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A simple and distinctive microbiota associated with honey bees and bumble bees

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Abstract

Specialized relationships with bacteria often allow animals to exploit a new diet by providing a novel set of metabolic capabilities. Bees are a monophyletic group of Hymenoptera that transitioned to a completely herbivorous diet from the carnivorous diet of their wasp ancestors. Recent culture-independent studies suggest that a set of distinctive bacterial species inhabits the gut of the honey bee, *Apis mellifera*. Here we survey the gut microbiotae of diverse bee and wasp species to test whether acquisition of these bacteria was associated with the transition to herbivory in bees generally. We found that most bee species lack phylotypes that are the same or similar to those typical of *A. mellifera*, rejecting the hypothesis that this dietary transition was symbiont-dependent. The most common bacteria in solitary bee species are a widespread phylotype of *Burkholderia* and the pervasive insect associate, *Wolbachia*. In contrast, several social representatives of corbiculate bees do possess distinctive bacterial phylotypes. Samples of *A. mellifera* harboured the same microbiota as in previous surveys, and closely related bacterial phylotypes were identified in two Asian honey bees (*Apis andreniformis* and *Apis dorsata*) and several bumble bee (*Bombus*) species. Potentially, the sociality of *Apis* and *Bombus* species facilitates symbiont transmission and thus is key to the maintenance of a more consistent gut microbiota. Phylogenetic analyses provide a more refined taxonomic placement of the *A. mellifera* symbionts.

Keywords: *Apis mellifera*, bacterial microbiota, insect symbiosis, microbiology

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Introduction

Recent non-culture-based 16S rRNA and metagenomic surveys have revealed that the guts of honey bees (*Apis mellifera*) from the United States, Australia, South Africa, Germany, Sweden, and Switzerland harbour representatives of the same eight bacterial phylotypes (Fig. 1) (Jeyaprakash *et al.* 2003; Mohr & Tebbe 2006; Babendreier *et al.* 2007; Cox-Foster *et al.* 2007; Olofsson & Vasquez 2008). This result is contrary to results of previous culture-based surveys, which showed a complex and inconsistent microbiota of over 6000 bacterial strains in *A. mellifera* guts (Gilliam 1997). The eight characteristic phylotypes constitute ~95% of bacterial 16S rRNA sequences cloned from *A. mellifera* abdomens, and represent five bacterial classes (Fig. S1, Supporting information) (Cox-Foster *et al.* 2007). The association between these bacteria and *A. mellifera* is highly conserved despite environmental, geographic, and subspecies differences of hosts (Jeyaprakash *et al.* 2003; Mohr & Tebbe 2006; Babendreier *et al.* 2007; Olofsson & Vasquez 2008). These observations suggest that *A. mellifera* has a coevolved symbiotic relationship with some or all

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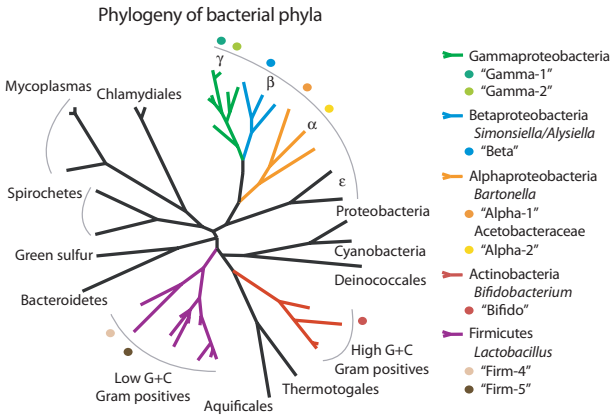

Fig. 1 Phylogenetic positions of *A. mellifera* bacterial gut microbiota associates. Phylogeny based on Brochier *et al.* (2002).

of these bacteria, and that the associations are maintained across generations of the host.

Many insects (i.e. termites, leaf-cutter ants, aphids, etc.) have evolved non-pathogenic, persistent associations with microorganisms that provide benefits to both partners (Hongoh *et al.* 2008; Moran *et al.* 2008; Pinto-Tomas *et al.* 2009). Nutritional symbioses represent the majority of known insect-microbe mutualisms, and are found in multiple insect lineages that subsist on unusual or low-nutrient diets (e.g. sap, blood, detritus, wood) (Moran *et al.* 2008). For example, acquisition of a nutrient-provisioning bacterial symbiont in a sharpshooter (Hemiptera: Cicadellidae: Cicadellinae) ancestor coincided with the transition from a diet of phloem sap to the comparatively nutrient-poor diet of xylem sap (Moran 2007), illustrating the potential impact microbial symbiosis can have on the ecology of the host insect.

Bees arose from predatory apoid wasps in the early to mid-Cretaceous, transitioning from the ancestral carnivorous life-style to herbivory, depending upon plant pollen as their sole protein source (Danforth *et al.* 2006). Pollen cytoplasm is nutrient-rich but is protected by a carbohydrate exine that is refractory to most digestive systems; nevertheless, diverse insects and vertebrates are able to use pollen as food (Roulston & Cane 2000). One hypothesis for the origin of the distinct dietary habits and ecology observed in bees is that these were facilitated by the acquisition of a distinctive microbiota capable of supplementing the host with necessary nutrients, or assisting in the digestion of pollen. If so, bacteria closely related to the lineages found in *A. mellifera* might be ubiquitous across all bee species, which comprise a monophyletic clade within the superfamily Apoidea. Outside of *A. mellifera*, few bee species (and specimens) have been screened, so the distribution of these bacterial types in bees has not been clear (Mohr & Tebbe 2006). In the current study, we further characterize the *A. mellifera* microbiota and determine the distribution of the same or related lineages in gut communities from members of all but one bee family, the wasp clade sister to all bees, and a distantly related wasp that has independently shifted to an exclusively pollen and nectar diet.

