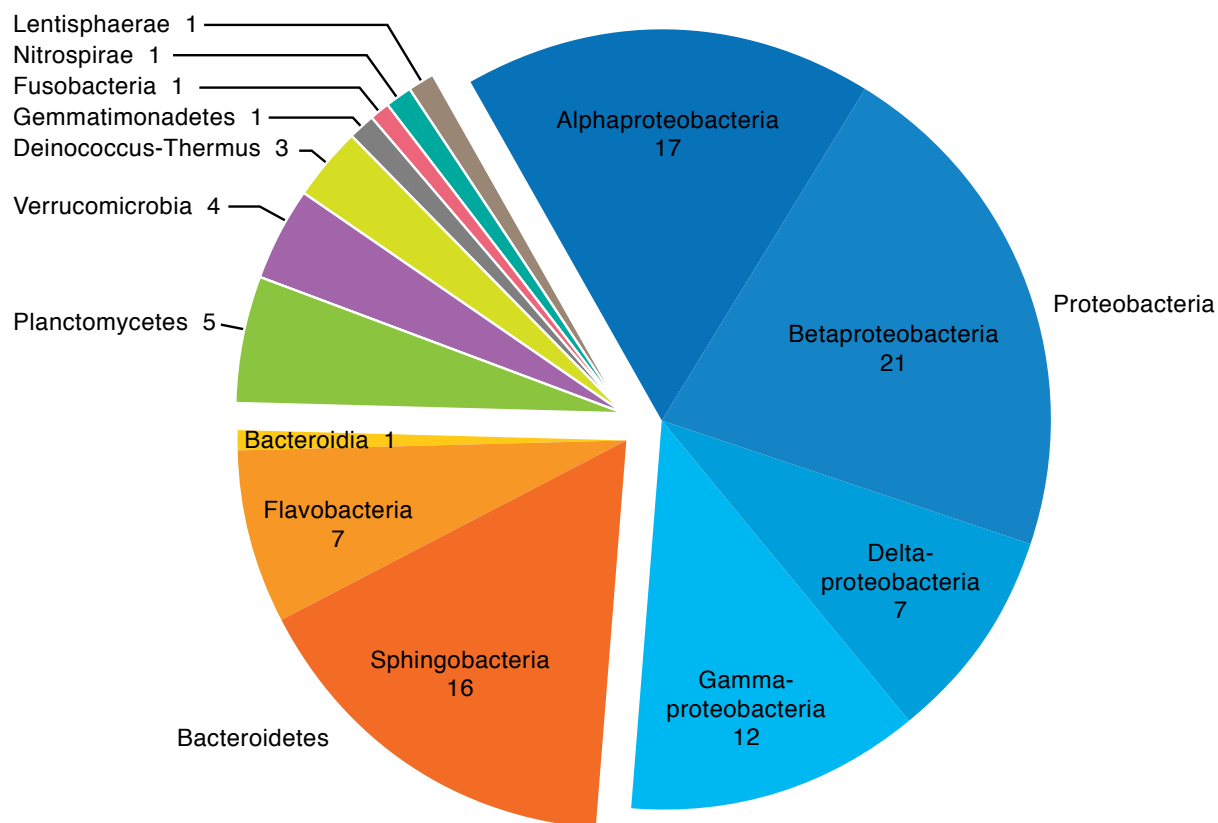
**Figure A2.2. Chamaesiphon incrustans epiphytic on Cladophora glomerata.** A.
Epifluorescence microscopy view after staining with the DNA-specific fluorochrome DAPI, in
UV excitation. Characteristic exospores are present (arrow). Frustules of the flat, oval-shaped
pennate diatom genus *Cocconeis* (arrowheads). B. SEM showing *Chamaesiphon* (arrow),
Cocconeis, and a cluster of putative filamentous cyanobacteria (arrowheads).
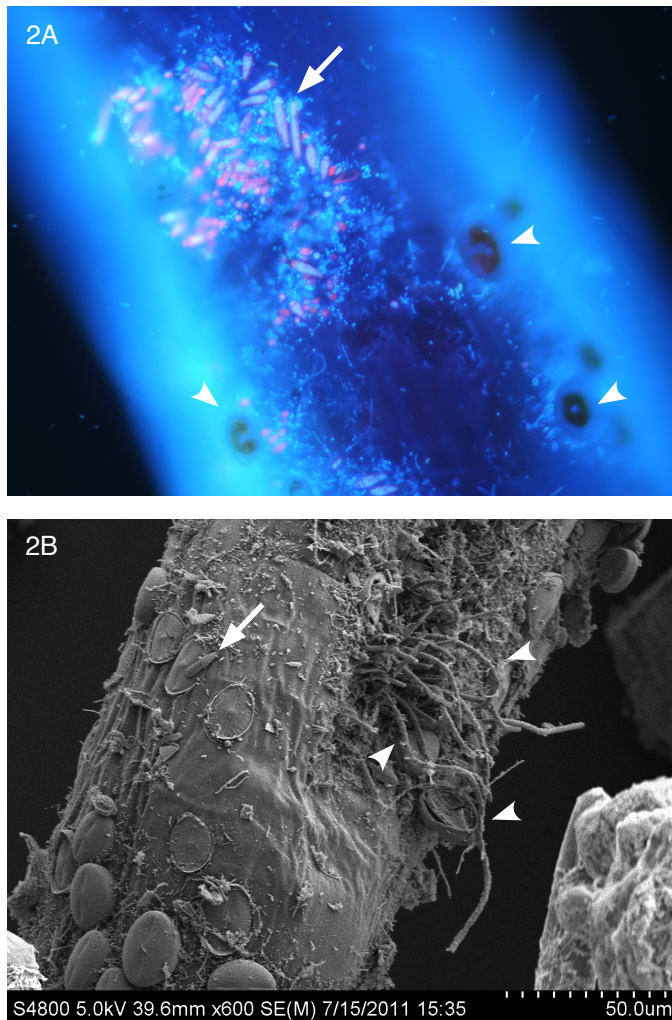
Figure A2.3. Elongate filament bacterial morphotype. A. Epiluorescence microscopy view after staining with the DNA-specific fluorochrome DAPI, in UV excitation. The elongate bacteria stand erect on the surface of the *C. glomerata*. B. SEM showing elongate bacteria lying on the algal surface with numerous smaller bacteria, a *Chamaesiphon incrustans* cell with exospore (arrow) and a *Cocconeis* diatom (arrowhead).
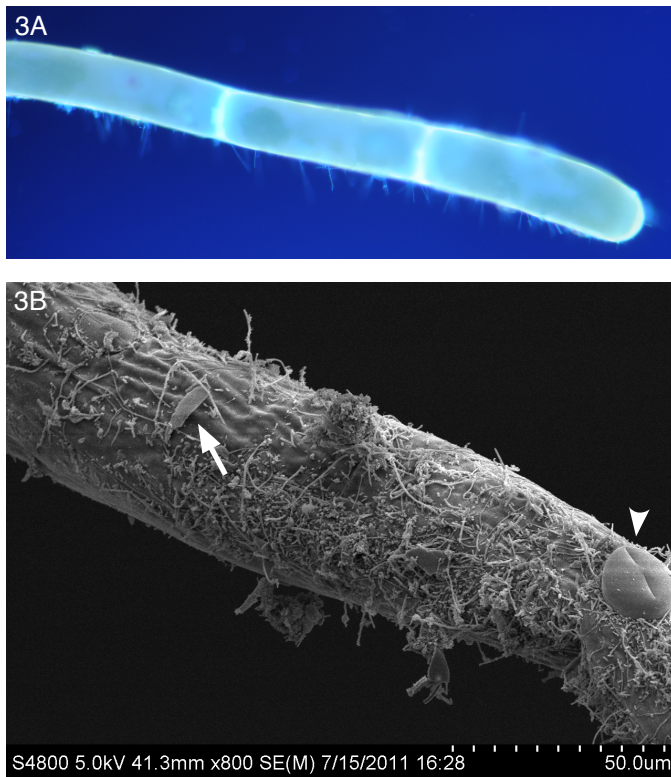
Figure A2.4. A bushy bacterial morphotype consisting of agglomerated tiny cocci. A. Lobed colony viewed with epifluorescence microscopy. B. Lobed colony viewed with SEM (arrow). Note that the cocci are smaller in diameter than the many nearby short rods and longer filament morphotypes.
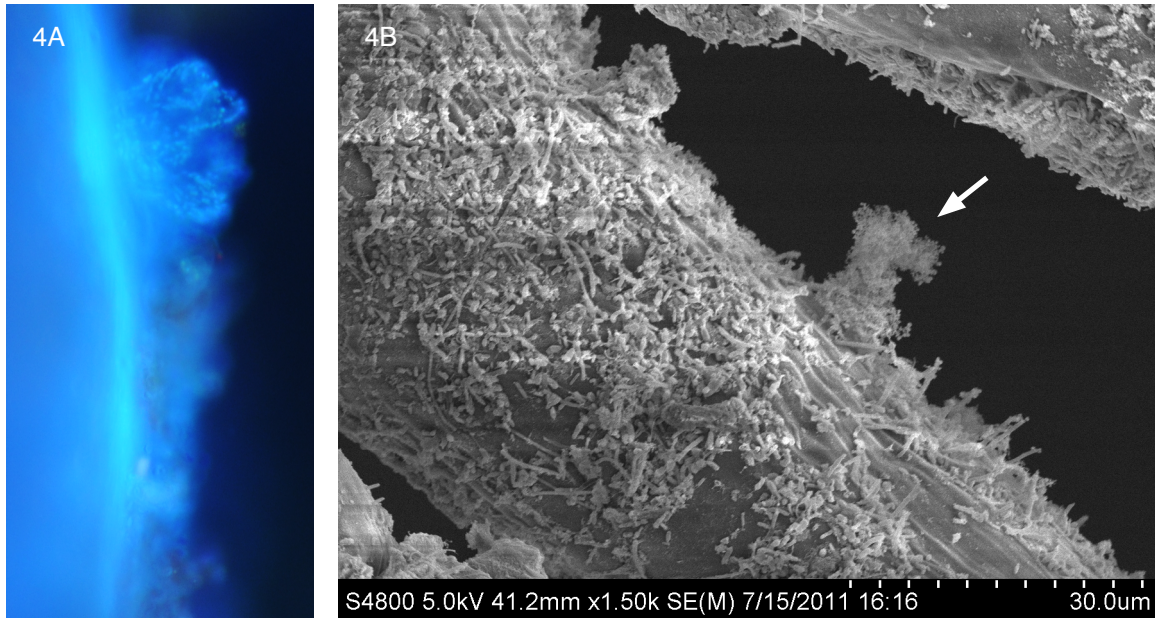
Figure A2.5. "Branched" rod bacterial morphotype (arrows). A. Viewed with DAPI epifluorescence microscopy. B. Viewed with SEM (circled). Note that the diatom *Cocconeis* does not accumulate a coating of bacterial cells, as does the macroalgal host *Cladophora*.
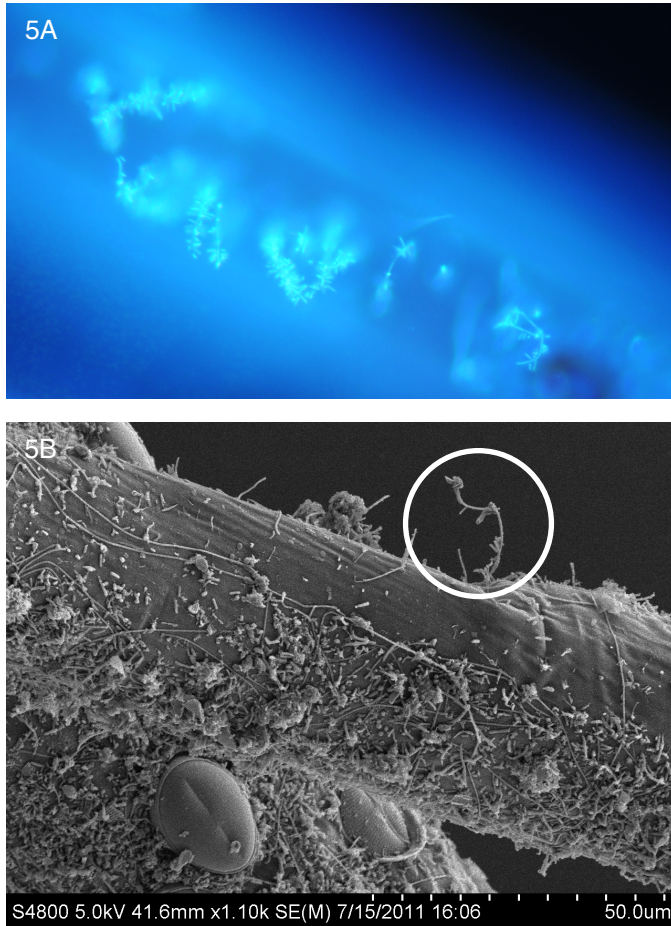
Supplemental Table 2.1. Bacterial genera inferred from 16S rRNA gene sequences associated with *Cladophora glomerata* in replicate samples taken from Lake Mendota, WI, through the growth season of 2011, with referenced functionalities.

