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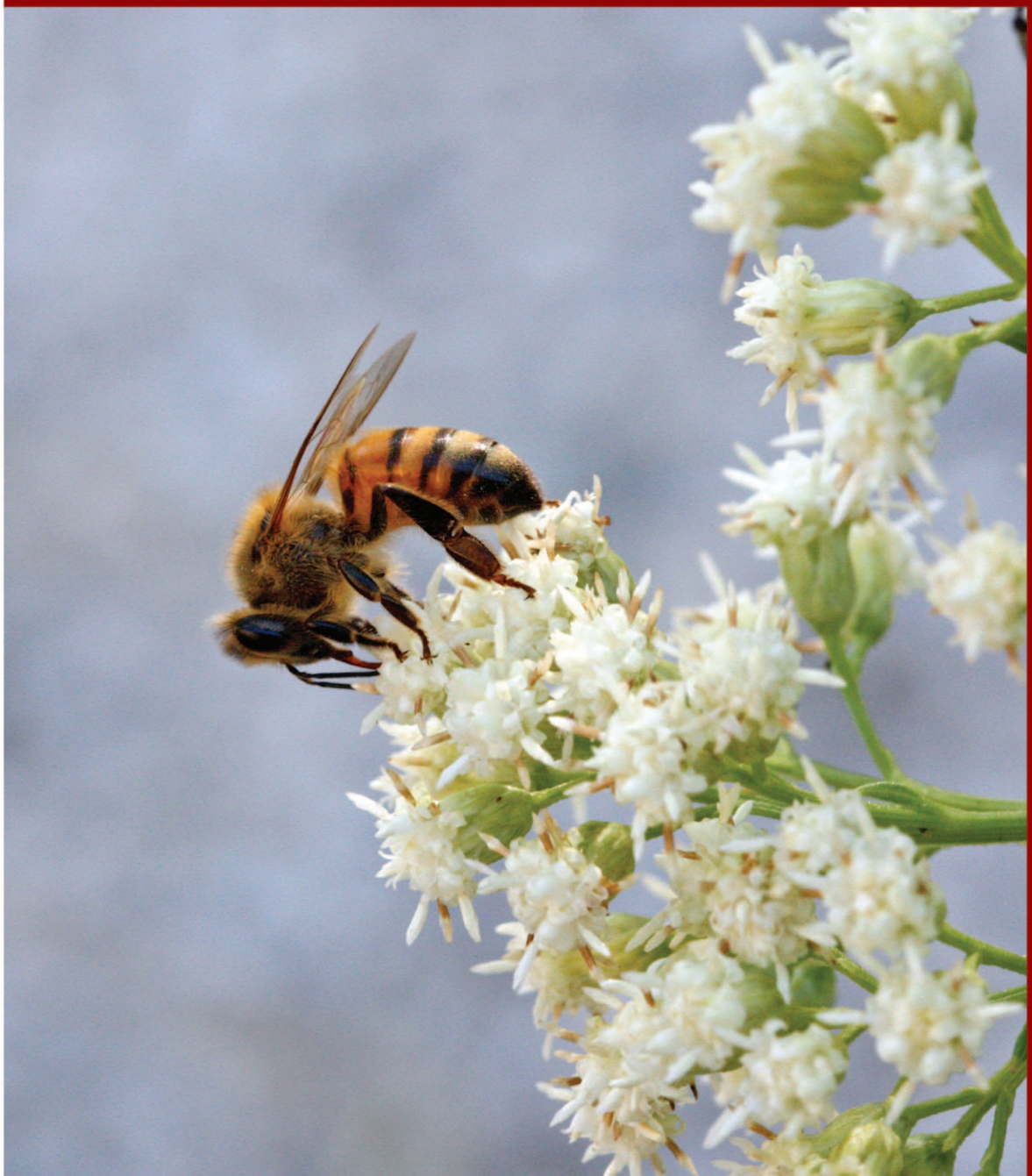

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Establishment of Characteristic Gut Bacteria during Development of the Honeybee Worker

