Applied and Environmental Microbiology, June 2007, p. 3470–3479 Vol. 73, No. 11
0099-2240/07/$08.00+0 doi:10.1128/AEM.02120-06
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Geographical Distribution and Diversity of Bacteria Associated with Natural Populations of *Drosophila melanogaster*▽†

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Received 7 September 2006/Accepted 24 March 2007

***Drosophila melanogaster* is one of the most widely used model systems in biology. However, little is known about its associated bacterial community. As a first step towards understanding these communities, we compared bacterial 16S rRNA gene sequence libraries recovered from 11 natural populations of adult *D. melanogaster*. Bacteria from these sequence libraries were grouped into 74 distinct taxa, spanning the phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, which were unevenly spread across host populations. Summed across populations, the distribution of abundance of genera was closely fit by a power law. We observed differences among host population locations both in bacterial community richness and in composition. Despite this significant spatial variation, no relationship was observed between species richness and a variety of abiotic factors, such as temperature and latitude. Overall, bacterial communities associated with adult *D. melanogaster* hosts are diverse and differ across host populations.**

Insects harbor diverse microbial communities (11, 29, 34, 65), and interactions between hosts and their microbes can range from mutualistic, such as the interaction between termites and their gut microbes (8, 65), to parasitic, such as the interaction of the bacterium *Paenibacillus larvae* (American foulbrood) in honeybees (62). Some of these interactions are relatively well characterized, owing to their economic importance or because of their remarkable biology. However, the exact nature of many other potentially interesting and experimentally tractable insect-microbe interactions, specifically those between microbes and the major insect model systems, remains poorly understood.

In addition to the immediate association between insect hosts and the bacterial communities they harbor, the bacteria that insects carry can also associate with and affect the fitness of other hosts through vector transmission. The most common vector-borne zoonotic inflammatory disease in the United States, Lyme disease (caused by *Borrelia burgdorferi*), is transmitted by the deer tick, *Ixodes scapularis*, and infected more than 23,000 people in 2002 (26). In addition, *Erwinia carotova*, responsible for soft rot in many species of plants and for significant economic losses, can be vector transmitted by a variety of insects, including *Drosophila melanogaster* (38). Clearly, vector-borne bacterial infections can have large economic and health impacts and are important determinants of fitness for a variety of potential hosts.

It is estimated that approximately 99% of the bacteria in nature are unculturable (3). With the advent of molecular techniques, such as PCR and genome sequencing, and metagenomic approaches, researchers have uncovered an astonishing level of microbial diversity in natural habitats, ranging from soil (7, 54, 69) and marine environments (23, 64, 69, 70) to the human gut (25). The same techniques are currently being applied to understanding the microbiota of a range of insects (11, 29, 34, 47, 53, 55, 56, 65). For example, using such sequence-based approaches, Dunn and Stabb (20) found that the ant lion, *Myrmeleon mobilis*, harbors a relatively simple microbial community, represented mostly by *Enterobacteriaceae*- and *Wolbachia*-like microorganisms. In contrast, Campbell et al. (11) found a more diverse microbiota in the biting midge, *Culicoides sonorensis*, comprised of genera from five different bacterial divisions. From the literature on the microbial communities of insects, two salient points emerge. First, these communities differ greatly among host species. Second, researchers are only beginning to understand the taxa comprising these microbial communities and how these microbes interact with their hosts. Considering the estimated 30 million insect species worldwide (21), the potential for uncovering new species of bacteria and for understanding interesting features of these insect-microbe interactions is staggering.

Despite their widespread use in the laboratory, relatively little is known about the interaction between model laboratory insect species and their associated microbial communities. Due to its experimental and genetic tractability, the fruit fly, *Drosophila melanogaster*, provides an ideal system for studying these interactions more closely. Recently, there have been numerous studies on *Drosophila* immunity, ranging from understanding the molecular basis of resistance (28, 32, 41) to the evolutionary ecology of the immune response (57, 61, 63). Some of these studies use bacteria isolated from natural populations of *Drosophila* (42, 71). However, in many other cases, researchers use bacteria that are not yet known to naturally cooccur with or infect *Drosophila*. One plausible reason that naturally occurring bacteria are not used to study immunity is that, to date, there are few comprehensive studies of the bacteria that associate with natural *D. melanogaster* populations (but see reference 19). Knowledge of the actual interactions

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 30 March 2007.

taking place between insect hosts and their microbial communities is critical to those studying insect immunity. However, before characterizing these interactions, we must characterize the bacterial communities and identify taxa that are of potential ecological interest.

Specific species of microbes, as well as traits of the microbial population overall, such as community richness or composition, have the potential to greatly affect the ecology and evolution of their *Drosophila* hosts. Here we characterize this composition and richness across 11 natural populations of *D. melanogaster*. We first identify the bacterial microbes present in host populations collected across a latitudinal cline by using a sequence-based approach. We then compare microbial community richness among these host populations and ask whether richness is associated with latitude or climate. This is the first study to characterize microbial communities associating with *D. melanogaster* hosts within and among natural host populations. These data will provide an important first step in understanding host-microbe interactions in this widely studied model system.

MATERIALS AND METHODS

Fly collections. Flies were collected from 11 sites along a latitudinal transect on the east coast of the United States between June and September 2005 (Table 1), using a combination of sweep netting behind fruit stands and sweep netting over fruit bucket traps. Details of the 11 collection sites are presented in Table 1.

Following collection, flies were anesthetized over ice. Male *D. melanogaster* flies were separated from other drosophilid species on the basis of morphological characteristics, such as size, color, body patterning, wing shape, and genital morphology (4, 45). These individuals were set aside and preserved in groups of five in 70% ethanol. After our return to the laboratory from the collection sites, the ethanol-preserved samples were kept at −80°C. In preparation for DNA isolation, the ethanol-preserved flies were shaken before being removed from the ethanol. While this method will not remove all surface-associated bacteria, those that are loosely associated will likely wash off.

DNA isolation. Total DNAs from flies and bacteria were isolated from one group of five male flies at each of the 11 different locations. Flies were removed from the ethanol and homogenized using a pestle in 200 μl of STE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, and 150 mM NaCl), with lysozyme added (final concentration, 4 mg/ml). The sample was then incubated for 30 min at 37°C. Following incubation, 20 μl of 10% sodium dodecyl sulfate and proteinase K (final concentration, 0.2 mg/ml) were added. These samples were then vortexed and incubated at 55°C overnight. After the overnight incubation, RNase A was added (final concentration, 0.1 mg/ml), and samples were incubated for 1 hour at 37°C. The samples were then extracted with equal volumes of phenol-chloroform-isoamyl alcohol (24:24:1) and chloroform-isoamyl alcohol (24:1), and the DNAs were ethanol precipitated. The DNA pellets were resuspended in 50 μl of Tris-EDTA (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]).