Domain Bacteria	Genus	Habitat, phenotype
Phylum Proteobacteria		
Class Alphaproteobacteria	*Erythromicrobium*	Photosynthetic (Yurkov et al., 1997)
	Hyphomicrobium	Aquatic, soil, plant leaves, genome (Vuilleumier et al., 2011)
	Porphyrobacter	Marine, hot springs, photosynthetic (Rainey et al., 2003; Yoon et al., 2004)
	Sandarakinorhabdus	Freshwater lakes, photosynthetic (Gich and Overmann, 2006)
	Croceicoccus	Deep sea sediments (Xu et al., 2009)
	Devosia insulae	Soil; *D. neptuniae* is a N-fixing symbiont in root nodules of aquatic legume *Neptunia natans*, *Nif* gene on plasmid (Rivas et al., 2002; Yoon et al., 2007)
	Blastobacter	Aquatic; one species is the nitrogen-fixer symbiotic in nodules of the flood-tolerant legume *Aeschynomene indica*, related to *Bradyrhizobium* (van Berkum and Eardly, 2002)
	Pseudorhodobacter	Marine (Jung et al., 2012)
	Altererythrobacter	Desert sand, marine sediments (Matsumoto et al., 2011; Xue et al., 2012)
	Sphingopyxis	Diverse habitats including cold marine, proteome (Ting et al., 2010)
	Roseomonas	Freshwater sediments (Jiang et al, 2006)
	Sphingomonas	Degrades refractory contaminants, mycoantagonist, secretes gellan exopolysaccharides (White et al., 1996; Abrusci et al., 2011)
	Novosphingobium	Pollutant degrader (Gupta et al., 2009)
	Rhodobacter	Swine waste lagoon, photosynthetic, non-sulfur, some have cellulose synthesis operon (Do et al., 2003; Medie et al., 2012)
	Brevundimonas	Soil (Yoon et al., 2007)
	Blastomonas	Freshwater, photosynthetic (Hiraishi et al., 2000)
	Enhydrobacter	Water, eutrophic lake (Kawamura et al., 2012).
Class Betaproteobacteria	*Leptothrix*	Wetlands, Fe(II) oxidation, sheath long and 1-2 μm wide (Fleming et al., 2011)
	Hydrogenophaga	Water, soil, H-oxidation (Kim et al., 2010)