Methods

Specimen collection and DNA extraction

Bee and wasp specimens were collected into 95% ethanol and stored at −80 °C until DNA was extracted (Table S1, Supporting information). Specimens were collected randomly as they were foraging at flowers. The two *A. mellifera* samples were prepared from adults collected within a single healthy hive in January 2008 at the Carl Hayden Bee Research Center (Tucson, AZ). For the *A. mellifera* hive sample a total of eighty adult digestive tracts (crop, midgut, ileum, and rectum) were dissected in 10 mM $MgSO_4$, homogenized with iris dissection scissors followed by a 1.5-mL tube mini-pestle, and passed through an 8-μm filter to remove most host cells.

Whole abdomens were removed and macerated with sterile dissection scissors for all specimens except larger bees (i.e. *Xylocopa* sp. and *Bombus* sp.). For large specimens, the abdominal exoskeleton was burdensome to fit into a 1.5-mL tube and prevented complete homogenization of the digestive tract so it was not used in the DNA extraction. For specimens stored in ethanol, DNA was extracted using the Gentra PureGene Kit (Qiagen Inc.) preceded by a 30-min lysozyme incubation (outlined in the DNeasy kit, Qiagen Inc.) to lyse Gram-positive bacterial cells. For specimens stored in lysis buffer (0.1 M Tris–HCl, 0.1 M EDTA, 0.01 M NaCl, 0.005% SDS, 500 μg/mL Proteinase K), DNA was extracted with a standard phenol/chloroform reaction, followed by ethanol precipitation. Resulting DNA was analysed for molecular weight and quantity on a 1.0% agarose gel (80 V, 2 h).

16S rRNA PCR and cloning

DNA samples for two *A. mellifera* samples, 11 other bee species, and three wasp species were sent to the Joint Genome Institute (JGI, Walnut Creek, CA). At JGI, partial 16S rRNA gene sequences were amplified from each specimen using universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1391R (5′-GACGGGCRGTGWGTRCA-3′) with reaction conditions listed in the JGI SOP 16S18S rRNA PCR Library Creation protocol as in Warnecke *et al.* (2007). PCR products were screened on a 1% agarose gel (100 V, 1 h) for the expected size along with a size ladder and positive

(known bacterial DNA) and negative (no template DNA) controls. Resulting PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen), and 384 clones were chosen for sequencing (forward and reverse). Sequences were automatically edited and assembled in the JGI sequencing pipeline.

Corbiculate 16S rRNA PCR and cloning

The corbiculate bees lack scopa (a dense mass of long, branched setae for pollen collection) on their hind legs, instead their tibia is modified into a corbicula, which is also known as the 'pollen basket'. Corbiculates form a clade within the bee family Apidae that contains several of the most economically important bee species (i.e. *A. mellifera* the honey bee) and the most complex eusocial societies among the bees. There are four tribes within the corbiculates; the solitary/communal orchid bees (Euglossini), the primitively eusocial bumble bees (Bombini), and the highly eusocial stingless (Meliponini) and honey bees (Apini) (Kawakita *et al.* 2008). We obtained additional sequences from the microbiota of several corbiculate bee species. A universal 16S rRNA PCR was performed at 20-μL reaction volume containing template DNA (~75 ng), 0.8 U Taq DNA Polymerase (New England BioLabs, Ipswich, MA, USA), 0.25 mM of each dNTP, 1x PCR buffer, 0.1 μM 27F-short primer (5′-GAGTTTG ATCCTGGCTCA-3′) and 0.1 μM 1507R primer (5′-TAC-CTTGTTACGACTTCACCCCAG-3′). Cycling conditions were as follows: 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s; and a final extension at 72 °C for 10 min. PCR products were cloned into *E. coli* JM109 competent cells using the pGEM-T Easy Vector System (Promega Corp.) per manufacturer's instructions, and grown on LB agar containing 100 μg/mL ampicillin, and appended with X-gal and IPTG for blue/white screening. Colony PCR was performed on randomly selected transformed colonies with vector primers M13F (5′-CAGGAAACAGCTATGAC-3′) and M13R (5′-GTA-AAACGACGGCCAG-3′) using the previously mentioned cycling conditions except with an annealing temperature of 55 °C. PCR products were screened for the expected size on a 1% agarose gel (95 V, 50 min), and cleaned with a 15-min incubation at 37 °C with 0.2 μL of Exo I and 0.2 μL CIP (New England BioLabs) followed by 15 min at 80 °C. For each specimen, 94 clones were sequenced at the Arizona Research Labs, Genetics Core (University of Arizona) using both forward and reverse M13 primers.

Chimeric sequence removal, classification, and heatmap construction

The 16S rRNA library from each specimen was aligned in the Green Genes online platform using the NAST alignment program (DeSantis *et al.* 2006), and potential chimeric sequences were identified with Bellerophon (Huber *et al.* 2004). Chimeric sequences were manually verified with the online RDP Chimera Detection program (Cole *et al.* 2003) and removed from the data set. The Ribosomal Database Project's Pyrosequencing Pipeline Infernal aligner (Nawrocki *et al.* 2009) (Bacteria alignment model) was used to align the resulting non-chimeric sequences generated in the current study, as well as those collected from previous publications exploring the *A. mellifera* microbiota.

Each clone library for the 16 JGI samples yielded between 182 and 360 near full-length 16S rRNA sequences, providing a total of 4799 sequences after the initial quality control. This data set was expanded with an additional 395 sequences from five species of corbiculate bees; these sequences were obtained at the DNA sequencing facility at the University of Arizona. In addition, we included 628 published 16S rRNA sequences, mostly shorter in length and mostly sampled from *A. mellifera*.

Resulting alignments were clustered into operational taxonomic units (OTUs) with RDP's Complete Linkage Clustering method (Cole *et al.* 2009), using a 0.97 sequence similarity cutoff for designating phylotypes. OTUs were classified with the RDP classifier, and resulting sequences with less than 70% bootstrap support at their Class assignment were removed as well as any chloroplast sequences. OTUs were compared to GenBank with BLASTn to identify their top hit. The OTU frequency within bee and wasp specimens was visualized in a heatmap, created with the 'heatmap.2' program within the gplots package for R.