PCR amplification. Bacterial 16S rRNA gene sequences were selectively PCR amplified from the isolated DNA samples for the construction of clone libraries. Each reaction tube contained 50 to 100 μg/ml of template genomic DNA, forward primer 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′), reverse primer 1522r (5′-AAG GAG GTG ATC CAG CCG CA-3′), and one Ready-to-Go PCR bead (GE Healthcare Life Science). The PCR program was as follows: 9 min at 95°C; 15 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C; and a final extension step of 60°C for 10 min. PCR products were resolved on a 1% agarose gel, and the gel was stained with SYBR green (Invitrogen) in order to visualize the relatively weak bands on a dark reader transilluminator. The approximately 1.5-kb 16S rRNA gene fragment was extracted from the agarose gel by using a QiaQuick gel extraction kit (QIAGEN) according to the manufacturer's directions and was eluted in 30 μl of sterile distilled water.

Construction of clone libraries. Clone libraries of bacterial PCR products were constructed using a TOPO TA cloning kit (Invitrogen) with TOPO One Shot electrocompetent cells. Successful transformants were plated onto Luria-Bertani plates containing kanamycin (final concentration, 50 μg/ml). Plasmids were extracted from the bacteria by using standard techniques (59).

Sequencing of 16S rRNA genes. Partial-length 16S rRNA gene fragments were sequenced in one direction, using an ABI 3700 capillary sequencer with T7

TABLE 1. Characteristics of 11 collection sites and 16S rRNA gene sequence clone libraries

Site[a]	Population name[b]	Location	Latitude	Date collected	Ambient temp (°C)	Source[c]	Site description[d]	n[e]	No. of OTUs[f]	Chao1 estimate[g]	ACE estimate[h]	Coverage[i]
A	Oakland	Oakland, NJ	41.01	28 August 2005	29.4	Cantaloupe traps	Behind private residence	76	16	15.3 ± 0.78	15.7 ± 0.78	100
B	Inwood	Inwood, WV	39.22	13 July 2005	29.5	Dumpster	Local fruit market	74	30	56.2 ± 3.2	61.3 ± 3.2	53.4
C	Woodstock	Woodstock, VA	38.87	2 September 2005	27	Peaches, tomatoes, cantaloupe	Roadside fruit stand	51	12	15.7 ± 1.8	21.3 ± 1.8	76.4
D	Ikenberry	Daleville, VA	37.41	2 September 2005	28	Banana traps	Local fruit market	78	15	18.0 ± 3.2	18.9 ± 3.2	83.3
E	Layman	Daleville, VA	37.41	2 September 2005	28	Apples, peaches, tomatoes	Local fruit market	80	12	17.0 ± 1.6	42.9 ± 1.6	70.6
F	Hillsborough	Hillsborough, NC	36.07	19 July 2005	32.2	Cantaloupe, peaches, tomatoes	Roadside fruit stand	58	13	49.0 ± 9.7	42.9 ± 9.7	26.5
G	Raleigh	Raleigh, NC	35.82	20 July 2005	36	Peaches, cantaloupe	Open-air farmer's market	86	9	14.0 ± 5.8	14.2 ± 5.8	64.3
H	Thomas	Watkinsville, GA	33.86	11 August 2005	32.2	Peaches	Roadside fruit stand	76	11	21.0 ± 6.3	17.0 ± 6.3	52.4
I	Horticulture farm	Watkinsville, GA	33.87	6 September 2005	26.7	Cantaloupe traps	Wooded area near fruit orchard	31	7	12.0 ± 5.4	20.8 ± 5.4	58.3
J	GA 441	Bishop, GA	33.81	11 August 2005	32.2	Peaches, tomatoes	Roadside fruit stand	46	8	9.5 ± 0.81	11.1 ± 0.81	84.2
K	Macon	Macon, GA	32.83	10 August 2005	32.2	Dumpster	Open-air farmer's market	69	19	35.5 ± 3.0	47.8 ± 3.0	53.5

[a] Five male flies were sampled from each of the 11 collection sites.
[b] As used in the text.
[c] Description of the medium that *Drosophila* hosts were congregating over.
[d] Roadside fruit stands are small, open-air businesses; local fruit markets are enclosed businesses selling fruit from local farms; and open-air farmer's markets host large collections of local farmers.
[e] Number of nonchimeric 16S rRNA gene sequences isolated from the host population.
[f] Number of OTUs identified in the 16S rRNA gene clone library. OTUs were defined based on 3% sequence divergence.
[g] Chao1 estimate of community richness (13, 15), using 3% sequence divergence, ± standard error around the estimate. Calculations for standard errors were done as described previously (13, 15).
[h] ACE estimate of community richness (14, 15), using 3% sequence divergence, ± standard error around the estimate (14, 15).
[i] The proportion of OTUs observed out of the estimated total number of OTUs in the population. As an estimate of the total number of OTUs in the population, the Chao1 estimate of community richness was used.

primers and ABI Big Dye Terminator chemistry. Sequences were examined visually, and vector and low-quality bases (20-bp window with an average PHRED score for quality of <16) were trimmed from the libraries by using LUCY (16). After trimming of these regions, the average sequence length was 827 base pairs.

Chimera detection. All clones were checked for chimeras, using both Chimera Check from the Ribosomal Database Project II (RDP II) (18) and Bellerophon (31). All sequences resembling chimeras by either program were removed from further analyses.

Determination of OTUs. Sequences were aligned separately for each clone library by using ClustalW (68) in BioEdit, version 7.0.5, with the default settings, with a gap-opening penalty of 10.0 and gap extension penalties of 0.1 and 0.2, for pairwise and multiple alignments, respectively. As points of reference, published sequences for *Escherichia coli* 16S rRNA (GenBank accession number L10328; base position numbers 131193 to 132733), *Borrelia burgdorferi* (accession number X85189), and *Bacillus subtilis* (accession number AY553095) were used in aligning the sequences. After the alignments were performed, the sequences were truncated at the 5′ and 3′ ends, and ambiguous areas of the alignment were removed manually. The number of common bases for each was as follows: GA 441, 617 bp; Hillsborough, 440 bp; Horticulture Farm, 578 bp; Ikenberry, 371 bp; Inwood, 318 bp; Layman, 550 bp; Macon, 502 bp; Oakland, 413 bp; Raleigh, 661 bp; Thomas, 546 bp; and Woodstock, 248 bp. Distance matrices were constructed using the DNADIST program within BioEdit, version 7.0.5, using the Jukes-Cantor correction for multiple substitutions. Using the DOTUR software package (60) with the default settings, operational taxonomic unit (OTU) groupings were determined at 97% sequence identity. In further phylogenetic analyses, a consensus sequence was generated to represent each OTU.