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Previous surveys have shown that adult honeybee (*Apis mellifera*) workers harbor a characteristic gut microbiota that may play a significant role in bee health. For three major phylotypes within this microbiota, we have characterized distributions and abundances across the life cycle and among gut organs. These distinctive phylotypes, called Beta, Firm-5, and Gamma-1 (BFG), were assayed using quantitative PCR, fluorescent *in situ* hybridization (FISH) microscopy, and the experimental manipulation of inoculation routes within developing bees. Adult workers (9 to 30 days posteclosion) contained a large BFG microbiota with a characteristic distribution among gut organs. The crop and midgut were nearly devoid of these phylotypes, while the ileum and rectum together contained more than 95% of the total BFG microbiota. The ileum contained a stratified community in which the Beta and Gamma-1 phylotypes dominated, filling the longitudinal folds of this organ. Deep sequencing of 16S rRNA genes showed clear differences among communities in midgut, ileum, and rectum. In contrast with older workers, larvae and newly emerged workers contain few or no bacteria, and their major food source, bee bread, lacks most characteristic phylotypes. In experiments aimed at determining the route of inoculation, newly emerged workers (NEWs) sometimes acquired the typical phylotypes through contact with older workers, contact with the hive, and emergence from the brood cell; however, transmission was patchy in these assays. Our results outline a colonization pattern for the characteristic phylotypes through *A. mellifera* ontogeny. We propose the names "*Candidatus* Snodgrassella alvi" and "*Candidatus* Gilliamella apicola" for the Beta and Gamma-1 phylotypes, respectively.

Animal health is greatly influenced by the microbial community within the digestive tract (12, 32). Whereas gut pathogens can negatively influence health, commensal microorganisms can prime immune responses, confer resistance to invading pathogens, and augment nutrition (43). Both vertebrates and invertebrates have consistent nonpathogenic associations with a microbial gut community (33, 38), including defined mutualistic relationships (e.g., with termites [22], broad-headed bugs [28], and plataspid stinkbugs [24]). Gut communities change with host environment, diet, and age and among gut compartments (3, 23, 41). Most studies of gut microbiota reflect a single sample and thus yield no information on temporal and spatial dynamics.

The social insect *Apis mellifera* (honeybee) is the most important pollinator globally, and the health of *A. mellifera* colonies has been a major concern following colony losses in the last decade (7, 29). In *A. mellifera*, adult workers harbor a characteristic gut microbiota consisting of nine distinct bacterial phylotypes, which account for >95% of their total bacterial microbiota and which have been observed repeatedly using several non-culture-based methods on samples representing different environments, continents, and host genotypes (2, 7, 25, 34, 36, 40). The repeated observation of the same distinctive bacterial phylotypes in non-culture-based studies implies that most members of this gut microbiota are maintained by transmission between individuals within a hive and not by selective acquisition from the extrahive environment (25, 34). Additionally, these observations suggest a symbiotic relationship that may be critical to bee health (34, 40). To date, no studies have addressed which organs of the gut these bacteria colonize or how the gut microbiota changes during *A. mellifera* ontogeny.

The *A. mellifera* gut bacteria encounter a physically and nutritionally variable environment due to the complex development and social behavior of this insect. Furthermore, the adult gut is divided into four major organs (crop, midgut, ileum, and rectum), providing different functions in the catabolism and absorption of food and different environments for bacterial symbionts (5, 51). Adult workers perform a succession of tasks as they age, which may expose them to different microorganisms: young bees nurse larvae within the hive, whereas older bees forage pollen and nectar from flowers outside the hive (1, 17, 49). In contrast to adults, larval *A. mellifera* have a discontinuous gut in which the foregut (crop and midgut) is not connected to the hindgut (ileum and rectum) until just before pupation, when they excrete dietary waste for the first time (51). Larvae reside within a single brood cell where nurse workers feed them a highly nutritional glandular secretion with small amounts of pollen and honey (62).

In this study, we use culture-independent methods to enumerate and visualize the microbiota of different gut organs and of bees of different ages. We focus on three abundant phylotypes within the *A. mellifera* gut microbiota.

MATERIALS AND METHODS

qPCR to estimate bacterial abundance. Workers were collected from two colonies at the USDA Carl Hayden Bee Research Center (USDA-CHBRC)

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TABLE 1 Quantitative PCR primers and FISH probes for the Beta, Firm-5, and Gamma-1 phylotypes[a]