Diagnostic PCR screening

DNA was extracted from bee and wasp samples (Table S1, Supporting information) using the methods described above. The following PCR primers (with annealing temperatures and extension times) were used to selectively amplify seven of the bacterial types: Alpha-1 (5′-CAAGTCGAACGCACTYTTCG-3′) and 1507R (5′-TACCTTGTTACGACTTCACCCCAG-3′), 58 °C, 1.5 min; Alpha-2.1-120F (5′-GTAGGGATCTGTCCATAAGAG-3′) and Alpha-2.1-806R (5′-GCTCCGACACTAAACAAC-TAGG-3′), 54 °C, 0.5 min; Alpha-2.2-180F (5′- GCCTGA-GGGCCAAAGGAG-3′) and Alpha-2.2-702R (5′-GCGT-CAGTTCCGAGCCAGG-3′), 61 °C, 0.5 min; Beta (5′-CTTAGAGATAGGAGAGTG-3′) and 1507R, 50 °C, 0.5 min; Firm-4 (5′-AGTCGAGCGCGGGAAGTCA-3′) and 1507R, 58 °C, 1.5 min; Firm-5qtF (5′-GGAATACT-TCGGTAGGAA-3′) and Firm-5qtR (5′-CTTATTTGG TATTAGCACC-3′), 52 °C, 0.5 min; Gamma1 (5′-GTATC-TAATAGGTGCATCAATT-3′) and 1507R, 54 °C, 1.5 min.

The lack of sufficiently specific primer sites for both the Gamma-2 and Bifido phylotypes precluded us from screening for these phylotypes in diagnostic PCR screens. PCRs were performed in 20 μL reactions with cycling conditions as above with positive (DNA from an *A. mellifera* gut) and negative (distilled water) control reactions. PCR products were screened for the expected size on a 1% agarose gel (95 V, 50 min).

Phylogenetic analysis of the A. mellifera *phylotypes*

Sequences were aligned in the RDP Pyrosequencing Pipeline Infernal aligner (Nawrocki *et al.* 2009) and manually refined using Mesquite (Maddison & Maddison 2009). Phylogenetic trees (GTR +I + γ model and 100 bootstrap replicates) were computed using a maximum likelihood framework in RAxML (Stamatakis 2006) with the CIPRES Portal (Miller *et al.* 2009).

Nucleotide accession numbers

The 16S rRNA gene sequences determined in this study are in GenBank under the numbers HM108361 through HM113359, and the Cox-Foster *et al.* (2007) sequences are HM107875 through HM108360.

Results

Bacterial microbiota profiles of diverse bee and wasp species

After culling chimeric, chloroplast, and unassigned sequences, we retained a final data set of 5604 16S rRNA sequences, representing 4999 near-full length sequences acquired in this study, plus 605 published sequences, mostly from *A. mellifera* and mostly shorter in length. The 4999 sequences were collected from a total of 20 bee species representing the phylogenetic diversity of bees, with more intensive sampling of the corbiculates, the clade containing *A. mellifera*. In addition, three wasp species were included as outgroups; one of these (*Paragia vespiformis*, Masaridae) represents an independent origin of pollen-feeding, allowing us to further examine whether particular bacterial types are associated with this feeding habit. A total of 146 OTUs with a 3% distance cutoff were obtained after clustering. Of those, 62 OTUs (42%) were singletons, and 91 OTUs (62%) were found in only one specimen.

Major constituents of the communities

Almost half of the sequences (2416/5604) fell within a single OTU with >99% identity to database sequences of *Burkholderia cepacia*. The *B. cepacia* OTU was recovered from all specimens surveyed in this study except for the *A. mellifera* hive sample (20/21). *Wolbachia* was also abundant in the apoid wasp, *Philanthus gibbosus* (Crabronidae), and in several bees: *Rediviva saetigera* (Melittidae), *Agapostemon virescens* (Halictidae), and *Colletes inaequalis* (Colletidae).

Apis mellifera

Almost all (>98%, 267/271 & 263/267 sequences) bacterial 16S rRNAs detected in both *A. mellifera* samples corresponded to one of the eight major groups of the previously described *A. mellifera* microbiota (Cox-Foster *et al.* 2007). The Alpha-2.2 and the Gamma-2 phylotypes were not detected in this screen. Sequences with top BLASTn hits in GenBank to the Beta, Bifido, Firm-4, Firm-5, and the Gamma-1 phylotypes were the most abundant. Both *A. mellifera* communities were dominated by the Firm-5 phylotype, in contrast to previous surveys of the *A. mellifera* microbiota, which were dominated by the Gamma-1 (Cox-Foster *et al.* 2007). This difference may reflect our use of lysozyme in DNA extractions, resulting in more efficient extraction of Gram-positive bacteria, or it may reflect differences in developmental stages sampled. We note that even though sampling methods were very different for the two *A. mellifera* samples, the profiles of symbiont abundances were similar.

The genera Apis *and* Bombus *(corbiculate clade)*

Some of the typical *A. mellifera* OTUs were recovered from other *Apis* and from *Bombus* species (both in the corbiculate clade), though none had the profile characteristic of *A. mellifera* itself. The *A. dorsata* microbiota included three major OTUs, a sequence with a top BLASTn hit in GenBank to the *A. mellifera* Alpha-1 phylotype, plus two phylotypes of *Burkholderia*. The majority of sequences from *A. andreniformis* (63%, 49/78 sequences) corresponded to a single OTU nearly identical (>96% sequence identity) to the *A. mellifera* Alpha-1 phylotype. The *Bombus sonorus* microbiota was dominated (96%, 75/78 sequences) by a single OTU closely related to the *A. mellifera* Beta phylotype (94% sequence identity). *Bombus* sp. from Montana and *Bombus impatiens* from California were both dominated (87%, 70/80 sequences and 76%, 54/71 sequences) by the *B. cepacia* OTU.