	Inhella	Freshwater (Song et al., 2009; Chen et al., 2011)
	Methylotenera	Obligate methanotroph, proteome (Bosch et al., 2009)
	Pseudorhodoferax	Produces secondary compounds (Bruland et al., 2009)
	Rhodoferax	Most species are photosynthetic (Hochkoeppler et al., 1995)
	Ferribacterium	Freshwater lake sediments, Fe(III) reducer (Cummings et al., 1999)
	Chitinbacter	Stream water, chitin-degrader (Yang, et al., 2010)
	Methylibium	Methanotroph (Stackebrandt et al., 2009)
	Dechloromonas	Soil, aromatic degrader, putative N-fixation, genome, metabolome (Salinero et al., 2009)
	Macromonas	Lakes (Matsuyama, 2004)
	Vogesella	Pond (Chou et al., 2009)
	Thiobacter	Subsurface hot aquifer, chemolithoautotrophic (sulfur oxidizing) (Hirayama et al, 2005)
	Malikia	Wastewater, long rod (Springer et al., 2005)
	Mitsuaria	Plant associations, suppresses plant pathogens, genome (Rong et al., 2012)
	Curvibacter	Wellwater (Ding and Yokota, 2004)
	Ideonella	Chlorate-respiration (Bäcklund et al., 2009)
	Massilia	Drinking water, soil (Zul et al., 2008)
	Undibacterium	Water (Eder et al., 2011)
	Limnobacter	Freshwater sediment, chemolithoheterotrophic, thiosulfate oxiders (Spring et al., 2001)
	Aquabacterium	Water (Lin et al., 2009)
Class Deltaproteobacteria	*Byssovorax*	Cellulose-degrading myxobacterium Reichenbach et al., 2006)
	Enhygromyxa	Marine myxobacterium (Iizuka, et al., 2003)
	Geobacter	Fe(III) oxide reduction, extracellular reduction of U(IV) to tetravalent uranium U(IV), electrically-conductive pili (Cologgi et al., 2011)
	Anaeromyxobacter	Myxobacterium, long rod, aryl-halorespiring facultative anaerobe, several species possess cellulose synthesis operon (Sandford et al., 2002; Medie et al., 2012)
	Kofleria	Rods in slime sheet that swarms
	Sorangium	Myxobacterium, bushy fruiting bodies, anti-cancer drug epothilone B, *S. cellulosum*

		degrades cellulose and genome encodes 17 cellulases, source of industrial enzymes, genome (Schneiker et al., 2007; Cao et al., 2011; Medie et al., 2012)
	Bdellovibrio	Predator on other bacteria (Rendulic et al., 2004; Mahmoud and Koval, 2010; Lambert et al., 2011)
	Nannocystis	Myxobacterium, produces secondary metabolites of potential technological application (Ohlendorf et al., 2008)
	Desulfobulbus	Aquatic sediments, sulfur cycling, genome for *D. proprionicus* (Pagani et al., 2011)
Class Gammaproteobacteria	*Haliea*	Marine (Lucena et al., 2010)
	Pseudomonas	Water, genomes of some species display cellulose operons or encode cellulases (Zablotowicz et al., 2001; Medie et al., 2012)
	Silanimonas	Slightly thermophilic, alkaliphilic hot spring (Lee et al., 2005)
	Lysobacter	Rice and pepper rhizospheres, antimicrobial (Park et al., 2008; Aslam et al., 2009)
	Alishewanella	Tidal flat, lake (Roh et al, 2009; Tahrhiz et al., 2011)
	Legionella	Water, live within ameobae, genome of *L. longbeachae* encodes 2 cellulases (Huang et al., 2011; Medie et al., 2012)
	Luteimonas	Marine, tidal flat (Park et al., 2011)
	Alkanindiges	Alkane degrader isolated from oil fields, activated sludge (Bogan et al., 2003; Klein et al., 2007)
	Rheinheimera	Alkaline lake (Brettar et al., 2006)
	Plesiomonas	Vitamin B_{12} producer (Sugita et al., 1994)
	Methylobacter	Arctic permafrost, methanotroph, genome (Svenning et al., 2011)
	Cellvibrio	Soils, genome of *C. japonicus* encodes 21 cellulases (Mergaert et al., 2003; Medie et al., 2012)
Phylum Bacteroidetes Class Sphingobacteria	*Sediminibacterium*	Freshwater eutrophic reservoir (Qu and Yuan, 2008)
	Terrimonas	Soil, freshwater spring (Sheu et al., 2010)
	Aquiflexum	Marine (Brettar et al., 2004a)
	Emticicia	Sludge (Saha and Chakrabarti, 2006)
	Flavisolibacter	Soil (Yoon and Im, 2007)
	Lactibacter	Aquatic

	Leadbetterella	Said to degrade starch and gelatine but not cellulose or chitin, though *L. byssophila* genome encodes 3 cellulases (Abt et al., 2011; Medie et al, 2012)
	Arcicella	Streamwater, freshwater lake (Nikitin et al., 2004; Sheu et al., 2010)
	Filimonas	Freshwater (Shiratori et al., 2009)
	Ferruginibacter	Freshwater sediments (Lim et al., 2009)
	Haliscomenobacter	Freshwater, "imprisoned rod" has a long sheath, genome (Daligault et al., 2012)
	Niabella	Soil (Kim et al., 2007)
	Segetibacter	Soil (An et al., 2007)
	Runella	Sludge, wastewater, long rod (Lu et al., 2007)
	Flectobacillus	Eutrophic pond (Hwang and Cho, 2006)
	Flexibacter	Aquatic, cells up to 100 μm long
Class Flavobacteria	*Crocinitomix*	Polar, marine, small rods to filaments >100 μm long, produce catalase (Bowman et al, 2003)
	Flavobacterium	Water, long rod, some fish pathogens, proteome (Dumpala et al., 2010)
	Wautersiella	Heterotrophic rod (Kämpfer et al., 2006)
	Formosa	Marine, agariphilic, from *Fucus* and *Acrosiphonia*, budding (Ivanova et al., 2004; Nedashkovskaya et al., 2006)
	Fluviicola	River, genome (O'Sullivan et al., 2005; Woyke et al., 2011)
	Mariniflexile	Marine, degrades sulfated galactans from brown algae (Jung et al., 2011)
	Cloacibacterium	Freshwater lake sediments (Cao et al., 2010)
Class Bacteroidia	*Porphyromonas*	Fish and human gut, obligate anaerobe (van Kessel et al., 2011)
Phylum Planctomycetes	*Planctomyces*	Freshwaters, genome for *P. brasiliensis* encodes 2 cellulases (LaButti et al., 2010; Jogler et al., 2011; Medie et al., 2012)
	Gemmata	Compartmented (Lee et al., 2009)
	Rhodopirellula	Marine, attached, budding, *R. baltica* genome encodes 1 cellulase (Winkelmann et al., 2010; Medie et al., 2012)
	Pirellula	Marine, genome (Glöckner et al., 2008)
	Zavarzinella	Acid *Sphagnum* bog, rosette, budding, doesn't consume cellulose (Kulichevskya et al., 2009)
Phylum Verrucomicrobiae	*Luteolibacter*	Marine from red algae, arctic tundra (Yoon et al., 2008; Jiang et al., 2011)