Primer/probe and target	Primer/probe name	Primer/probe sequence	qPCR product/probe length (bp)	FISH probe fluorophore (5′ attachment)
qPCR primers				
Beta	Beta-1009-qtF	CTTAGAGATAGGAGAGTG	128	
	Beta-1115-qtR	TAATGATGGCAACTAATGACAA		
Firm-5	Firm5-81-qtF	GGAATACTTCGGTAGGAA	114	
	Firm5-183-qtR	CTTATTTGGTATTAGCACC		
Gamma-1	Gamma1-459-qtF	GTATCTAATAGGTGCATCAATT	210	
	Gamma1-648-qtR	TCCTCTACAATACTCTAGTT		
FISH probes				
Beta	beta-572	TTAACCGTCTGCGCTCGCTT	20	Cy5
Firm-5	fir5-575	TCCCGCCTGCGTTCG	15	Cy3
Gamma-1	gam1-1246	CGAGGTCGCCTCCCTTTGTA	20	Texas Red-X
Universal bacteria	eub339	TGCTGCCTCCCGTAGGAG	18	Alexa Fluor 488

[a] The following touchdown reaction protocol was used for all reactions: 95°C for 10 min; 40 cycles of 95°C for 5 s, variable annealing temperature for 15 s, and 72°C for 5 s. The annealing temperature was 68°C for the first three cycles and was then lowered by 1°C for each cycle until it reached 55°C, with remaining cycles at 55°C.

in Tucson, AZ, in November 2009. To obtain known-age adults, a frame that contained a capped brood was cleared of adult bees, caged, and held at 34°C for 24 h in a dark, humidified incubator (mimicking hive conditions). For each colony, five newly emerged workers (NEWs) were collected (day 1 bees), and 50 NEWs were marked with Testors enamel paint (Testor Corp., Rockford, IL), returned to their colony, and allowed to mature naturally. Subsequently, five marked bees were collected at days 9 and 19 and three at day 30. Samples were stored in 75% ethanol at −20°C. For DNA extraction, gut organs (i.e., crop, midgut, ileum, and rectum) were dissected and separated with sterile forceps and dissection scissors. DNA was extracted and quantified as in Martinson et al. (34) and diluted to ~75 ng/μl to standardize PCR and quantitative PCRs (qPCRs). To verify that DNA was of sufficient quality for PCR, a control PCR was performed for an ~600-bp fragment of the *A. mellifera* elongation factor 1-alpha gene (ef1α) with primers efs599 and efa923 (56).

Primer pairs that amplified 100- to 250-bp products from the 16S rRNA gene were designed for each of the three most consistently observed and most abundant bacterial phylotypes found in the *A. mellifera* microbiota (Table 1). These specific phylotypes (or species) are referred to as Beta, Firm-5, and Gamma-1 as in previous studies (7, 34), and we use BFG phylotypes for the set of these three phylotypes. Standard curves were created and reactions were run as described in Oliver et al. (39) on a LightCycler (Roche Applied Science, Indianapolis, IN).

The qPCR results were expressed as the total number of bacterial 16S rRNA gene copies per sample by multiplying by the total DNA amount in each sample, and values were normalized with log transformation. To preserve a normal distribution, samples that were below the detection level of the qPCR curve were given a value of 1,000. Least-square-mean analysis was performed using JMP version 8 (SAS Institute Inc., Cary, NC) to identify effects that correlated with estimates of the BFG phylotypes. Variables (i.e., *A. mellifera* age, gut organ, and bacterial phylotype) and all interaction terms were added into the model at the start. After each run, the factor with the highest *P* value was removed until only factors with a *P* value of <0.05 remained. Significant differences within and between samples were determined by a Tukey's honestly significant difference (HSD) test. Data were back transformed for presentation. The Morisita index (a quantitative measure of beta diversity) was calculated for the community profiles of each gut organ and compared statistically using ANOSIM in PAST v2.06 (20).

Fluorescence *in situ* hybridization (FISH) microscopy. Two individuals were collected from each of four age groups within a hive at the USDA-CHBRC (Tucson, AZ, August 2010): uncapped fifth-instar larvae, NEWs, nurse bees, and foragers. Adult digestive tracts were dissected and placed onto a strip of filter paper to facilitate the positioning of the specimen. Tissue was fixed in 4% paraformaldehyde for 2 to 3 h (adult guts) or overnight (larvae) at room temperature, rinsed in 1× phosphate-buffered saline (PBS) buffer for 20 s, and held in 75% ethanol at 4°C until embedding. Samples were embedded into paraffin with vacuum infiltration using a Tissue-Tek VIP tissue processor (Sakura Finetek USA Inc., Torrance, CA), and 5-μm sections were cut with disposable blades and mounted onto Epic Plus slides (Epic Scientific, Tualitin, OR).