Survey of the A. mellifera *gut microbiota in phylogenetically diverse bee species*

In order to further examine the host range of phylotypes corresponding to typical *A. mellifera* associates,

we used diagnostic PCR on a panel of bee species to survey the presence or absence of seven of the common associates. Positive amplifications for any of these sequences were obtained only within the genera *Apis* and *Bombus* (Fig. S2, Supporting information). *A. mellifera* specimens had the highest percent of positive reactions of any species (82%, 46/56 reactions). Most negative results for *A. mellifera* were from the Alpha-2.2, which was identified in only two of eight specimens. *A. dorsata* specimens were universally infected with all symbionts except the Alpha-2.1, Firm-4, and Firm-5, while the *A. andreniformis* specimens were universally infected with Alpha-1 and had high infection rates of the Beta (5/6), Firm-4 (5/6), and Gamma-1 (4/6). Ten of the twelve *Bombus* specimens were positive for at least one of these bacterial phylotypes, but the set of typical *A. mellifera* bacteria was less ubiquitous in *Bombus* than in *Apis* specimens. Specifically, among *Bombus* samples screened, only the Gamma-1 (8/12), Beta (4/12), and Firm-5 (4/12) were detected. Because these diagnostic screens depended upon specific primers to amplify sequences closely related to the *A. mellifera* associates, mismatches due to sequence divergence within those primer regions could yield negative results. Nevertheless, results of the PCR surveys are consistent with the near-universal absence of related sequences from non-corbiculate bee species in the 16S rRNA libraries, and both provide evidence that members of the characteristic *A. mellifera* microbiota are absent from most bee species.

Phylogenetic relationships of the A. mellifera *gut microbiota*

We analysed the phylogenetic relationships of each of the *A. mellifera* bacterial types (Fig. S3, Supporting information). Six of the eight phylotypes correspond to single distinct clades, exclusively sampled from *A. mellifera* or close relatives; the Alpha-2 and Bifido types each correspond to two closely related lineages.

Alpha-1. The Alpha-1 sequences form a highly supported clade related to the genus *Bartonella* within the Rhizobiales. This clade also includes sequences from several herbivorous ant species (Russell *et al*. 2009).

Alpha-2. Two distinct clades were identified in the Alpha-2 phylogeny. Clade 1 clustered outside the Acetobacteraceae and included two published sequences associated with *Drosophila*, as well as sequences from the *Xylocopa* specimen. Clade 2 grouped within the genus *Gluconobacter* and contained only sequences from European *A. mellifera*. This clade contained sequences from each of the *A. dorsata*, *Xylocopa*, *Caupolicana*, and *Calliopsis* specimens as well as *Saccharibacter floricola*, a bacterium isolated from pollen.

Beta. A well-supported clade corresponding to the Beta symbiont fell within Neisseriaceae and grouped with the genera *Simonsiella* and *Alysiella*.

Bifido. The Bifido bacterial sequences from *A. mellifera* fell into two sister clusters within the clade representing the genus *Bifidobacterium*. Distributed among the *A. mellifera* sequences were several sequences from cultured isolates, originally obtained from *A. mellifera* guts. The cultured *B. asteroides* sequences form a clade with one set of *A. mellifera* sequences, while *B. coryneforme* and *B. indicum* cluster with the sister clade.

Firm-4 and Firm-5. Firm-4 and Firm-5 both fell within the genus *Lactobacillus* with high support. Firm-4 forms a clade sister to the *acidophilus* clade, whereas the Firm-5 fell within the *acidophilus* clade. The Firm-5 cluster contains several sequences (GenBank accession numbers AY667698, AY667699, and AY667701) that are described as cultured from the guts of Italian *A. mellifera*.

Gamma-1 and Gamma-2. The Gamma-1 and Gamma-2 taxa together form a highly supported clade branching between the Enterobacteriaceae and Pasteurellaceae. Within this separate clade are bacterial sequences cloned from an aphid, a fly, a ground beetle, and a wild boar.

Discussion

Our results confirm that *A. mellifera* is consistently colonized by a distinctive set of bacterial species. Both *A. mellifera* samples in the 16S rRNA survey were dominated by these bacteria (Fig. 2), as in previous surveys based on different methodologies and on samples from different localities. Furthermore, our surveys using diagnostic PCR primers revealed consistent presence of these bacteria in a panel of *A. mellifera* specimens (Fig. S2, Supporting information) and of many of these bacterial types in other corbiculate bees sampled, including members of the genera *Apis* and *Bombus*. In contrast, most other bee and wasp species completely lacked these phylotypes; a few bee species harboured one or two phylotypes associated with *A. mellifera*, but these phylotypes represented less than 10% of their microbiota profile (Fig. 2, Fig. S2, Supporting information). Thus, we can dismiss the hypothesis that the *A. mellifera* microbiota represents an ancestral set of bacterial associates that aided in the transition from predaceous wasp to pollenivorous (herbivorous) bee. Within the corbiculate clade, however, the phylogenetically