Phylogenetic analyses of 16S rRNA gene sequences. Using the BLAST (2) and Seqmatch (18) tools, the RDP II (18) and GenBank sequence databases were screened for published sequences that closely matched the 74 consensus sequences generated to represent each of the OTUs isolated from the host populations. All analyses were performed between June 2006 and January 2007. These published sequences (see Fig. 2 and 3), along with the 74 OTU consensus sequences, were then used in a series of phylogenetic analyses designed to understand the relationship that the 16S rRNA gene sequences we recovered from *D. melanogaster* shared both with published 16S rRNA sequences and with each other. To begin, sequences were aligned using ClustalW (68) in BioEdit, version 7.0.5, with the default settings, with a gap-opening penalty of 10.0 and gap extension penalties of 0.1 and 0.2, for pairwise and multiple alignments, respectively. After the alignments were performed, ambiguous areas of alignment were removed using Gblocks (12), yielding 649 common bases. A distance matrix and a neighbor-joining tree were constructed under default parameters, using 10,000 bootstrap replicates and the PHYLIP v. 3.6 software package (J. Felsenstein, University of Washington, Seattle, 2005). After determining how sequences grouped with each other, they were divided into the following seven subcategories for further analysis: *Wolbachia*, *Bacteroidetes*, *Firmicutes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Epsilonproteobacteria*. For each of the seven individual categories, the published sequences and OTUs corresponding to that category were compiled, and distance matrices were again constructed using the methods described above. Neighbor-joining trees were generated in MEGA 3.1 (39) for each subcategory, using the Kimura two-parameter model for nucleotide substitutions and 10,000 bootstrap replicates. Neighbor-joining trees for the *Wolbachia* subcategory were unrooted, and trees for the remaining subcategories were rooted with *Synechococcus elongata* (AF132930).

Estimates of species richness. Using DOTUR (60) and a 97% level of sequence identity to define the OTUs, rarefaction curves were generated to ask if our libraries approached a level of taxonomic diversity that represented the true diversity present in these 11 locations, and the nonparametric species richness estimators Chao1 (13, 15) and ACE (14, 15) were obtained. Library coverage was estimated by calculating the ratio of the actual number of OTUs observed to the Chao1 estimate of species richness.

Statistical test for clinal variation in richness. To determine if there was significant clinal variation in microbial species richness, we tested for significant correlations between latitude and the Chao1 estimate of species richness and between latitude and the ACE estimate at 97% sequence identity. All data were analyzed using the Spearman nonparametric correlation test in JMP, version 5.0.1a (SAS, Cary, NC).

Statistical test for correlation between richness and climatic factors. Because latitude is not the only factor that may determine differences in microbial community richness, we also tested for a significant correlation between the Chao1 and ACE estimates of richness and several other climatic factors, namely, mean annual temperature, monthly temperature range (defined as the 12-month average of the differences between the monthly mean maximum and minimum temperatures), mean annual precipitation, and mean January low temperature. These climatic data were published by the National Oceanic and Atmospheric Administration and represent 30-year averages recorded between 1971 and 2000 at various locations across the United States (48–52). In cases where the exact collection site location was not listed in the report, the listed location that was closest to the collection site was used. All data were analyzed using the Spearman nonparametric correlation test in JMP, version 5.0.1a (SAS, Cary, NC).

Species abundance distribution. Recent studies found that microbial species abundance distributions are well described by the power law distribution $f(S_N) = \zeta(g)N^g$, where S_N is the number of genera that are found in N sequence samples, g is the power coefficient, and ζ is Reimann's zeta function, a normalizing constant. When data are plotted on a log-log scale, if the distribution of microbial genera fits a power law, then $\log(S_N)$ versus $\log(N)$ should be a straight line with a slope of g.

The data were fit to a power law distribution by using maximum likelihood (Microsoft Excel program [available upon request]). A recent study of species abundance distributions in soil microbes (24) compared power law distributions with other related distributions. Given the relatively small size of our data set, we do not have sufficient statistical power to make these comparisons. Our interest in fitting a power law distribution to these data is primarily a heuristic one.

Nucleotide sequence accession numbers. All unique 16S rRNA gene sequences are available in the GenBank database under accession numbers DQ980639 through DQ981381.

RESULTS

A total of 11 libraries were constructed to represent the bacterial communities present in hosts at 11 locations. From these 11 libraries, we obtained 992 sequences. Of these 992 sequences, 264 (26.6%) were discarded as chimeric.

Using DOTUR, the 728 remaining sequences from the 11 locations were grouped into 74 OTUs at the 3% level of sequence divergence (see the supplemental material). No OTUs were present in all libraries, and there were no populations containing all 74 OTUs. The OTUs were spread unevenly both within and across host populations (see Fig. 1 through 4; the supplemental material), with many appearing in only 1 host population among the 11 sampled. The mean number of OTUs per host population was 13.8 (Table 1).

A wide range of bacterial species were present in the gene clone libraries recovered from the 11 locations. One prominent feature of these libraries was the high prevalence of *Wolbachia* sequences (453 of the 728 total sequences [62.2%]). The two most abundant groups of bacteria fell into the *Alphaproteobacteria* (125 of the 728 total sequences [17.2%]) and *Gammaproteobacteria* (59 of the 728 total sequences [8.1%]) classes. In addition, these libraries contained a significant proportion (39 of 728 [5.4%]) of sequences with matches in the RDP II and GenBank databases that were taxonomically unclassified environmental samples. Many of the sequences showed relatively low similarity to published 16S rRNA gene sequences in the RDP II database. Across all 11 libraries, 31 of the 728 sequences (4.3%) shared <97% sequence identity with published 16S rRNA gene sequences in the RDP database. The lowest of these, assigned to OTU 46, shared only 59.4% identity with its next closest relative in the database (see the supplemental material).