A specific and exclusively binding FISH probe was designed for each 16S rRNA gene of the Beta, Firm-5, and Gamma-1 phylotypes (Table 1). Sections were cleared of paraffin prior to probe hybridization by melting the wax in a 60°C incubation for 30 min, followed by two 5-min washes in xylene, a 7-min wash in 95% ethanol, a 7-min wash in 75% ethanol, and a 5-min rinse in double-distilled water (ddH_2O). The hybridization of probes was performed simultaneously by following the protocol presented in Daims et al. (11). Spectral imaging was used to view sections on a Zeiss 510 Meta confocal microscope. Autofluorescence was assayed for each tissue type by imaging sections as described above, without FISH probes. Observations were made on each gut organ for adult specimens and at two locations along the larval midgut.

Diagnostic PCR screen for bacteria in larvae. To determine the presence and identity of bacterial microbiota in larvae, DNA samples were screened with universal and specific primer sets. Specimens (third instar, fifth instar, and NEW) were collected from Beltsville, MD, at the USDA Bee Research Laboratory (USDA-BRL), from two healthy hives and one hive infected with European foulbrood (*Melissococcus plutonius*). Collections were made at the USDA-CHBRC in Arizona (5th instar and nurse adult) and at West Campus (Yale University, West Haven, CT) from healthy hives. DNA was subsequently extracted from surface-sterilized larvae and screened with eukaryotic ef1α primers to verify DNA quality using previously mentioned methods. Universal bacterial 16S rRNA gene primers 27f-short and 1507r (34) were used to screen for bacterial presence/absence. Specimens that produced an amplicon with the universal bacterial primers were further screened with seven primer pairs that each amplify specific phylotypes (Alpha-1, Alpha-2.1, Alpha-2.2, Beta, Firm-4, Firm-5, and Gamma-1) found in the *A. mellifera* microbiota (primer sequences and reaction conditions are listed in Martinson et al. [34]). All PCR screens were run with a positive (adult *A. mellifera* gut DNA) and a negative (ddH_2O) control on a 1% agarose gel (100 V, 50 min).

Assessing the source of the characteristic microbiota in NEWs. To determine the source of the microbiota in NEWs, two cage formats were used: frame cages (cages around hive frames containing brood and bee bread) and cup cages (caged worker bees with hand-collected bee bread). For cup cage analyses, NEWs that had not been exposed to the hive environment or older workers were collected by transferring late-stage pupae from sealed brood cells to a cage and incubated at 34°C with high humidity (to mimic hive conditions), and pupae were allowed to eclose natu-

TABLE 2 Statistical analysis of qPCR results[a]

Factor	Result including individuals from days:			
	1, 9, 19, and 30		9, 19, and 30	
	F	*P* value	*F*	*P* value
Colony	$F_{1,432}$	0.3724	$F_{1,312}$	0.5438
Age (A)	$F_{3,432}$	2.4×10^{-106}*	$F_{3,312}$	0.0361*
Gut Section (GS)	$F_{3,432}$	6.6×10^{-84}*	$F_{3,312}$	9.8×10^{-80}*
Bacterial Phylotype (BP)	$F_{2,432}$	0.0011*	$F_{2,312}$	0.2391
A−GS	$F_{9,432}$	1.6×10^{-33}*	$F_{9,312}$	0.2821
A−BP	$F_{6,432}$	0.074	$F_{6,312}$	0.2386
A−GS−BP	$F_{18,432}$	0.8135	$F_{18,312}$	0.992
GS−BP	$F_{6,432}$	3.7×10^{-5}*	$F_{6,312}$	2.4×10^{-5}*

[a] All factors were analyzed with the standard least-squares test. Asterisks indicate significant effects on bacterial abundances.

rally. Resulting NEWs were marked with paint. Six NEWs were placed into each of three cup cages (constructed as described in Evans et al. [13]). All cup cages were provided sterile 1:1 sucrose-H_2O solution (0.25-μm filter) and raw bee bread *ad libitum*. In both cage types, bees were allowed to survive until day 9 and were then collected into 95% ethanol. Individuals that died before day 9 were collected into 95% ethanol during daily inspections. DNA was extracted and screened with diagnostic primers by following described protocols (34, 55).