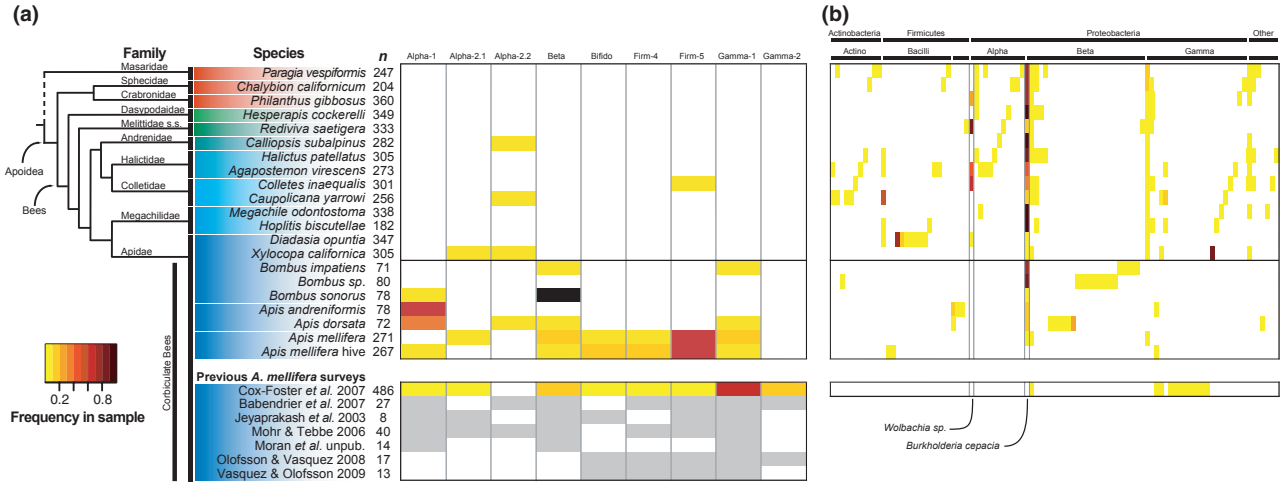

Fig. 2 Frequency of bacterial types in the microbiotae of 21 specimens (three wasps and 16 bees) represented as a heatmap. (a) OTUs correspond to one of the distinctive *A. mellifera* bacterial phylotypes. Column '*n*' denotes number of bacterial 16S rRNA sequences for each specimen. Lower section displays the presence or absence of each bacterial type in previously published studies. (b) OTUs recovered that did not have a top BLASTn hit to the *A. mellifera* bacterial types.

restricted host range of these bacteria suggests an interdependent, coevolved association, as observed in other associations between insects and mutualistic microbes (Hosokawa *et al.* 2006; Warnecke *et al.* 2007; Moran *et al.* 2008; Ohkuma *et al.* 2009).

The other *Apis* species and the *Bombus* species screened in this study frequently contained only a subset of the phylotypes common in the *A. mellifera* microbiota (Fig. S2, Supporting information). These associations were detected even though specimens were collected from distant geographic locations, suggesting that the bacterial microbiota is transferred between generations and not acquired from the environment. While the majority of bee species are solitary, those within the genus *Apis* are highly eusocial and those within the genus *Bombus* are primitively eusocial, living in colonies from tens of workers up to several thousand individuals (Wilson 1971). A potential mechanism for younger generations to acquire the characteristic microbiota could be the oral transfer of food between nestmates (trophallaxis), found in eusocial Apinae (Michener 1974). *Apis* species have more frequent trophallaxis in comparison to *Bombus* (Wilson 1971). Another variable that may affect maintenance of the microbiota is colony establishment. *Apis* colonies split approximately in half to form two new colonies by colony fission (Michener 1974). In contrast, most *Bombus* colonies are established by individual females; these colonies grow and completely disband on an annual cycle (Michener 1974). This life cycle may impose a greater chance of microbiota loss possibly resulting in higher variation of infection frequency among *Bombus* colonies than among *Apis* colonies (Fig. S2, Supporting information). Overall, eusocial behaviour may be one of the most important variables affecting the inheritance and maintenance of the corbiculate-specific microbiota.

While *Apis* and *Bombus* species may maintain a consistent microbiota, the other specimens in our study have strikingly different profiles of bacterial taxa in their guts (Fig. 2) with no evident connection to phylogenetic position, collection location, or natural history. Most bee species are solitary and burrow into soil or plant materials to make nests, which are provisioned with pollen and nectar to provide the necessary nutrition for the offspring to mature (Michener 2007). Bees, thus, come into contact with many plant- and soil-associated microorganisms. In our 16S rRNA survey, a single phylotype of *Burkholderia cepacia* was present in nearly all bee specimens (20/21) and was prominent (>10% of sequences) in most samples (16/21) surveyed in depth. *B. cepacia* is commonly found in the soil rhizosphere and on plant surfaces (Compant *et al.* 2008); hence, this bacterium may be acquired from the environment. The other major group identified in bee and wasp specimens was *Wolbachia*, a common intracellular bacterium of insects, estimated to infect between 20% and 66% of all species (Werren & Windsor 2000; Duron *et al.* 2008; Hilgenboecker *et al.* 2008) and present in five of the bee and wasp species we surveyed.

Apis mellifera *microbiota phylogenies*

Each member of the *A. mellifera* characteristic gut microbiota forms one or two distinct clades, and sequence