	Verrucomicrobium	Compartmented, interacts with eukaryotes (Sait et al., 2011)
	Prosthecobacter	Compartmented, degrades the *Leptothrix* sheath (Jenkins et al., 2002; Pilhofer et al., 2007)
	Opitutus	Uses plant-derived polysaccharides to make proprionate and interact with methanogens, *O. terrae* genome encodes 14 cellulases (van Passell et al., 2011; Medie et al., 2012).
Phylum Deinococcus-Thermus		
	Meiothermus	Hot springs, genome (Tindall et al., 2010; Zhang et al., 2010)
	Truepera	Sarcinoid, extreme resistance to ionizing radiation and high irradiance, genome of *T. radiovictrix* encodes 1 cellulase (Ivanova et al, 2011; Medie et al., 2012)
	Deinococcus	Water, sediment, soil, sludge, compost; strictly aerobic, high resistance to ionizing radiation (Im et al., 2008)
Phylum Lentisphaerae	*Lentisphaera*	Marine, slime, genome (Cho et al., 2004; Thrash et al., 2010)
Phylum Nitrospirae	*Nitrospira*	Nitrite oxidizer (Lücker et al., 2010)
Phylum Fusobacteria	*Cetobacterium*	Fish and human gut, obligate anaerobe, Vitamin B_{12} producer (van Kessel et al., 2011)
Phylum Gemmatimonadetes	*Gemmatimonas*	Wastewater, polyphosphate-accumulator (Zhang, et al., 2003)

Additional Literature Cited:

Abrusci, C., J. Palomar, J. L. Pablos, F. Rodriguez, and F. Catalina. 2011. Efficient biodegradation of common ionic liquids by *Sphingomonas paucimobilis* bacterium. *Green Chem*. 13: 709-717.

Abt, B., H. Teshima, S. Lucas, A. Lapidus, T. G. Del Rio, M. Nolan, H. Tice, et al. 2011. Complete genome sequence of *Leadbetterella byssophila* type strain (4M15[T]). *Standards in Genomic Sciences* 4: 2-12.

An, S.-S., H.-G. Lee, W.-T. Im, Q.-M. Liu, and S.-T. Lee. 2007. *Segetibacter koreensis* gen. nov., sp. nov., a novel member of the phylum Bacteroidetes, isolated from the soil of a ginseng field in South Korea. *International Journal of Systematic and Evolutionary Microbiology* 57:1828-1833.

Aslam, Z., M. Yasir, C. O. Jeon, and Y. R. Chung. 2009. *Lysobacter oryzae* sp. nov., isolated from the rhizosphere of rice (*Oryza sativa* L.). *International Journal of Systematic and Evolutionary Microbiology* 59: 675-680.

Bäcklund, A. S., J. Bohlin, N. Gustavsson, and T. Nilsson. 2009. Periplasmic c cytochromes and chlorate reduction in *Ideonella dechloratans*. *Applied and Environmental Microbiology* 75: 2439-2445.

Bogan, B. W., W. R. Sullivan, K. J. Kayser, K. D. Derr, H. C. Aldrich, and J. R. Paterek. 2003. *Alkanindiges*

illinoisensis gen. nov., sp. nov., an obligately hydrocarbonoclastic, aerobic squalane-degrading bacterium isolated from oilfield soils. *International Journal of Systematic and Evolutionary Microbiology* 53: 1389-1395.

Bosch, G., T. Wang, E. Latypova, M. G. Kalyuzhnaya, M. Hackett, and L. Chistoserdova. 2009. Insights into the physiology of *Methylotenera mobilis* as revealed by metagenome-based shotgun proteomic analysis. *Microbiology* 155: 1103-1110.

Bott, T. L., and K. Rogenmuser. 1980. Fungal pathogen of *Cladophora glomerata* (Chlorophyta). *Applied and Environmental Microbiology* 40: 977-980.

Bowman, J. P., C. M. Nichols, and J. A. E. Gibson. 2003. *Algoriphagus ratkowskyi* gen. nov., sp. nov., *Brumimicrobium glaciale* gen. nov., sp. nov., *Cryomorpha ignava* gen. nov., sp. nov. and *Crocinitomix catalasitica* gen. nov., sp. nov., novel flavobacteria isolated from various polar habitats. *International Journal of Systematic and Evolutionary Microbiology* 53: 1343-1355.

Brettar, I., R. Christen, and M. G. Höfle. 2004. *Aquiflexum balticum* gen. nov., sp. nov., a novel marine bacterium of the Cytophaga-Flavobacterium-Bacteroides group isolated from surface water of the central Baltic Sea. *International Journal of Systematic and Evolutionary Microbiology* 54: 2335-2341.

Brettar, I, R. Christen, and M. G. Höfle. 2006. *Rheinheimera perlucida*, sp. nov., a marine bacterium of the Gammaproteobacteria isolated from surface water of the Central Baltic Sea. *International Journal of Systematic and Evolutionary Microbiology* 56: 2177-2183.

Bruland, N., S. Bathe, A. Willems, and A. Steinbüchel. 2009. *Pseudorhodoferax soli* gen. nov., sp. nov. and *Pseudorhodoferax caeni* sp. nov., two members of the class Betaproteobacteria belonging to the family Comamonadaceae. *International Journal of Systematic and Evolutionary Microbiology* 59: 2702-2707.

Cao, S.-J., C.-P. Deng, B.-Z. Li, X.-Q. Dong, and H.-L. Yuan. 2010. *Cloacibacterium rupense* sp. nov., isolated from freshwater lake sediment. *International Journal of Systematic and Evolutionary Microbiology* 60: 2023-2026.

Cao, W.-R., G.-L. Gong, X.-L. Liu, W. Hu, Z.-F. Li, H. Liu, and Y.-Z. Li. 2011. Optimization of epothilone B production by *Sorangium cellulosum* using multiple steps of the response surface methodology. *African Journal of Biotechnology* 10: 11058-11070.

Chen, W.-M., F.-S. Sheu, C.-C. Young, and S.-Y. Sheu. 2011. *Inhella fonticola* sp. nov., isolated from spring water and emended description of the genus *Inhella*. *International Journal of Systematic and Evolutionary Microbiology* doi: 10.11099/ijs.0.034884-0

Cho, J.-C., K. L. Vergin, R. M. Morris, and S. J. Giovannoni. 2004. *Lentisphaera araneosa* gen. nov., sp. nov., a transparent exopolymer producing marine bacterium, and the description of a novel bacterial phylum, Lentisphaerae. *Environmental Microbiology* 6: 611-621.

Chou, J.-H., Y.-J. Chou, A. B. Arun, C.-C. Young, C. A. Chen, J.-T. Wang, and W.-M. Chen. 2009. *Vogesella lacus* sp. nov., isolated from a soft-shell turtle culture pond. *International Journal of Systematic and Evolutionary Microbiology* 59: 2629-2632.

Cologgi, D. L., S. Lampa-Pastirk, A. M. Speers, S. D. Kelly, and G. Reguera. 2011. Extracellular reduction of uranium via *Geobacter* conductive pili as a protective cellular mechanism. *Proceedings of the National Academy of Sciences, USA* 108: 15248-15252.

Cummings, D. E., F. Caccavo Jr., S. Spring and R. F. Rosenzweig. 1999. *Ferribacterium limneticum*, gen. nov., sp. nov., an Fe(III)-reducing microorganism isolated from mining-impacted freshwater lake sediments. *Archves of Microbiol*ogy 171: 183-188.

Ding, L., and A. Yokota. 2004. Proposals of *Curvibacter gracilis* gen. nov., sp. nov. and *Herbaspirillum putei* sp.

nov. for bacterial strains isolated from well water and reclassification of [*Pseudomonas*] *huttiensis*, [*Pseudomonas*] *lanceolata*, [*Aquaspirillum*] *delicatum* and [*Aquaspirillum*] *autotrophicum* and *Herbaspirillum huttiense* comb. nov., *Curvibacter lanceolatus* comb. nov., *Curvibacter delicatus* comb. nov. and *Herbaspirillum autotrophicum* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 54: 2223-2230.