Based on sequence separation at the 3% level of sequence divergence, our samples contained 10 OTUs that grouped with *Wolbachia* sequences (Fig. 1). The neighbor-joining tree had a wide range of bootstrap support values (10% to 100%) but qualitatively captured aspects of previous studies (44, 46) in the manner that taxa grouped into supergroups. All 10 *Wolba-*

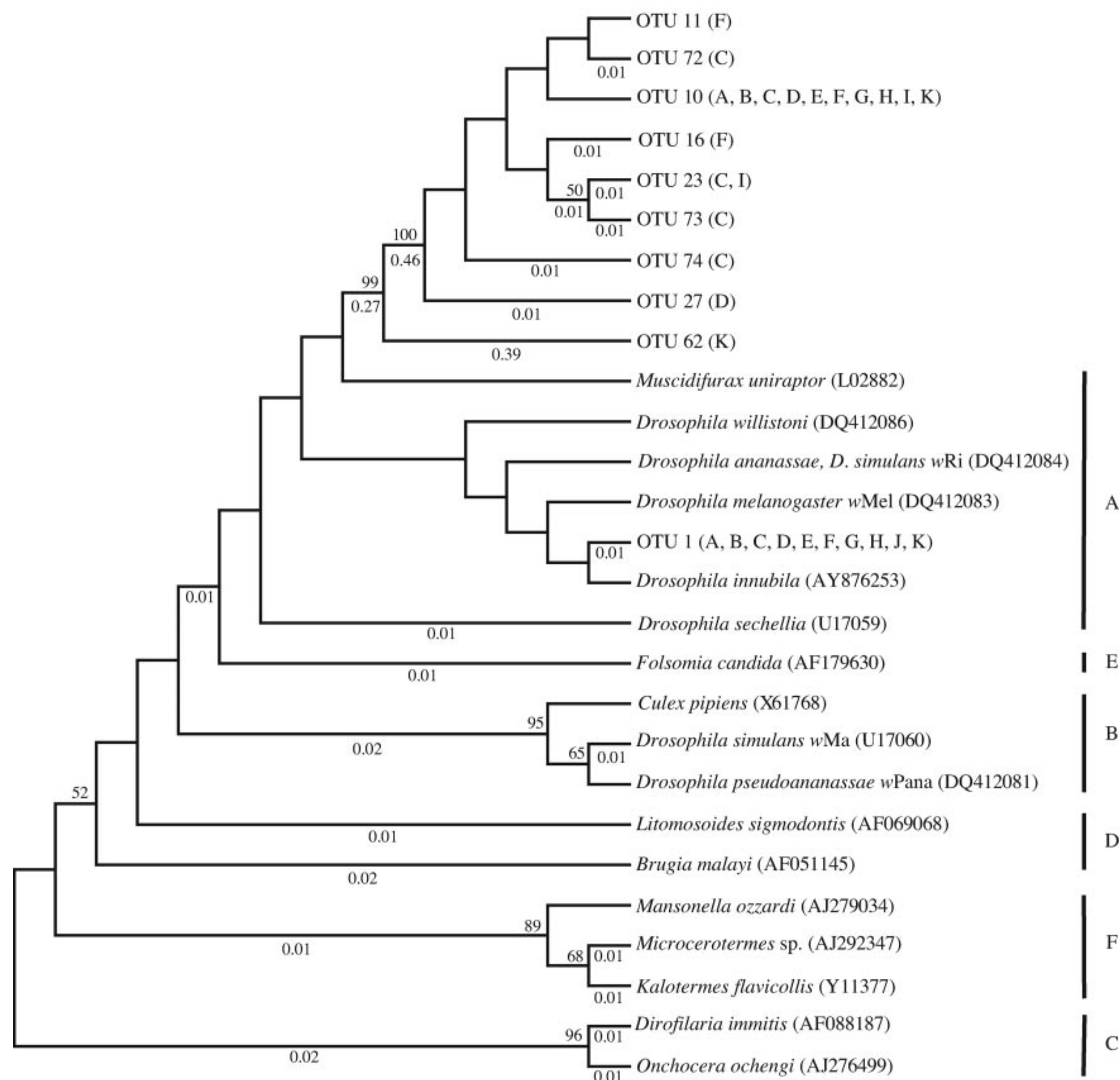

FIG. 1. Unrooted phylogenetic tree of *Wolbachia* sequences based on 16S rRNA gene sequences showing the positions of members of the six *Wolbachia* supergroups (A to F), as described previously (44), and the 10 *Wolbachia* OTUs isolated from the 11 *D. melanogaster* host populations. Letters in parentheses to the right of the OTUs represent the populations where the OTUs were observed. Populations corresponding to each letter are presented in Table 1. The neighbor-joining tree is the result of 10,000 bootstrap replicates, using 1,313 nucleotides. Bootstrap values of ≥50% are posted above the branches at the nodes. Branch lengths with more than zero nucleotide substitutions per site are indicated below the corresponding branches. Scientific names correspond to the invertebrate host species where the *Wolbachia* sequences are found and are followed by GenBank accession numbers. Letters to the right of the phylogeny represent *Wolbachia* supergroup designations (44).

chia OTUs formed a monophyletic group with supergroup A (Fig. 1). Two of the *Wolbachia* OTUs, OTUs 1 and 10, were widespread, while the remaining OTUs were limited to specific populations (Fig. 1).

The remaining 275 sequences were grouped into 64 OTUs (see Fig. 2 through 4; the supplemental material). Forty-two OTUs grouped with the *Proteobacteria* (15 *Alphaproteobacteria*, 5 *Betaproteobacteria*, 21 *Gammaproteobacteria*, and 1 *Epsilonproteobacteria* OTU) (Fig. 2). Seventeen of these OTUs grouped within the *Firmicutes* (Fig. 3). The remaining five OTUs grouped within the *Bacteroidetes* (Fig. 4). Bootstrap values for the neighbor-joining trees varied widely, with many OTUs assigned to clades defined by a published sequence with high bootstrap support (Fig. 2, 3, and 4).

Our analyses suggest that we have not sampled all of the taxa present in these *Drosophila* hosts, because the rarefaction curves do not plateau as more sequences are sampled from the library (Fig. 5). Using the Chao1 estimator of richness for comparison, the average coverage was 65.7% ± 6.06% (mean ± standard error) across all 11 libraries (Table 1). The Oakland population had the highest coverage (100%) (Table 1), whereas the Hillsborough population had the lowest coverage (26.5%) (Table 1).