identities are high (>96%) within each such clade. Thus, these lineages correspond to coherent bacterial species with wide geographic distribution (Fig. 1, Fig. S3, Supporting information). Some of these clades (such as the Bifido) may consist of two or three distinct sister species. Phylogenetic classification may aid in future cultivation attempts, as the genera identified as close relatives have defined morphologies, biochemical profiles, and habitats. The Alpha-1 phylotype forms a sister clade to the genus *Bartonella*, a group of intracellular pathogens that can infect many insects (Minnick & Battisti 2009), raising the possibility that these bacteria are at least partially intracellular within *A. mellifera*. Closely related to the *A. mellifera* Alpha-1 were sequences associated with the evolution of herbivory in phylogenetically diverse ant lineages (Russell *et al.* 2009). The Alpha-2 phylotype sequences are divided into two distinct clades within Acetobacteraceae. The first clade groups with several sequences found in the *Xylocopa* specimen and with two sequences previously found in a survey of *Drosophila*-associated bacteria (Roh *et al.* 2008), forming a distinct clade separate from other named genera. The second clade clusters within the genus *Gluconobacter* and contains only sequences originating from European *A. mellifera* (Babendreier *et al.* 2007), suggesting a geographically heterogeneous distribution of these Alphaproteobacteria. *Saccharibacter floricola*, a bacterium isolated from pollen, was nested within this second clade in addition to sequences from the *A. dorsata*, *Xylocopa*, *Caupolicana*, and *Calliopsis* specimens, suggesting that this phylotype is associated with flowers (Jojima *et al.* 2004). The Beta symbiont forms a distinct clade close to the *Simonsiella/Alysiella* cluster within Neisseriaceae. *Simonsiella/Alysiella* species have been isolated from the oral cavities of several mammals in which they are thought to be non-pathogenic commensals (Kuhn *et al.* 1978; Hedlund & Kuhn 2006). The Bifido phylotype forms two sister clades within the genus *Bifidobacterium* and includes three *Bifidobacterium* species previously cultured from the *A. mellifera* gut (Scardovi & Troatelli 1969). Thus, the Bifido phylotype may correspond to two or three known species that can be cultured in the lab. The Firm-4 and Firm-5 phylotypes comprise two clades within and sister to the *acidophilus* group of the genus *Lactobacillus*. Several unpublished database sequences corresponding to the Firm-5 clade are described as being cultured from the *A. mellifera* gut (NCBI accession nos AY667698, AY667699, and AY667701). The Gamma-1 and Gamma-2 phylotypes are sister groups forming a clade that is sister to the family Pasteurellaceae and separate from Enterobacteriaceae. This new clade encompasses several database sequences associated with animals (*Drosophila*, DQ980728; aphid, EU348326; beetle, EF608532; and wild boar, FJ612598). Potentially, members of this clade are widespread associates within insect guts (boars may consume insects).

Co-evolution of gut bacteria and corbiculate bees

Sequences amplified from microbiota of *Apis* and *Bombus* formed clades together (Fig. S3, Supporting information), raising the possibility of a co-evolutionary association between these bee genera and a specific assemblage of bacteria. Screening several representative species from other members of this clade (the solitary/communal Euglossini and the highly eusocial Meliponini) would provide further insights into the effects of social behaviour upon this bacterial community.

Two Firm-5 sequences were amplified from the *Colletes inaequalis* specimen (Fig. 2, Fig. S3, Supporting information), implying that this bacterial type is found in more disparate hosts. However, these sequences were minor constituents in the *C. inaequalis* sample, raising the possibility that these bacteria were transients acquired at flowers, a shared habitat of diverse bee species.

Possible functions: mutualist vs. pathogen

The consistency of the association between *A. mellifera* and its characteristic microbiota suggests mutualistic relationships, at least with some members, though pathogenic effects have not been excluded.

Relatives of several members of the *A. mellifera* microbiota (Acetobacteraceae, *Bifidobacterium*, *Lactobacillus*, and *Simonsiella*) produce short chain fatty acids such as lactic or acetic acid as waste products during the metabolism of carbohydrates (Biavati & Mattarelli 2006; Hammes & Hertel 2006; Hedlund & Kuhn 2006; Kersters *et al.* 2006). Assimilation of these compounds could supplement bee nutrition, just as short chain fatty acids produced by rumen microbes supply nearly all the energy requirements of ruminant mammals (Dehority 1997). Short chain fatty acids can be absorbed through the rectal wall in insects (Bradley 2008), and we have observed that the majority of the pollen and bacterial biomass within an adult *A. mellifera* is contained inside the rectum. Overwintering *Apis* may obtain additional nutrition from these rectal bacteria, as consumed food is stored for longer periods of time within the rectum during winter months (Spivak & Gilliam 1998; Lindstrom *et al.* 2008).

Osmotic pressure change within the gut has been suggested as a main digestive mechanism capable of breaking pollen cells and releasing their contents (Roulston & Cane 2000). Unlike many animals, *A. mellifera* mix pollen with a nectar/glandular solution before feeding (Michener 1974), which might facilitate digestion by introducing enzymes and/or microorganisms

that can degrade resistant carbohydrates. Further, *A. mellifera* pollen is stored for long periods of time, which changes its texture and nutritive qualities (Human & Nicolson 2006).

A. mellifera colonies offer a favourable environment for viral, bacterial, fungal, and protist pathogens because of the high density of individuals and the exchange of food among nestmates. Due to the risk of infection spreading through an entire hive, the *A. mellifera* immune system is expected to be highly developed. In contrast with this expectation, a comparison of the *A. mellifera* genome to *Anopheles gambiae* and *Drosophila melanogaster* revealed a substantial decrease in recognizable immune pathway genes (71 in *A. mellifera*, 209 in *A. gambiae*, 196 in *D. melanogaster*) (Evans *et al.* 2006). This decrease in gene number was not observed in other *A. mellifera* gene families, suggesting that *A. mellifera* has a reduced immune flexibility (Evans *et al.* 2006; Weinstock *et al.* 2006). However, social behaviours (i.e., grooming and removal of diseased brood) provide a major defensive barrier against pathogens (Spivak & Reuter 2001; Evans & Spivak 2010). Additionally, the *A. mellifera* gut microbiota could provide further protection against invading pathogens by producing inhibitory compounds or by monopolizing nutrients within the gut (Corr *et al.* 2007; Round & Mazmanian 2009).

Conclusions

In this survey of bees from a broad sample of families and subfamilies, members of the characteristic bacterial microbiota of *A. mellifera* were found to be absent from most species outside of the corbiculate clade, represented in our study by *Apis* and *Bombus*. An earlier survey of short 16S rRNA segments from *A. mellifera*, *Bombus terrestris*, and *Osmia bicornis* raised the possibility of a broader distribution in bees (Mohr & Tebbe 2006). However, our more extensive survey suggests that most of the bacterial species identified from *A. mellifera* have a narrow association with *Apis* and *Bombus*. Co-evolution between the corbiculate bees and a characteristic microbiota may explain this association, but further screening of the other corbiculate tribes is needed to accept or reject this hypothesis.