Do, Y. S., T. M. Schmidt, J. A. Zahn, E. S. Boyd, A. de la Mora, and A. A. DiSpirito. 2003. Role of *Rhodobacter* sp. strain PS9, a purple non-sulfur photosynthetic bacterium isolated from an anaerobic swine waste lagoon, in odor remediation. *Applied and Environmental Microbiology* 69: 1710-1720.

Dumpala, P. R., N. Gülsoy, M. L. Lawrence, and A. Karsi. 2010. Proteomic analysis of the fish pathogen *Flavobacterium columnare*. *Proteome Science* 8:26.

Eder, W., G. Wanner, W. Ludwig, H.-J. Busse, F. Ziemke-Kägeler, and E. Lang. 2011. Description of *Undibacterium oligocarboniphilum* sp. nov., isolated from purified water, and *Undibacterium pigrum* strain CCUG 49012 as the type strain of *Undibacterium parvum* sp. nov., and emended descriptions of the genus *Undibacterium* and the species *Undibacterium pigrum*. *International Journal of Systematic and Evolutionary Microbiology* 61: 384-391.

Fleming, E. J., A. E. Langdon, M. Martinez-Garcia, R. Stepanauskas, N. J. Poulton, E. D. P. Masland, and D. Emerson. 2011. What's new is old: Resolving the identity of *Leptothrix ochracea* using single cell genomics, pyrosequencing and FISH. *Public Library of Science One* 6:e17769.

Gich, F., and J. Overmann. 2006. *Sandarakinorhabdus limnophila* gen. nov., sp. nov., a novel bacteriochlorophyll a-containing, obligately aerobic bacterium isolated from freshwater lakes. *International Journal of Systematic and Evolutionary Microbiology* 56: 847-854.

Glöckner, F. O., M. Kube, M. Bauer, H. Teeling, T. Lombardot, W. Ludwig, D. Gade, et al. 2003. Complete genome sequence of the marine planctomycete *Pirellula* sp. strain 1. *Proceedings of the National Academy of Sciences, USA* 100: 8298-8303.

Gupta, S. K., D. Lal, and R. Lal. 2009. *Novosphingobium panipatense* sp. nov. and *Novosphingobium mathurense* sp. nov., from oil-contaminated soil. *International Journal of Systematic and Evolutionary Microbiology* 59: 156-161.

Hiraishi, A., H. Kuraishi, and K. Kawahara. 2000. Emendation of the description of *Blastomonas natatoria* (Sly 1985) Sly and Cahill 1997 as an aerobic photosynthetic bacterium and reclassification of *Erythromonas ursincola* Yurkov et al. 1997 as *Blastomonas ursincola* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 50: 1113-1118.

Hirayama, H., K. Takai, F. Inagaki, K. H. Nealson, and K. Horikoshi. 2005. *Thiobacter subterraneus* gen. nov., sp. nov., an obligately chemolithoautotrophic, thermophilic, sulfur-oxidizing bacterium from a subsurface hot aquifer. *International Journal of Systematic and Evolutionary Microbiology* 55: 467-472.

Hochkoeppler, A., S. Ciurli, G. Venturoli, and D. Zannoni. 1995. The high potential iron-sulfur protein (HiPIP) from *Rhodoferax fermentans* is competent in photosynthetic electron transfer. *Federation of European Biochemical Societies Letters* 357: 70-74.

Huang, S.-W., B.-M. Hsu, N.-H. Chen, C.-C. Huang, K.-H. Huang, J.-S. Chen, and P.-M. Kao. 2011. Isolation and identification of *Legionella* and their host ameobae from weak alkaline carbonate spring water using a culture method combined with PCR. *Parasitology Research* 109: 1233-1241.

Hwang, C. Y., and B. C. Cho. 2006. *Flectobacillus lacus* sp. nov., isolated from a highly eutrophic pond in Korea. *International Journal of Systematic and Evolutionary Microbiology* 56: 1197-1201.

Iizuka, T., Y. Jojima, R. Fudou, M. Tokura, A. Hiraishi, and S. Yamanaka. 2003. *Enhygromyxa salina* gen. nov.,

sp. nov. a slightly halophytic myxobacterium isolated from the coastal seas of Japan. *Systematic and Applied Microbiology* 26: 189-196.

Im, W.-T., H.-M. Jung, L. N. Ten, M. K. Kim, N Bora, M Goodfellow, S. Lim, et al. 2008. *Deinococcus aquaticus* sp. nov., isolated from fresh water, and *Deinococcus caeni* sp. nov., isolated from activated sludge. *International Journal of Systematic and Evolutionary Microbiology* 58: 2348-2353.

Ivanova, E. P., Y. V. Alexeeva, S. Flavier, J. P. Wright, N. V. Zhukova, N. M. Gorshkova, V. V. Miklailov, D. V. Nicolau, and R. Christen. 2004. *Formosa algae* gen. nov., sp. nov., a novel member of the family Flavobacteriaceae. *International Journal of Systematic and Evolutionary Microbiology* 54: 705-711.

Ivanova, N., C. Rohde, C. Munk, M. Nolan, S. Lucas, T. G. Del Rio, H. Tice, et al. 2011. Complete genome sequence of *Truepera radiovictrix* type strain (RQ-24[T]). *Standards in Genomic Sciences* 4: 91-99.

Jenkins, C., R. Samudrala, I. Anderson, B. P. Hedlund, G. Petroni, N. Michailova, N. Pinel, et al. 2002. Genes for the cytoskeletal protein tubulin in the bacterial genus *Prosthecobacter. Proceedings of the National Academy of Sciences, USA* 99: 17049-17054.

Jiang, C.-Y., X. Dai, B.-J. Wang, Y.-G. Zhou, and S.-J. Liu. 2006. *Roseomonas lacus* sp. nov., isolated from freshwater lake sediment. *International Journal of Systematic and Evolutionary Microbiology* 56: 25-28.

Jiang, F., W. Li, M. Xiao, J. Dai, W. Kan, L. Chen, W. Li, et al. 2011. *Luteolibacter luojiensis* sp. nov., isolated from Arctic tundra soil, and emended description of the genus *Luteolibacter. International Journal of Systematic and Evolutionary Microbiology* doi:10.1099/ijs.0.037309-0.

Jogler, C., F. O. Glöckner, and R. Kolter. 2011. Characterization of *Planctomyces limnophilus* and development of genetic tools for its manipulation establish it as a model species for the Phylum Planctomycetes. *Applied and Environmental Microbiology* 77: 5826-5829.

Jung, Y.-T., J. H. Kim, T.K. Oh, and J. H. Yoon. 2011. *Mariniflexile aquimaris* sp. nov., isolated from seawater, and emended description of the genus *Mariniflexile* Nedashkovskaya et al.. 2006. *International Journal of Systematic and Evolutionary Microbiology* doi:10.1099/ijs.0.031492-0.

Jung, Y.-T., K.-H. Oh, T.-K. Oh, and J.-H. Yoon. 2012. *Pseudorhodobacter aquimaris* sp. nov., isolated from seawater, and emended description of the genus *Pseudorhodobacter* Uchino et al. 2002. *International Journal of Systematic and Evolutionary Microbiology* 62: 100-105.

Kämpfer, P., V. Avesani, M, Janssens, J., Charlier, T. De. Baere, and M. Vaneechoutte. 2006. Description of *Wautersiella falsenii* gen., nov., sp. nov., to accommodate clinical isolates phenotypically resembling members of the genera *Chryseobacterium* and *Empedobacter. International Journal of Systematic and Evolutionary Microbiology* 56: 2323-2329.