Species richness varied across host locations, as measured by both the Chao1 and ACE estimators (Table 1). The two estimators of richness were positively correlated with each other (Spearman's rho value = 0.76; P = 0.006). Despite spatial variation in richness, using either of the two estimators, there

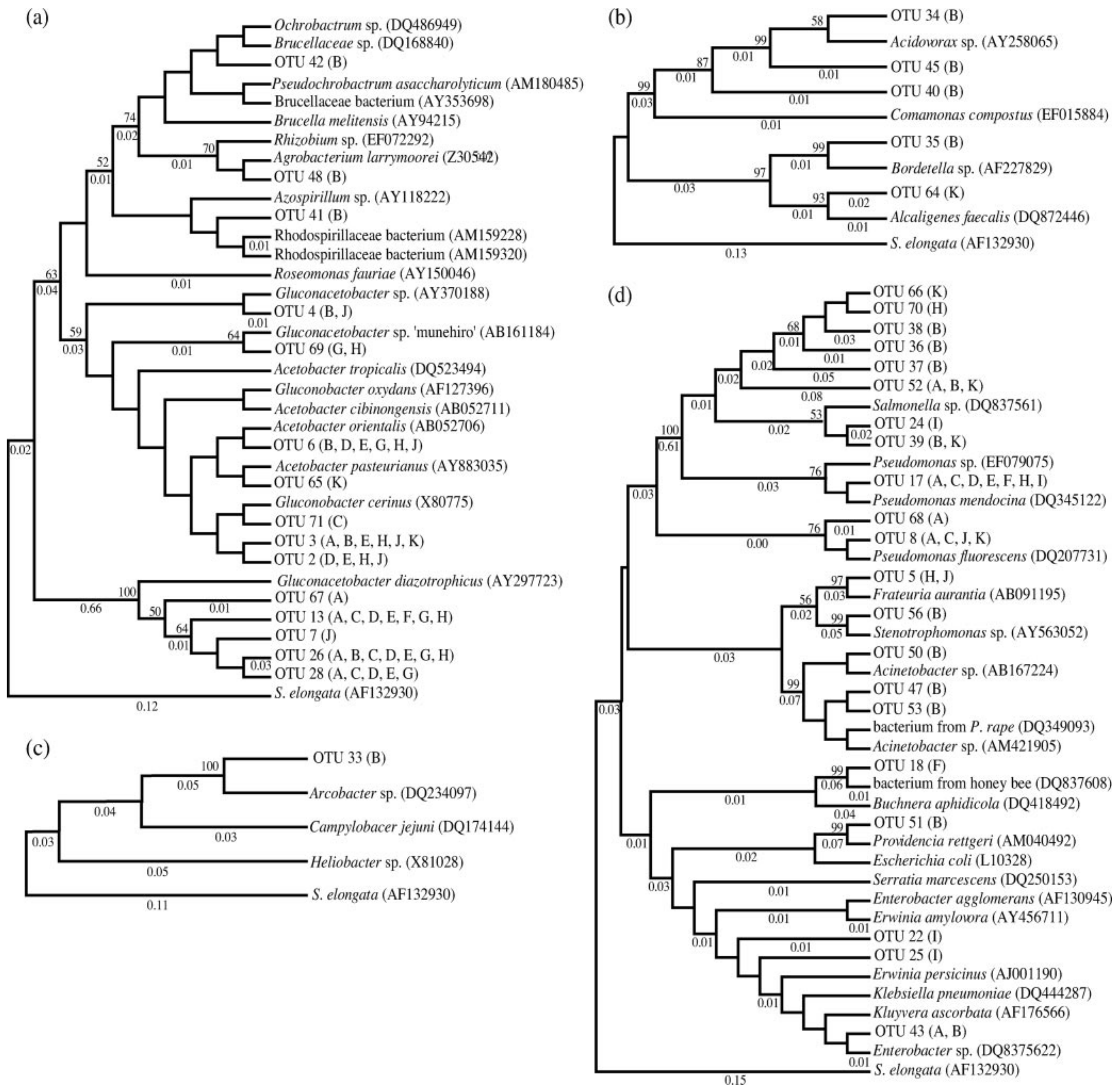

FIG. 2. Phylogenetic trees representing the taxonomic positions of proteobacterial OTUs isolated from 11 *D. melanogaster* host populations. (a) *Alphaproteobacteria*; (b) *Betaproteobacteria*; (c) *Epsilonproteobacteria*; (d) *Gammaproteobacteria*. Phylogenies were inferred using the neighbor-joining method and were bootstrapped for 10,000 replicates. The number of bases used for analysis was (a) 670, (b) 427, (c) 253, and (d) 690. Numbers above branch points represent bootstrap values of ≥50%. Numbers below branches indicate branch lengths (nucleotide substitutions per site) of greater than zero. Trees are rooted with the 16S rRNA gene sequence for *Synechococcus elongata* (AF132930), a member of the phylum *Cyanobacteria*. Letters in parentheses to the right of each OTU indicate the *D. melanogaster* host populations where that OTU was observed. A key for these letters is presented in Table 1.

was no clear relationship between microbial species richness and latitude for the Chao1 (Spearman's rho value = 0.06; P = 0.87) or ACE (Spearman's rho value = −0.05; P = 0.89) estimate of species richness.

Species richness across host populations was not correlated with climate, using either of the two richness estimators. There was no correlation between the Chao1 estimate of richness and mean annual temperature (Spearman's rho value = −0.05; P = 0.89), monthly temperature range (Spearman's rho value = 0; P = 1.0), mean annual precipitation (Spearman's rho value = −0.21; P = 0.56), or mean January low temperature (Spearman's rho value = 0; P = 1.0). There was also no significant correlation between the ACE estimate of species richness and mean annual temperature (Spearman's rho value = 0.13; P =