This survey offers a closer view of the bacteria associated with bees; however, more sampling within and between bee species could provide a more complete understanding of the relationship between bees and their bacterial microbiota. We note that a similar set of bacterial phylotypes has been recovered from all non-culture-based studies of the *A. mellifera* microbiota, despite extremely different methods of sampling, amplification and sequencing (Babendreier *et al.* 2007; Cox-Foster *et al.* 2007; Jeyaprakash *et al.* 2003; Mohr & Tebbe 2006; Olofsson & Vasquez 2008; this study). Thus, although our sampling of other bee species is limited, and could be influenced by preservation method or PCR primers, our failure to retrieve the same or related phylotypes from most species sampled is striking.

Our results support the existence of a relatively simple microbiota in *A. mellifera*, an eminent model organism (Weinstock *et al.* 2006) and an ecologically influential and economically important insect (Morse & Calderone 2000). Learning more about this little known, but potentially essential, microbiota may have wide-reaching implications for our understanding of the basic biology of honey bees and for current practices in apiculture and agriculture.

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References

Babendreier D, Joller D, Romeis J, Bigler F, Widmer F (2007) Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. *FEMS Microbiology Ecology*, **59**, 600–610.

Biavati B, Mattarelli P (2006) The Family Bifidobacteriaceae. In: *The Prokaryotes*, 3rd edn, Vol. 3 (eds Dworkin M, Falkow S, Rosenberg E *et al.*), pp. 322–382. Springer-Verlag, New York, NY.

Bradley TJ (2008) Active transport in insect recta. *Journal of Experimental Biology*, **211**, 835–836.

Brochier C, Bapteste E, Moreira D, Philippe H (2002) Eubacterial phylogeny based on translational apparatus proteins. *Trends in Genetics*, **18**, 1–5.

Cole JR, Chai B, Marsh TL *et al.* (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Research*, **31**, 442–443.

Cole JR, Wang Q, Cardenas E *et al.* (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research*, **37**, D141–D145.

Compant S, Nowak J, Coenye T, Clement C, Barka EA (2008) Diversity and occurrence of *Burkholderia* spp. in the natural environment. *FEMS Microbiology Reviews*, **32**, 607–626.

Corr SC, Li Y, Riedel CU *et al.* (2007) Bacteriocin production as a mechanism for the antfinfective activity of *Lactobacillus*

salivarius UCC118. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 7617–7621.

Cox-Foster DL, Conlan S, Holmes EC *et al.* (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. *Science*, **318**, 283–287.

Danforth BN, Sipes S, Fang J, Brady SG (2006) The history of early bee diversification based on five genes plus morphology. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 15118–15123.

Dehority BA (1997) Foregut fermentation. In: *Gastrointestinal Microbiology* (eds Mackie RI, White BA, Isaacson RE). pp. 39–83, Chapman & Hall, New York, NY.

DeSantis TZ, Hugenholtz P, Keller K *et al.* (2006) NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Research*, **34**, W394–W399.

Duron O, Bouchon D, Boutin S *et al.* (2008) The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biology*, **6**, 12.

Evans JD, Spivak M (2010) Socialized medicine: individual and communal disease barriers in honey bees. *Journal of Invertebrate Pathology*, **103**, S62–S72.

Evans JD, Aronstein K, Chen YP *et al.* (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Molecular Biology*, **15**, 645–656.

Gilliam M (1997) Identification and roles of non-pathogenic microflora associated with honey bees. *FEMS Microbiology Letters*, **155**, 1–10.

Hammes WP, Hertel C (2006) The Genera *Lactobacillus* and *Carnobacterium*. In: *The Prokaryotes*, 3rd edn, Vol. 4 (eds Dworkin M, Falkow S, Rosenberg E *et al.*), pp. 320–403. Springer-Verlag, New York, NY.

Hedlund BP, Kuhn DA (2006) The Genera *Simonsiella* and *Alysiella*. In: *The Prokaryotes*, 3rd ed., Vol. 5 (eds Dworkin M, Falkow S, Rosenberg E *et al.*), pp. 828–839. Springer-Verlag, New York, NY.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2008) How many species are infected with *Wolbachia*? – a statistical analysis of current data. *FEMS Microbiology Letters*, **281**, 215–220.

Hongoh Y, Sharma VK, Prakash T *et al.* (2008) Genome of an endosymbiont coupling N-2 fixation to cellulolysis within protist cells in termite gut. *Science*, **322**, 1108–1109.

Hosokawa T, Kikuchi Y, Nikoh N, Shimada M, Fukatsu T (2006) Strict host–symbiont cospeciation and reductive genome evolution in insect gut bacteria. *PLoS Biology*, **4**, 1841–1851.

Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics*, **20**, 2317–2319.

Human H, Nicolson SW (2006) Nutritional content of fresh, bee-collected and stored pollen of *Aloe greatheadii* var. *davyana* (Asphodelaceae). *Phytochemistry*, **67**, 1486–1486.

Jeyaprakash A, Hoy MA, Allsopp MH (2003) Bacterial diversity in worker adults of *Apis mellifera capensis* and *Apis mellifera scutellata* (Insecta: Hymenoptera) assessed using 16S rRNA sequences. *Journal of Invertebrate Pathology*, **84**, 96–103.

Jojima Y, Mihara Y, Suzuki S *et al.* (2004) *Saccharibacter floricola* gen. nov., sp nov., a novel osmophilic acetic acid bacterium isolated from pollen. *International Journal of Systematic and Evolutionary Microbiology*, **54**, 2263–2267.

Kawakita A, Ascher JS, Sota T, Kato M, Roubik DW (2008) Phylogenetic analysis of the corbiculate bee tribes based on 12 nuclear protein-coding genes (Hymenoptera: Apoidea: Apidae). *Apidologie*, **39**, 163–U118.