Bee bread was experimentally assessed as a source of the characteristic microbiota in a cup cage of laboratory-reared NEWs with no nurse workers and a diet of raw bee bread (see Table S1, cups 1.1 and 1.2, in the supplemental material). Additionally, bee bread was directly PCR screened for the characteristic phylotypes using DNA from two samples of bee bread combined from seven comb cells. Older workers were assessed as a source of the characteristic microbiota in a cup cage of laboratory-reared NEWs with three nurse workers (in-hive workers collected from the same hive as NEWs) and a diet of raw bee bread (see Table S1, cup 2). We assessed whether nurse gut homogenate, added to bee bread, could introduce the characteristic microbiota in a cup cage with laboratory-reared NEWs (see Table S1, cup 3).

To assess the ability of hive materials (i.e., comb and brood cell cap) to introduce the microbiota, pupae were allowed to emerge in the laboratory in frame cages lacking adult workers. Resulting NEWs were marked with paint and used in the frame cage tests (see Table S1 in the supplemental material). To assess the transfer of the characteristic microbiota from hive materials to NEWs, 20 marked NEWs were placed back into a frame cage (see Table S1, frame 1). To assess transfer from hive materials in combination with nurse workers, 20 NEWs and 20 older nurse workers (collected from within the same hive and marked with a different color) were placed into a frame cage (see Table S1, frame 2).

454 Pyrotag analysis of *A. mellifera* gut bacterial community. DNA from distinct gut organs of 3 day 9 workers and 1 day 30 worker, dissected and extracted for the qPCR analysis described above, were selected for 454 Pyrotag sequencing. An ~450-bp portion of the bacterial 16S rRNA gene was amplified from each sample using the universal primers 926f and 1492r. Each sample was given a barcode sequence (see Table S2 in the supplemental material), and reactions were run as described in Ochman et al. (38) with 454 FLX Titanium sequencing (Roche Applied Science, Indianapolis, IN). The finished sequence was analyzed with the default QIIME parameters (except for the alterations listed) (4). Raw 454 output was split into samples, the barcode and primer sequence were removed, and resulting sequences were filtered for quality and length (minimum quality score of 25, retained sequences between 460 to 600 bp). Sequences were deionized, 97% sequence-similarity operational taxonomic units (OTUs) were picked and aligned, nonbacterial OTUs unable to properly align were removed, and chimeric OTUs were identified. The manual inspection of the alignment identified more chimeras that were removed with the QIIME-identified chimeras. The resulting data set was processed in QIIME for OTU abundance and jackknifed beta diversity (with even taxon sampling). Alpha diversity was obtained in Mothur (46). OTUs were classified as one of the characteristic phylotypes or "other" with a Blastn search in GenBank. The Heatmap.2 program within the Gplots package for R was used to display OTUs with a frequency of >0.05% of one sample or a top Blastn hit to a characteristic *A. mellifera* phylotype in the GenBank nucleotide database.

Phylogenetic analysis for proposed "*Candidatus*" names. Representative sequences of the Beta and Gamma-1 phylotypes and closely related genera were aligned with Infernal in RDP (6). A maximum likelihood phylogeny was constructed for each with RAxML using the GTRGAMMA parameter and 100 bootstrap replicates (52).

RESULTS

qPCR results. The colony of origin had no significant effect on the numbers of bacteria (Table 2), so bees from different colonies were pooled for subsequent analyses. Worker age had a large effect on BFG abundance, mainly because NEWs collected on day 1 were nearly devoid of the BFG phylotypes, with fewer than 10^4 copies (Fig. 1a). Day 1 individuals had at least 3 orders of magnitude fewer 16S rRNA gene copies than older workers, which contained more than 10^6 copies ($P = 2.4 \times 10^{-106}$ by Tukey's HSD) (Fig. 1a and Table 2). Because day 1 individuals possess very few gut bacteria and therefore are not representative of mature honeybee workers, subsequent analyses were performed both with and with-