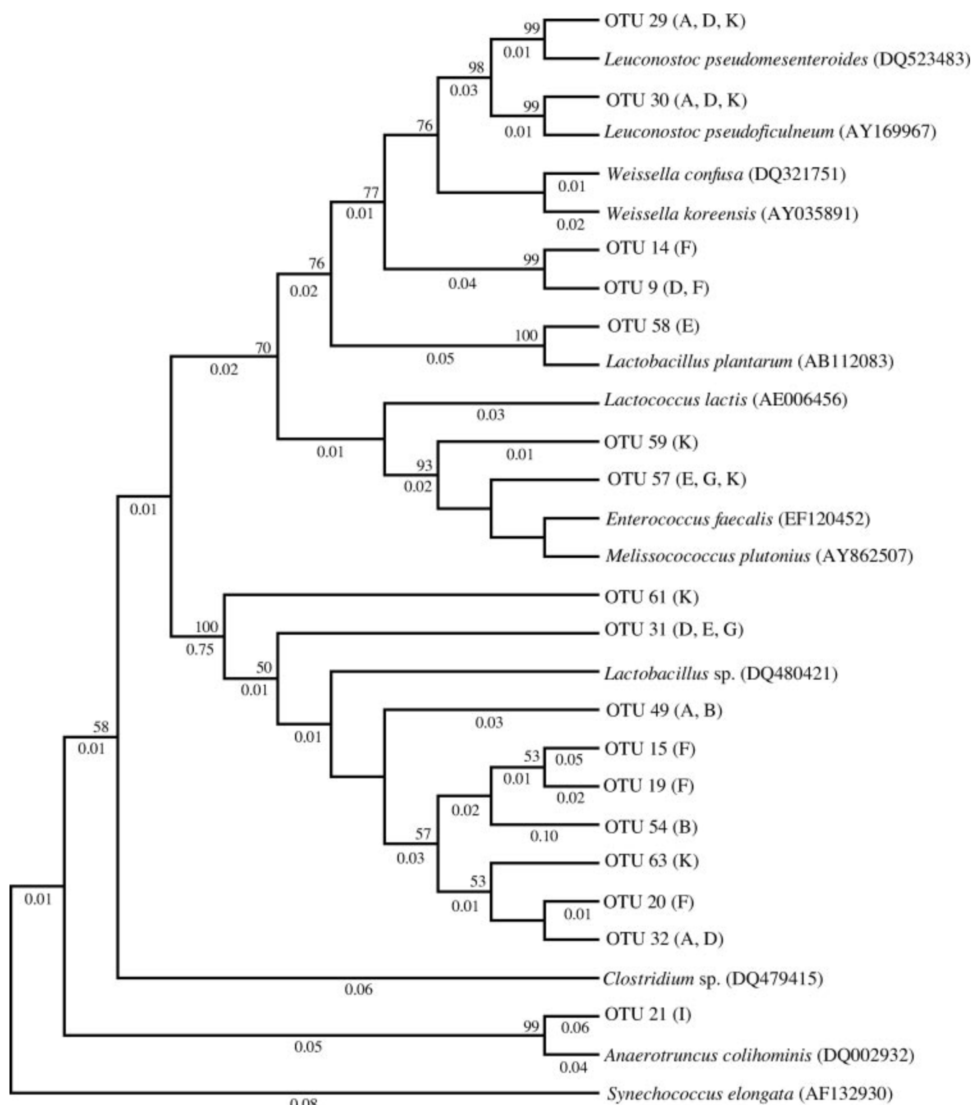

FIG. 3. Phylogenetic trees representing the taxonomic positions of *Firmicutes* OTUs isolated from 11 *D. melanogaster* host populations. Phylogenies were inferred using the neighbor-joining method, using 371 bases, and were bootstrapped for 10,000 replicates. Numbers above branch points represent bootstrap values of ≥50%. Numbers below branches indicate branch lengths (nucleotide substitutions per site) of greater than zero. Trees are rooted with the 16S rRNA gene sequence for *Synechococcus elongata* (AF132930), a member of the phylum *Cyanobacteria*. Letters in parentheses to the right of each OTU indicate the *D. melanogaster* host populations where that OTU was observed. A key for these letters is presented in Table 1.

0.71), monthly temperature range (Spearman's rho value = 0.02; $P = 0.95$), mean annual precipitation (Spearman's rho value = −0.20; $P = 0.59$), or mean January low temperature (Spearman's rho value = 0.11; $P = 0.76$).

The frequency distribution of species abundance appeared to approximately fit a power law distribution, with a g value of −1.46. This value is not far from the range of values for g reported by Gans et al. ($g = -1.96$ to -2.11) (24), although we used genera, as opposed to their use of species.

DISCUSSION

We used a sequence-based approach to study the microbial communities within natural host populations of *Drosophila melanogaster*. Our data suggest that there are many species of bacteria present in these *Drosophila* hosts, including a large number of *Wolbachia* species. Most of these species of bacteria were unevenly distributed among the host populations. The bacterial species richness of these microbial communities differed among host populations. However, despite significant spatial variation in microbial community richness, there was no clear relationship between latitude or climate and microbial species richness.

Our interpretation of these data comes with three caveats. First, our method of DNA extraction does not allow us to discern between bacteria associated with the inside or outside of the host. Second, because microbial DNAs were isolated from whole bodies of flies, we cannot draw conclusions about the tissue specificity of the microbes observed in these libraries. Last, we observed a large proportion of chimeric sequences in these libraries. This pattern could be due to our thorough

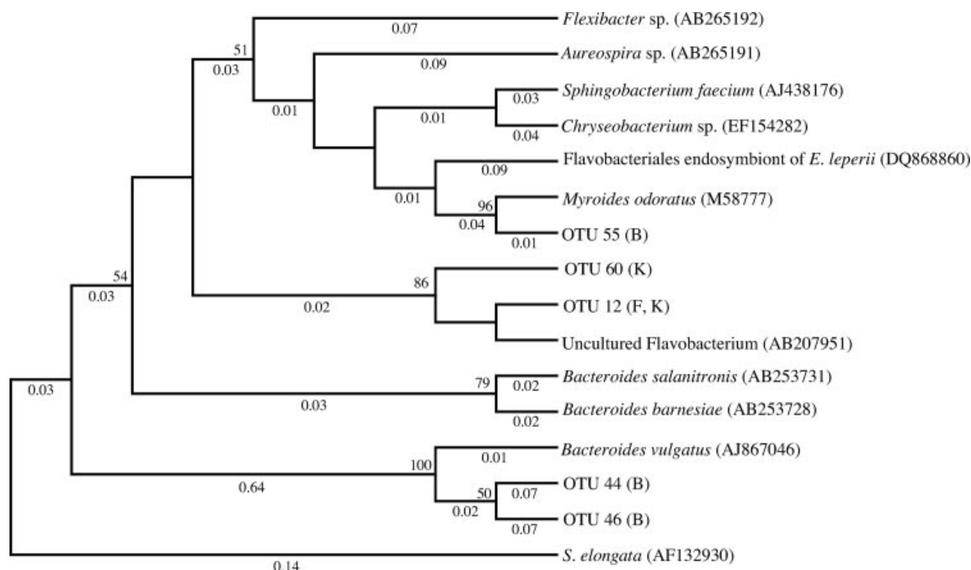

FIG. 4. Phylogenetic trees representing the taxonomic positions of *Bacteroidetes* OTUs isolated from 11 *D. melanogaster* host populations. Phylogenies were inferred using the neighbor-joining method, using 808 bases, and were bootstrapped for 10,000 replicates. Numbers above branch points represent bootstrap values of ≥50%. Numbers below branches indicate branch lengths (nucleotide substitutions per site) of greater than zero. Trees are rooted with the 16S rRNA gene sequence for *Synechococcus elongata* (AF132930), a member of the phylum *Cyanobacteria*. Letters in parentheses to the right of each OTU indicate the *D. melanogaster* host populations where that OTU was observed. A key for these letters is presented in Table 1.

methods of identifying chimeras or to inappropriate concentrations of $MgCl_2$ or deoxynucleoside triphosphates in the PCR beads used to amplify the DNAs. With these three caveats in mind, we highlight several interesting characteristics of these libraries.