Kersters K, Lisdiyanti P, Kmagata K, Swings J (2006) The Family Acetobacteraceae: The Genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*. In: *The Prokaryotes*, 3rd edn, Vol. 5 (eds Dworkin M, Falkow S, Rosenberg E *et al.*), pp. 163–200. Springer-Verlag, New York, NY.

Kuhn DA, Gregory DA, Buchanan GE, Nyby MD, Daly KR (1978) Isolation, characterization, and numerical taxonomy of *Simonsiella* strains from oral cavities of cats, dogs, sheep, and humans. *Archives of Microbiology*, **118**, 235–241.

Lindstrom A, Korpela S, Fries I (2008) The distribution of *Paenibacillus larvae* spores in adult bees and honey and larval mortality, following the addition of American foulbrood diseased brood or spore-contaminated honey in honey bee (*Apis mellifera*) colonies. *Journal of Invertebrate Pathology*, **99**, 82–86.

Maddison WP, Maddison DR (2009) Mesquite: a modular system for evolutionary analysis. Version 2.72. http://mesquiteproject.org.

Michener CD (1974). *The Social Behavior of the Bees: A Comparative Study*, The Belknap Press of Harvard University Press, Cambridge, MA.

Michener CD (2007) *The Bees of the World*, 2nd edn. Johns Hopkins University Press, Baltimore, MD.

Miller MA, Holder MT, Vos R *et al.* (2009) The CIPRES Portals. CIPRES. URL: http://www.phylo.org/sub_sections/portal. Accessed February 2010.

Minnick MF, Battisti JM (2009) Pestilence, persistence and pathogenicity: infection strategies of *Bartonella*. *Future Microbiology*, **4**, 743–758.

Mohr KI, Tebbe CC (2006) Diversity and phylotype consistency of bacteria in the guts of three bee species (Apoidea) at an oilseed rape field. *Environmental Microbiology*, **8**, 258–272.

Moran NA (2007) Symbiosis as an adaptive process and source of phenotypic complexity. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 8627–8633.

Moran N, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics*, **42**, 165–190.

Morse RA, Calderone NW (2000) The value of honey bees as pollinators of U.S. crops in 2000. *Bee Culture*, **128**, 1–15.

Nawrocki EP, Kolbe DL, Eddy SR (2009) Infernal 1.0: inference of RNA alignments. *Bioinformatics*, **25**, 1335–1337.

Ohkuma M, Noda S, Hongoh Y, Nalepa CA, Inoue T (2009) Inheritance and diversification of symbiotic trichonymphid flagellates from a common ancestor of termites and the cockroach *Cryptocercus*. *Proceedings of the Royal Society B-Biological Sciences*, **276**, 239–245.

Olofsson TC, Vasquez A (2008) Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. *Current Microbiology*, **57**, 356–363.

Pinto-Tomas AA, Anderson MA, Suen G *et al.* (2009) Symbiotic nitrogen fixation in the fungus gardens of leaf-cutter ants. *Science*, **326**, 1120–1123.

Roh SW, Nam YD, Chang HW *et al.* (2008) Phylogenetic characterization of two novel commensal bacteria involved with innate immune homeostasis in *Drosophila melanogaster*. *Applied and Environmental Microbiology*, **74**, 6171–6177.

Roulston TH, Cane JH (2000) Pollen nutritional content and digestibility for animals. *Plant Systematics and Evolution*, **222**, 187–209.

Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. *Nature Reviews Immunology*, **9**, 313–323.

Russell JA, Moreau CS, Goldman-Huertas B *et al.* (2009) Bacterial gut symbionts are tightly linked with the evolution of herbivory in ants. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 21236–21241.

Scardovi V, Troatelli LD (1969) New species of bifid bacteria from *Apis mellifera* L. and *Apis indica* F. a contribution to the taxonomy and biochemistry of the genus *Bifidobacterium*. *Zentralbl Bakteriol Parasitenkd Infektionskr Hyg*, **123**, 64–88.

Spivak M, Gilliam M (1998) Hygienic behaviour of honey bees and its application for control of brood diseases and varroa Part I. Hygienic behaviour and resistance to American foulbrood. *Bee World*, **79**, 124–134.

Spivak M, Reuter GS (2001) Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. *Apidologie*, **32**, 555–565.

Stamatakis A (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*, **22**, 2688–2690.

Warnecke F, Luginbuhl P, Ivanova N *et al.* (2007) Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature*, **450**, 560–U517.

Weinstock GM, Robinson GE, Gibbs RA *et al.* (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature*, **443**, 931–949.

Werren JH, Windsor DM (2000) *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? *Proceedings of the Royal Society of London Series B-Biological Sciences*, **267**, 1277–1285.

Wilson EO (1971) *The Insect Societies*, The Belknap Press of Harvard University Press, Cambridge, MA.

This study is part of V.G.M.'s doctoral research, which explores the ecological, evolutionary, and functional relationships between *Apis mellifera* and its bacterial microbiota. N.A.M. and V.G.M. are broadly interested in the complex interactions involving symbiotic microorganisms and their hosts and the diverse functions that these microbes can perform.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Proportional representation of bacterial 16S rRNA sequences from the individual sample (271 sequences) and hive sample (267 sequences) of adult *A. mellifera*. In each case, >98% of recovered sequences belong to one of the eight characteristic associates. Colour key is the same as in Fig. 1.

Fig. S2 Diagnostic screen based on PCR amplification with diagnostic primers for seven of the bacteria commonly associated with *A. mellifera*. Positives are only observed within corbiculate bees.

Fig. S3 Phylogenetic relationships of each of the bacterial species characteristic of *A. mellifera* (RAxML with 100 bootstrap replicates). Numbers on branches represent bootstrap support. Trees represent A. Alpha-1, B. Alpha-2, C. Beta, D. Bifido, E. Firm, and F. Gamma symbionts.

Table S1 Sample collection information

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