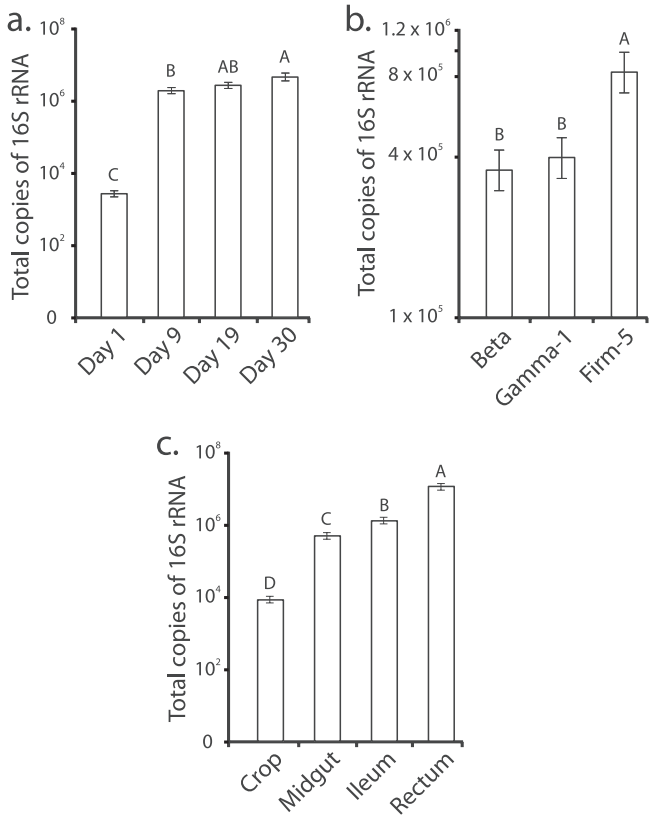

FIG 1 Abundances of the Beta, Firm-5, and Gamma-1 phylotypes (BFG) per adult worker, for different ages and gut organs, measured as copies of the 16S rRNA gene. (a) Total BFG abundance in workers at 1, 9, 19, and 30 days postemergence. (b) Average phylotype abundances in adult *A. mellifera* workers (days 1 to 30). (c) Average BFG abundances of the gut organs in adult *A. mellifera* workers (days 1 to 30). Letters above confidence intervals (1 standard deviation) represent significance levels (Tukey's HSD).

out day 1 individuals. After day 1, total BFG 16S rRNA gene copies continued to gradually increase with age, more than doubling between days 9 and 30.

Averaged across all ages, there were more 16S rRNA gene copies from the Firm-5 phylotype than from the Beta or Gamma-1 phylotypes ($P = 0.0011$ by Tukey's HSD) (Fig. 1b and Table 2). The Beta and Gamma-1 phylotypes represented 23 and 25% of the total BFG 16S rRNA gene copies, respectively, while the Firm-5 phylotype represented 52%. When individuals from day 1 were excluded, the average total copies of the 16S rRNA gene per bee increased 5- to 6-fold relative to the analysis including day 1 individuals, but relative proportions of the phylotypes were similar ($P = 0.2391$) (Table 2).

Numbers of total 16S rRNA gene copies varied greatly among the organs ($P = 6.6 \times 10^{-84}$ and 9.8×10^{-80} by Tukey's HSD) (Fig. 1c and Table 2). On average, of the total amount of BFG 16S rRNA gene copies within the entire digestive tract, the crop harbored 0.007 to 0.062%, the midgut harbored 1 to 4%, the ileum harbored 4 to 10%, and the rectum harbored 87 to 94%. Numbers of each phylotype were characteristic for a particular gut organ, regardless of host age or bacterial type (Fig. 2a and c).

Only day 1 samples failed to show a significant age-organ interaction ($P = 1.6 \times 10^{-33}$ by Tukey's HSD) (Fig. 2a and Table 2). All day 1 samples had very low abundance (Fig. 2a). Gut organs differed in relative abundances of the three phylotypes, and the organ-bacterial phylotype interaction was significant whether or not individuals from day 1 were excluded ($P = 3.7 \times 10^{-5}$ and 2.4×10^{-5} by Tukey's HSD) (Table 2). The total BFG amount varied by several orders of magnitude between organs (Fig. 2). In addition, the crop/rectum, midgut, and ileum had distinct community profiles according to ANOSIM ($P = <0.0001$; $R = 0.3575$) (Fig. 2b). The crop and rectum BFG communities were not significantly different (ANOSIM score of 0.3504) and consisted mostly of the Firm-5 phylotype (69 and 81%). The midgut was dominated by the Gamma-1 phylotype (47%), and the ileum was dominated by the Beta phylotype (42%) (Fig. 2b and c).