Kawamura, Y., N. Fujiwara, T. Naka, A. Mitani, H. Kubota, J. Tomida, Y. Norita, and J. Hitomi. 2012. Genus *Enhydrobacter* Staley et al., 1987 should be recognized as a member of the family Rhodospirillaceae within the class Alphaproteobacteria. *Microbiology and Immunology* 56: 21-26.

Kim, B.-Y., H.-Y. Weon, S.-H. Yoo, S.-B. Hong, S.-W. Kwon, E. Stackebrandt, and S.-J. Go. 2007. *Niabella aurantiaca* gen. nov., sp. nov., isolated from a greenhouse soil in Korea. *International Journal of Systematic and Evolutionary Microbiology* 57: 538-541.

Kim, Y.-J., M. K. Kim, H.-Y. Weon, H.-B. Kim, and D.-C. Yang. 2010. *Hydrogenophaga temperata* sp. nov., a betaproteobacterium isolated from compost in Korea. *Journal of General and Applied Microbiol*ogy 56: 419-425.

Klein, A. N., D. Frigon, and L. Raskin. 2007. Populations related to *Alkanindiges*, a novel genus containing obligate alkane degraders, are implicated in biological foaming in activated sludge systems. *Environmental Microbiology* 9:

1898-1912.

Kulichevskaya, I. S., O. I Baulina, P. L. E. Bodelier, W. I. C. Rijpstra, J. S. Sinninghe Damsté, and S. N. Dedysh. 2009. *Zavarzinella formosa* gen. nov., sp. nov., a novel stalked, *Gemmata*-like planctomycete from a Siberian peat bog. *International Journal of Systematic and Evolutionary Microbiology* 59: 357-364.

LaButti, K., J. Sikorski, S. Schneider, M. Nolan, S. Lucas, T. G. Del Rio, H. Tice, et al. 2010. Complete genome sequence of *Planctomyces limnophilus* type strain (Mü 290[T]). *Standards in Genomic Sciences* 3:1 doi: 10.4056/sigs.1052813.

Lail, K., J. Sikorski, E. Saunders, A. Lapidus, T. G. Del Rio., A. Copeland, H. Tice, et al. 2010. Complete genome sequence of *Spirosoma linguale* type strain (1[T]). *Standards in Genomic Science* 2:2.

Lambert, C., A. K. Fenton, L. Hobley, and R. E. Sockett. 2011. Predatory *Bdellovibrio* bacteria use gliding motility to scout for prey on surfaces. *Journal of Bacteriology* 193: 3139-3141.

Lee, E. M., C. O. Jeon, I. Choi, K.-S. Chang, and C.-J. Kim. 2005. *Silanimonas lenta* gen. nov., sp. nov., a slightly thermophilic and alkaliphilic gammaproteobacterium isolated from a hot spring. *International Journal of Systematic and Evolutionary Microbiology* 55: 385-389.

Lim, J. H., S.-H. Baek, and S.-T. Lee. 2009. *Ferruginibacter alkalilentus* gen. nov., sp. nov. and *Ferruginibacter lapsinanis* sp. nov., novel members of the family 'Chitinophagaceae' in the phylum Bacteroidetes, isolated from freshwater sediment. *International Journal of Systematic and Evolutionary Microbiology* 59:2394-2399.

Lin, M.-C., S.-R. Jiang, J.-H. Chou, A. B. Arun C.-C. Young, and W.-M. Chen. 2009. *Aquabacterium fontiphilum* sp. nov., isolated from spring water. International Journal of Systematic and Evolutionary Microbiology 59: 681-685.

Lu, S., J. R. Lee, S. H. Ryu, B. S. Chung, W.-S. Choe, and C. O. Jeon. 2007. *Runella defluvii*, sp. nov., isolated from a domestic wastewater treatment plant. *International Journal of Systematic and Evolutionary Microbiology* 57: 2600-2603.

Lucena, T., J. Pascual, E. Garay, D. R. Arahal, M. C. Macián, and M. J. Pujalte. 2010. *Haliea mediterranea* sp. nov., a marine gammaproteobacterium. *International Journal of Systematic and Evolutionary Microbiology* 60: 1844-1848.

Lücker, S., M. Wagner, F. Maixner, E. Pelletier, H. Koch, B. Vacherie, T. Rattei, et al. 2010. A *Nitrospira* metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *Proceedings of the National Academy of Sciences* 107: 13479-13484.

Mahmoud, K. K., and S. F. Koval. 2010. Characterization of type IV pili in the life cycle of the predator bacterium *Bdellovibrio. Microbiology* 156: 1040-1051.

Matsumoto, A., H. Kasai, Y. Matsuo, S. Omura, Y. Shizuri, and Y. Takahashi. 2009. Iluminobacter fluminus, gen. nov., sp. nov., a novel actinobacterium isolated from the sediment of an estuary. *Journal of General and Applied Microbiol*ogy 55: 201-205.

Matsumoto, M., D. Iwama, A. Arakaki, A. Tanaka, T. Tanaka, H. Miyashita, and T. Matsunaga. 2011. *Altererythrobacter ishigakiensis* sp. nov., an astaxanthin-producing bacterium isolated from a marine sediment. *International Journal of Systematic and Evolutionary Microbiology* 61: 2956-2961.

Matsuyama, M. 2004. Phylogenic status of a purple sulfur bacterium and its bloom in Lake Kaiike. *Limnology* 5: 95-101.

Mergaert, J., D. Lednická, J. Goris, M. C. Cnockaert, P. De Vos, and J. Swings. 2003. Taxonomic study of

Cellvibrio strains and description of *Cellvibrio ostraviensis* sp. nov., *Cellvibrio fibrivorans* sp. nov., and *Cellvibrio gandavensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 53: 465-471.

Nedashkovskaya, O. I., S. B. Kim, M. Vancanneyt, C. Snauwaert, A. M. Lysendo, M. Rohde, G. M. Frolova, et al. 2006. *Formosa agariphila* sp. nov., a budding bacterium of the family Flavobacteriaceae isolated from marine environments, and emended description of the genus *Formosa*. *International Journal of Systematic and Evolutionary Microbiology* 56: 161-167.

Nikitin, D. I., C. Strömpl, M. S. Oranskaya, and W.-R. Abraham. 2004. Phylogeny of the ring-forming bacterium *Arcicella aquatica* gen. nov., sp. nov. (ex Nikitin et al. 1994) from a freshwater neuston biofilm. *International Journal of Systematic and Evolutionary Microbiology* 54: 681-684.

Ohlendorf, B., S. Leyers, A. Krick, S. Kehraus, M. Wiese, and G. M. König. 2008. Phenylnannolones A-C: Biosynthesis of new secondary metabolites from the myxobacterium *Nannocystis exedens*. *ChemBioChem*. 9: 2997-3003.

O'Sullivan, L. A., J. Rinna, G. Humphreys, A. J. Weightman, and J. C. Fry. 2005. *Fluviicola taffensis* gen. nov., sp. nov., a novel freshwater bacterium of the family Cryomorphaceae in the phylum 'Bacteroidetes.' *International Journal of Systematic and Evolutionary Microbiology* 55: 2189-2194.

Pagani, I., A. Lapidus, M. Nolan, S. Lucas, N. Hammon, S. Deshpande, J.-F. Cheny, et al. 2011. Complete genome sequence of *Desulfobulbus propionicus* type strain (1pr3[T]). *Standards in Genomic Sciences* 4: 100-110.

Park, J. H., R. Kim, Z. Aslam, C. O. Jeon, and Y. R. Chung. 2008. *Lysobacter capsici* sp. nov., with antimicrobial activity, isolated from the rhizosphere of pepper, and emended description of the genus *Lysobacter*. *International Journal of Systematic and Evolutionary Microbiology* 58: 387-392.