Bacterial phyla present. Three phyla—*Proteobacteria*, *Firmicutes*, and *Bacteroidetes*—were present in these samples. OTUs falling within the *Proteobacteria* phylum were represented considerably more than OTUs grouping within the *Firmicutes* or *Bacteroidetes*, even after subtracting the highly prevalent gram-

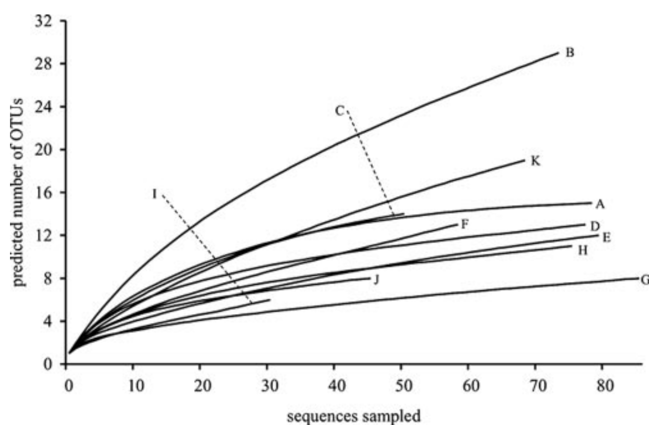

FIG. 5. Rarefaction analysis of bacterial 16S rRNA gene clone libraries recovered from 11 *D. melanogaster* host populations. Populations are labeled A through K. A key to the population labels is presented in Table 1. The predicted number of OTUs was calculated from the number of clones analyzed at the 3% level of sequence divergence. The slope of each curve indicates whether the diverse populations were completely sampled (zero or low slope) or whether new taxa are predicted if additional clones are analyzed (steep slope).

negative bacterium *Wolbachia*. The overabundance of proteobacteria we observed could be due simply to the limitations of using lysozyme, which can be ineffective against gram-positive anaerobic cocci (22), instead of bead beating to lyse the bacterial cells. Alternatively, the overabundance of proteobacteria in these samples could be due to the ecology of the host. Our findings are consistent with work using the deer tick, *Ixodes scapularis* (6). Benson et al. (6) noticed a high prevalence of proteobacteria even after subtracting intracellular bacteria such as *Wolbachia*. They hypothesized that this pattern could be due to the humid environments that ticks prefer, which are more permissive conditions for desiccation-sensitive microbes such as proteobacteria. The prevalence of proteobacteria has also been shown for several other species of insects, including *Culicoides sonorensis*, an orbivirus vector (11); the honeybee *Apis mellifera* (34); and the ant lion, *Myrmeleon mobilis* (20). In contrast, bacterial communities associated with certain species of wood- and soil-feeding termites tend to be biased towards gram-positive microorganisms (29, 65). In *D. melanogaster*, it is possible that abiotic and biotic factors, such as climate or the availability of certain food sources, affect the proportion of proteobacteria or bacteria from other phyla in the host.

Non-*Wolbachia* genera. Aside from *Wolbachia*, the libraries contained sequences from diverse bacterial communities. Many of these sequences have not been found in a cultured organism and may represent novel genera. A phylogenetic approach was used to classify many of the OTUs isolated from the *D. melanogaster* hosts. For many of the OTUs, low bootstrap support precludes taxonomic identification. However, tentative classifications could be made for many other cases, and some suggest potentially interesting host-bacterium inter-

actions taking place in this system that might be studied more rigorously in future experiments.

Most of the OTUs isolated from *D. melanogaster* host populations belonged to four classes of the *Proteobacteria*, a diverse phylum containing upwards of 460 genera and 1,619 species (36). These OTUs were located primarily within the *Alpha*- and *Gammaproteobacteria*, a feature consistent with both their ubiquity in nature (36) and their presence in many species of insect hosts (6, 11, 34, 65). Many OTUs were highly similar to taxa that interact with animals and plants in interesting ways, suggesting a possible role for these bacteria in the ecology of their *D. melanogaster* hosts and for *D. melanogaster* in mediating interactions between these bacteria and alternative hosts. For example, within the *Alphaproteobacteria*, many of the OTUs grouped closely within the *Gluconacetobacter* genus. Species in this genus are found primarily in sugary, acidic, and alcoholic habitats, such as flowers, fruits, plant tissues, and plant rhizospheres (37), and associate with insects such as the pink sugarcane mealybug (37) and honeybees (34). Three OTUs in the *Betaproteobacteria* grouped closely with published sequences from the genera *Acidovorax* and *Bordetella*, which are implicated in both plant (5) and animal (72) diseases. OTU 18 closely matched a gammaproteobacterium isolated from the intestine of the honeybee and could represent a generalist capable of cross-species horizontal transmission. OTUs 51, 68, and 8 grouped closely with the insect pathogens *Providencia rettgeri* (33) and *Pseudomonas fluorescens* (40, 62) and could therefore be pathogenic to *D. melanogaster* in nature.

Seventeen of the 74 OTUs were members of the gram-positive *Firmicutes* phylum. Many of these OTUs showed high similarity with published sequences from the *Leuconostoc* genus. Since some members of the *Leuconostoc* genus ferment fructose (43), these OTUs may play a role in host digestion of fruits or other plant materials or may live commensally in the host gut. OTU 58 was highly similar to the published sequences for members of the *Lactobacillus* genus. Although members of this genus are sometimes pathogenic to plants and animals (27), others are part of the normal nonpathogenic floras of plants, insects, and vertebrates (27) and have been shown to increase the life span in laboratory strains of *Drosophila* (10).