Park, Y.-J., M. S. Park, S.-H. Lee, W. Park, K. Lee, and C. O. Jeon. 2011. *Luteimonas lutimaris* sp. nov., isolated from a tidal flat. *International Journal of Systematic and Evolutionary Microbiology* 61: 2729-2733.

Pilhofer, M., G. Rosati, W. Ludwig, K.-H. Schleifer, and G. Petroni. 2007. Coexistence of tubulins and *ftsZ* in different *Prosthecobacter* species. *Molecular Biology and Evolution* 24: 1439-1442.

Qu, J.-H., and H.-L. Yuan. 2008. *Sediminibacterium salmoneum* gen. nov., sp. nov., a member of the phylum Bacteroidetes isolated from sediment of a eutrophic reservoir. *International Journal of Systematic and Evolutionary Microbiology* 58: 2191-2194.

Rainey, F. A., J. Silva, M. F. Nobre, M. T. Silva, and M. S. da Costa. 2003. *Porphyrobacter cryptus* sp. nov., a novel slightly thermophilic, aerobic, bacteriochlorophyll a-containing species. *International Journal of Systematic and Evolutionary Microbiology* 53: 35-41.

Reichenbach, H., E. Lang, P. Schumann, and C. Spröer. 2006. *Byssovorax cruenta* gen. nov., sp. nov., nom. rev., a cellulose-degrading myxobacterium: Rediscovery of '*Myxococcus cruentus*' Thaxter 1897. *International Journal of Systematic and Evolutionary Microbiology* 56: 2357-2363.

Rendulic, S., P. Jagtap, A. Rosinus, M. Eppinger, C. Baar, C. Lanz, H. Keller, et al. 2004. A predator unmasked: Life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. *Science* 303: 689-692.

Roh, S. W., Y, D. Nam, H. W. Chang, K. H. Kim, M. S. Kim, H. M. Oh., and J. W. Bae. 2009. *Alishewanella aestuarii* sp. nov., isolated from tidal flat sediment, and emended description of the genus *Alishewanella*. *International Journal of Systematic and Evolutionary Microbiology* 59: 421-424.

Rong, X, F. B. Gurel, T. Meulia, and B. B. M. Gardener. 2012. Draft genome sequence of the biocontrol bacterium *Mitsuaria* sp. strain H24L5A. *Journal of Bacteriology* 194: 734-735.

Saha, P. and T. Chakrabarti. 2006. *Emticicia oligotrophica* gen. nov., sp. nov., a new member of the family 'Flexibacteraceae.' Phylum Bacteroidetes. *International Journal of Systematic and Evolutionary Microbiology* 56: 991-995.

Sait, M., O. K. Kamneva, D. S. Fay, N. V. Kirienko, J. Polek, M. M. Shirasu-Hiza, and N., L. Ward. 2011. Genomic and experimental evidence suggests that *Verrucomicrobium spinosum* interacts with eukaryotes. *Frontiers in Microbiology* 2: 1-11.

Sanford, R. A., J. R. Cole, and J. M. Tiedje. 2002. Characterization and description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic myxobacterium. *Applied and Environmental Microbiology* 68: 893-890.

Schneiker, S., O. Perlova, O. Kaiser, K. Gerth, A. Alici, M. O. Altmeyer, D. Bartels, et al. 2007. Complete genome sequence of the myxobacterium *Sorangium cellulosum*. *Nature Biotechnology* 25: 1281-1288.

Sheu, S.-Y., N.-T. Cho, A. B. Arun, and W.-M. Chen. 2010a. *Terrimonas aquatica* sp. nov., isolated from a freshwater spring. *International Journal of Systematic and Evolutionary Microbiology* 60: 2705-2709.

Sheu, S.-Y., C.-S. Yang, M.-H. Chen, A. B. Arun, C.-C. Young, and W.-M. Chen. 2010b. *Arcicella aurantiaca* sp. nov., isolated from stream water. *International Journal of Systematic and Evolutionary Microbiology* 60: 2979-2983.

Shiratori, H., Y. Tagami, T. Morishita, Y. Kamihara, T. Bepu, and K. Ueda. 2009. *Filimonas lacunae* gen. nov. sp. nov., a member of the phylum Bacteroidetes isolated from fresh water. *International Journal of Systematic and Evolutionary Microbiology* 59: 1137-1142.

Song, J., H.-M. Oh, J.-S. Lee, S.-B. Woo, and J.-C. Cho. 2009. *Ihnella inkyongensis* gen. nov., sp. nov., a new freshwater bacterium in the order Burkholderiales. *Journal of Microbiology and Biotechnology* 19: 5-10.

Spring. S., P. Kämpfer, and K. H. Schleifer. 2001. *Limnobacter thiooxidans* gen. nov., sp. nov., a novel thiosulfate-oxidizing bacterium isolated from freshwater lake sediment. *International Journal of Systematic and Evolutionary Microbiology* 51: 1463-1470.

Spring, S., M. Wagner, P. Schumann and P. Kämpfer. 2005. *Malikia granosa* gen. nov., sp. nov., a novel polyhydroxalkanoate- and polyphosphate-accumulating bacterium isolated from activated sludge, and reclassification of *Pseudomonas spinosa* as *Malikia spinosa* comb. nov. *International Journal of Systematic and Evolutionary Microbiology* 55: 631-629.

Stackebrandt, E., S. Verbarg, A. Frühling, H.-J. Busse and B. J. Tindall. 2009. Dissection of the genus *Methylibium*: reclassification of *Methylibium fulvum* as *Rhizobacter fulvus* comb. nov., *Methylibium aquaticum* as *Piscinibacter aquaticus* gen. nov., comb. nov. and *Methylibium subsaxonicum* as *Rivibacter subsaxonicus* gen. nov., comb. nov. and emended descriptions of the genera *Rhizobacter* and *Methylibium*. *International Journal of Systematic and Evolutionary Microbiology* 59: 2552-2560.

Svenning, MM, A. G. Hestnes, I. Wartialinen, L. Y. Stein, M. G. Klotz, M. G. Kalyuzhnaya, A. Spang, et al. 2011. Genome sequence of the arctic methanotroph *Methylobacter tundripaludum* SV96. *Journal of Bacteriology* 193: 6418-6419.

Takeda, M., A. Yoneya, Y. Miyazaki, K. Kondo, H. Makita, M. Kondoh, E. Suzuki, and J.-I. Koizumi. 2008. *Prosthecobacter fluviatilis* sp. nov., which lacks the bacterial tubulin *btubA* and *btubB* genes. *International Journal of Systematic and Evolutionary Microbiology* 58: 1561-1565.

Tarhriz, V., G. Nematzadeh, V. S. Zununi, MA. A. Hejazi, and M. S. Hejazi. 2011. *Alishewanella tabrizica* sp. nov., isolated from Qurogol Lake in mountainous region of Azarbayjan. *International Journal of Systematic and Evolutionary Microbiology* doi: 10.1099/ijs.0.031567-0

Thrash, J. C., J.-C. Cho, K. L. Vergin, R. M. Morris, and S. J. Giovannoni. 2010. Genome sequence of *Lentisphaera araneosa* HTCC2155[T], the type species of the order Lentisphaerales in the Phylum Lentisphaerae. *Journal of Bacteriology* 192: 2938-2939.

Tindall, B. J., J. Sikorski, S. Lucas, E. Goltsman, A. Copeland, T. G. Del Rio, M. Nolan, et al. 2010. Complete genome sequence of *Meiothermus ruber* type strain (21[T]). *Standards in Genomic Sciences* 3: 26-36.

Ting, L., T. J. Williams, M. J. Cowley, F. M. Lauro, M. Guilhaus, M. J. Raftery and R. Cavicchioli. 2010. Cold adaptation in the marine bacterium, *Sphingopyxis alaskensis*, assessed using quantitiative proteomics. *Environmental Microbiology* 12: 2658-2676.

Urkov, V., E. Stackebrandt, O. Buss, A. Vermeglio, V. Gorlenko, and J. T. Beatty. 1997. Reorganization of the genus *Erythromicrobium*: Description of "*Erythromicrobium sibiricum*" as *Sandaracinobacter sibiricus* gen. nov., sp. nov., and of "*Erythromicrobium ursincola*" as *Erythromonas ursincola* gen. nov., sp. nov. *International Journal of Systematic Bacteriology* 47: 1172-1178.

Van Kessel, M. A. H. J., B. E. Dutilh, K. Neveling, M. P. Kwint, J. A. Velltman, G. Flik, M. S. M. Jetten, P. H. M. Klaren, and H. J. M. Op den Camp. 2011. Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio* L.) *AMB Express* 1:41

Van Passel, M. W. J., R. Kant, A. Palva, A. Copeland, S. Lucas, A. Lapidus, T. G. del Rio, et al. 2011. Genome sequence of the Verrucomicrobium *Opitutus terrae* PB90-1, an abundant inhabitant of rice paddy soil ecosystems. *Journal of Bacteriology* 193: 2367-2368.

Vuilleumier, S., T. Nadalig, M. F. U. Haque, G. Magdelenat, A. Lajus, S. Roselli, S. E. L. Muller, et al. 2011. Complete genome sequence of the chloromethane-degrading *Hyphomicrobium* sp. strain MC1. *Journal of Bacteriology* 193: 5035-5036.

White, D. C., S. D. Sutton, and D. B. Ringelberg. 1996. The genus *Sphingomonas*: physiology and ecology. *Current Opinion in Biotechnology* 7: 301-306.

Winkelmann, N., U. Jaekel, C. Meyer, W. Serrano, R. Rachel, R. Roselló-Mora, and H. Harder. 2010. Determination of the diversity of *Rhodopirellula* isolates from European seas by multilocus sequence analysis. *Applied and Environmental Microbiol*ogy 76: 776-785.

Woyke, T., O. Chertkov, A. Lapidus, M. Nolan, S. Lucas, T. G. Del Rio, H. Tice, et al. 2011. Complete genome sequence of the gliding freshwater bacterium *Fluviicola taffensis* type strain (RW262[T]). *Standards in Genomic Sciences* 5: 1 doi: 10.4056/sigs.2124912.

Xu, X.-W., Y.-H. Wu, C.-S. Wang, X.-G. Wang, A. Oren, and M. Wu. 2009. *Croceicoccus marinus* gen. nov., sp. nov., a yellow-pigmented bacterium from deep-sea sediment, and emended description of the family Erythrobacteraceae. *International Journal of Systematic and Evolutionary Microbiology* 59: 2247-2253.

Xue, X., K. Zhang, F. Cai, J. Dai, Y. Wang, E. Rahman, F. Peng, and C. Fang. 2012. *Altererythrobacter xinjiangensis* sp. nov., isolated from desert sand, and emended description of the genus *Altererythrobacter*. *International Journal of Systematic and Evolutionary Microbiology* 62: 28-32.

Yang, C.-S., S.-Y. Sheu, C.-C. Young, A. B. Arun, C.-Y. Cheng, and W.-M. Chen. 2010. *Chitinibacter alvei* sp. nov., isolated from stream water. *International Journal of Systematic and Evolutionary Microbiology* 60: 1760-1764.

Yoon, J.-H., M.-H. Lee, and T.-K. Oh. 2004. *Porphyrobacter donghaensis* sp. nov., isolated from sea water of the East Sea in Korea. *International Journal of Systematic and Evolutionary Microbiology* 54: 2231-2235.

Yoon, J.-H., S.-J. Kang, S. Park, and T.-K. Oh. 2007a. *Devosia insulae* sp. nov., isolated from soil, and emended description of the genus *Devosia*. *International Journal of Systematic and Evolutionary Microbiology* 57: 1310-1314.

Yoon, J.-H., S.-J. Kang, J.-S. Lee, H. W Oh, and T.-K. Oh. 2007. *Brevundimonas lenta* sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology* 57: 2236-2240.

Yoon, J., Y. Matsuo, K. Adachi, M. Nozawa, S. Matsuda, H. Kasai, and A. Yokota. 2008. Description of *Persicirhabdus sediminis* gen. nov., sp. nov., *Roseibacillus ishigakijimensis* gen. nov., sp. nov., *Roseibacillus ponti* sp. nov., *Roseibacillus persicicus* sp. nov., *Luteolibacter pohnpeiensis* gen. nov., sp. nov. and *Luteolibacter algae* sp. nov., six marine members of the phylum 'Verrucomicrobia', and emended descriptions of the class Verrucomicrobiae, the order Verrucomicrobiales and the family Verrucomicrobiaceae. *International Journal of Systematic and Evolutionary Microbiology* 58: 998-1007.

Yoon, M.-H., and W.-T. Im. 2007. *Flavisolibacter ginsengiterrae* gen. nov., sp. nov. and *Flavisolibacter ginsengisoli* sp. nov., isolated from ginseng cultivating soil. 2007. *International Journal of Systematic and Evolutionary Microbiology* 57: 1834-1839.

Zablotowicz, R. M., M. A. Locke, R. E. Hoagland, S. S. Knight, and B. Cash. 2001. Flurorescent *Pseudomonas* isolates from Mississippi Delta oxbow lakes: in vitro herbicide biotransformations. *Environmental Toxico*logy 16: 9-19.

Zhang, X.-Q., W.-J. Xhang, B.-P. Wei, X.-W. Xu, X.-F. Zhu, and M. Wu. 2010. *Meiothermus cateniformans* sp. nov., a slightly thermophilic species from north-eastern China. *International Journal of Systematic and Evolutionary Microbiology* 60: 840-844.

Zul, D., G. Wanner, and J. Overmann. 2008. *Massilia brevitalea* sp. nov., a novel betaproteobacterium isolated from lysimeter soil. *International Journal of Systematic and Evolutionary Microbiology* 58: 1245-1251.

Appendix 3. Additional work

In addition to the work described in this dissertation, I performed research that will be

incorporated into the following three pending publications.

1. Houck E, Hanshew A, and Currie CR. In preparation. The microbial communities of
   hemimetabolists versus holometabolists.

   The undergraduate, Erik Houck, who works with me has been exploring how bacterial

communities change over the life cycle of insects, comparing the shifts in hemimetabolists to

those in holometabolists. I have assisted him in: design of experiments, collection of samples,

design and setup of 454 pyrosequencing, data analysis, and contributed to writing the

manuscript.

2. Grubbs K, Pinto-Tomas A, Dugenske R, Hanshew A, Scott J, Kontnik R, Currie C, and
   Clardy J. In preparation. Apinimycin paper.

   During my rotation in the Currie lab I was tasked with collecting flowers visited by

honeybees and culturing Actinobacteria from them. This is part of a larger project of Kirk

Grubbs looking at the role of Actinobacteria in honeybee hives.

3. Jewell KA, Strassman E, Hanshew A, McMahon K, Goodrich-Blair H, Currie C, and Suen
   G. In preparation. Initial DNA polymerase selection alters 454 pyrosequencing of complex
   microbial communities.

   This is a collaboration between the Suen lab and I assessing the impact of polymerase choice

on the formation of chimeras during pyrosequencing. I have contributed in the following ways:

assisted in design of experiments, collection of insect samples, design and setup of 454

pyrosequencing, data analysis, and contributed to writing the manuscript.