***Wolbachia*.** One of the more striking characteristics of the 16S rRNA clone libraries was the large number of *Wolbachia* sequences. Because PCR can be biased, the frequency of *Wolbachia* sequences we observed cannot be a direct measure of the frequency of *Wolbachia* species in nature. However, the relatively strong bias towards *Wolbachia* species in our libraries probably reflects an abundance of these microbes relative to other bacteria. Although the exact frequency of *Wolbachia* infections in natural *D. melanogaster* populations is unclear and likely variable, it is estimated that approximately 30% to 75% of the *D. melanogaster* stocks housed at *Drosophila* stock centers are infected with this intracellular parasite (17, 46). Furthermore, preliminary studies in our lab suggest that 55 to 60% of wild-caught *D. melanogaster* isofemale lines, including lines derived from the populations used in this study, are infected with *Wolbachia* (V. Corby-Harris, unpublished data). Studies of other arthropods suggest that within-species infection rates range from 2 to 83% for *Solenopsis invicta* (66), 5 to 100% for *Acraea* species (35), and 25 to 100% for fig wasps (67). When sequences were grouped based on 3% sequence divergence, the *Wolbachia* and *Wolbachia*-like sequences were grouped into 10 distinct OTUs that were unevenly distributed across host locations. This pattern suggests distinct species or lineages of *Wolbachia* across host locations, a pattern similar to that demonstrated for *Wolbachia* in geographically distinct populations of *S. invicta* (1).

To understand how the *Wolbachia* sequences we isolated were related to each other and to published *Wolbachia* 16S rRNA gene sequences, we constructed a phylogeny consisting only of *Wolbachia*-like gene sequences we isolated and published *Wolbachia* 16S rRNA gene sequences. The phylogeny we constructed agrees qualitatively with previous work (44) in that the published sequences formed five distinct supergroups (A through F). However, the bootstrap support values varied widely, with many below 70%, and the relationships of the supergroups to each other were inconsistent with those in previous studies (44, 46). Such inconsistencies could be due to the slowly evolving nature of the 16S rRNA molecule, which may not provide adequate resolution between the clades (44). Indeed, Lo et al. (44) suggested that more rapidly evolving sequences, such as *ftsZ*, are more appropriate for understanding the phylogenetics of the *Wolbachia* genus. Nonetheless, the phylogeny we constructed suggests that there are two distinct lineages of *Wolbachia* in natural populations of *D. melanogaster* hosts. Both of these lineages appear to be monophyletic with published sequences from supergroup A, which is found in a variety of arthropod hosts, including drosophilids, *Tribolium*, and *Nasonia* (44). This monophyly is consistent with one or a few origins of *Wolbachia* in *D. melanogaster* hosts found in nature.

Species richness and composition. Aside from identifying species of bacteria associated with natural populations of *D. melanogaster*, we also aimed to characterize the richness of these microbial communities overall. Here it is important to point out that we sampled only five flies per location (pooled into one sample), and our estimates of species richness and composition could change with increased sampling effort. In addition, our ability to identify trends based on these data is weakened by the lack of multiple samples from each collection site. With these limitations in mind, however, there are nonetheless some intriguing features of the communities that were sampled.

The shapes of the rarefaction curves suggest that the taxonomically diverse populations present in these 11 host populations have not been sampled completely. Many taxa were found only once or twice. The fact that we were able to uncover these rare taxa with this sampling effort suggests that many unidentified and ecologically important species of bacteria living in natural populations of *D. melanogaster* have yet to be identified.

We observed that microbial species richness varied across host populations. Motivated by previous studies of plants and animals that demonstrated a negative correlation between species richness and latitude (9, 58), we sought to explain this variation in richness among locations by using latitudinal data from each location. In addition, because latitude is only one of the many characteristics of a geographic location, we also tested for a significant relationship between climatic factors and richness. While microbial species richness varied across

the host locations that we sampled, there was no evidence of a relationship between richness and latitude. The lack of a clear relationship between richness and latitude in these 11 host populations could simply be due to the fact that the range we sampled (approximately 10 degrees latitude) was not large enough to observe an effect. There was also no evidence for a significant correlation between richness and climatic factors, such as mean annual temperature, monthly temperature range, mean annual precipitation, and mean January low temperature, using either the Chao1 or ACE richness estimator. One reason for these nonsignificant results is that bacterial populations are responding to climatic factors over relatively short time scales or within microenvironments that the 30-year climate averages cannot adequately represent. Alternatively, since many biotic and abiotic factors account for the distributions of microbes in the environment (30), it is possible that other unidentified and ecologically important factors, or interactions between these factors, may account for the differences in richness that we observed across populations. Factors such as the types of fruit present in these habitats, host genetic structure, or the presence of alternative insect hosts could account for such variation and need to be studied empirically.

Cox and Gilmore recently completed a survey of bacteria isolated from wild and laboratory-reared *D. melanogaster* flies (19). Several features of their study agree with the data presented here. First, they showed that wild *D. melanogaster* flies harbor a wide range of bacterial species from the *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* phyla (19). Additionally, although the *Gammaproteobacteria* are the most diverse group isolated from flies, the *Alphaproteobacteria* are the most abundant, after the highly prevalent *Wolbachia* species are excluded (19). Last, Cox and Gilmore's estimates of species richness (19) fall well within the range of species richness estimates in the present study. Together with Cox and Gilmore's work, the data presented here move us towards a better understanding of the bacteria interacting with *Drosophila*.

Our study sheds light on the composition and richness of microbial communities present in natural populations of *D. melanogaster* hosts and highlights several important features of these communities. Although our findings have important consequences for how researchers understand the ecology and evolution of *Drosophila* hosts in nature and the dynamics of insect-associated bacterial communities, more work must be done to explicitly test hypotheses regarding the nature of the interactions between *Drosophila* and the microbes we identified. Fortunately, *D. melanogaster* is an experimentally tractable model organism that lends itself beautifully to such in-depth studies.

ACKNOWLEDGMENTS

We thank Judith Mank, DeEtte Walker, Justine Lyons, Mary Ann Moran, Chih-Horng Kuo, and Chris Lasher for technical assistance, Tracy Leskey, H. T. Melvin's Place, the Sokol family, Cynthia Marston, the Grove family, and Robert Cheves for access to collection sites, and members of the Promislow lab for comments on a previous version of the manuscript.

This work was supported by an NSF DDIG grant (DEB-0508785) to V.C.-H., an Alton Fellowship in Genetics to V.C.-H., a Senior Scholar Award to D.E.L.P. from the Ellison Medical Foundation, and the Georgia Research Alliance Giles Professorship to J.L.B.

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