FISH microscopy. Autofluorescence was observed in the gut tissues but did not obscure the FISH probe imaging of bacteria within the guts of *A. mellifera* workers. Both fifth-instar larvae and NEW gut samples lacked signal from any of the FISH probes (and therefore are not included in Fig. 3), while nurse and forager guts produced a signal for each probe (Fig. 3). These results indicate that the guts of larvae and very young workers contain very few or no bacteria. In FISH surveys, NEWs, nurses, and foragers are expected to correspond approximately to day 1, 9, and 30 individuals based on known behaviors of worker bees of various ages (e.g., see Ament et al. [1]).

In contrast to larvae and young NEWs, FISH images revealed that nurses and foragers had substantial numbers of bacteria within their guts. A similar pattern of bacterial colonization was observed in both nurses and foragers (Fig. 3). The nurse/forager crop was nearly devoid of bacteria. The nurse/forager midgut had a small number of bacteria, including the Beta phylotype, distributed along its entire length, but the bacterial load increased toward the posterior end of the midgut, where the Gamma-1 phylotype became dominant.

The nurse/forager ileum has a bacterial profile strikingly different from that of the midgut. The large invaginations along the ileum's length are filled with bacterial cells (Fig. 3c and d). This mass of bacteria is mainly composed of the BFG phylotypes. Additionally, some cells only stained with the universal bacterial probe (eub339). The Gamma-1 and Beta phylotypes are the most numerous cells in the ileum. The Beta phylotype is often directly associated with the ileum's intima, while the Gamma-1 phylotype is found throughout the ileum's invaginations (Fig. 3f and g). The Firm-5 phylotype is not dominant, but clusters of Firm-5 can be seen.

The nurse/forager rectum can contain pollen cells that auto-

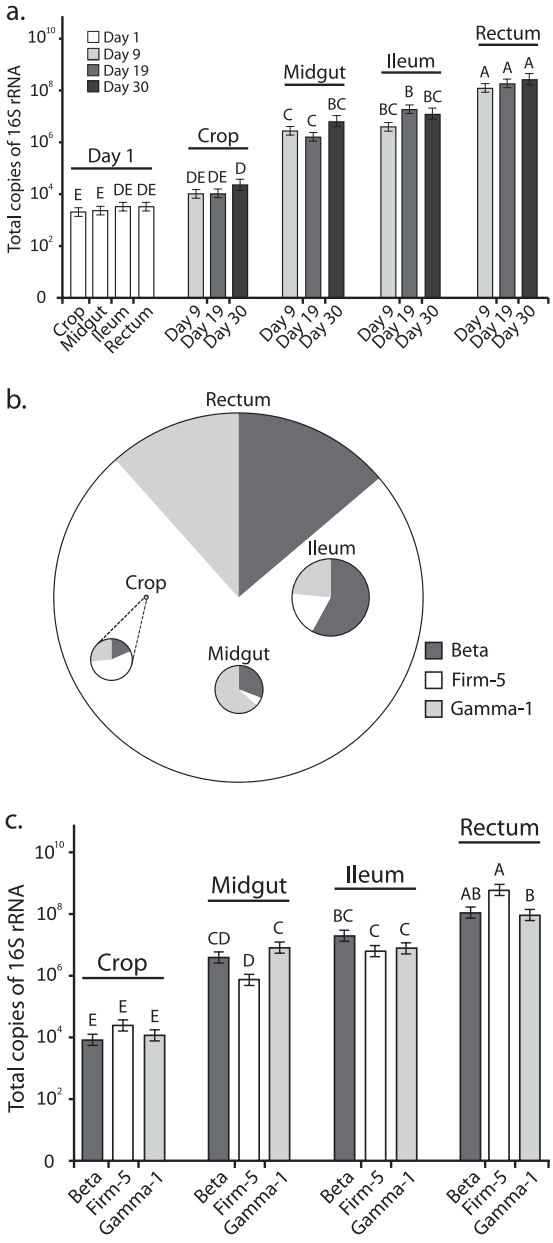

FIG 2 Comparison of the BFG community parsed by age, gut organ, and bacterial phylotype. (a) Numbers of 16S rRNA gene copies corresponding to BFG phylotypes in *A. mellifera* gut organs for worker adults of different ages. (b) Mean phylotype abundances relative to total BFG abundance for gut organs of adult workers, excluding day 1 workers. The circle's area is proportional to the organ's total BFG abundance, and the crop chart is expanded. (c) Abundances of phylotypes in each gut organ for adult workers, excluding day 1 workers. Letters above confidence intervals (1 standard deviation) represent significance levels (Tukey's HSD).

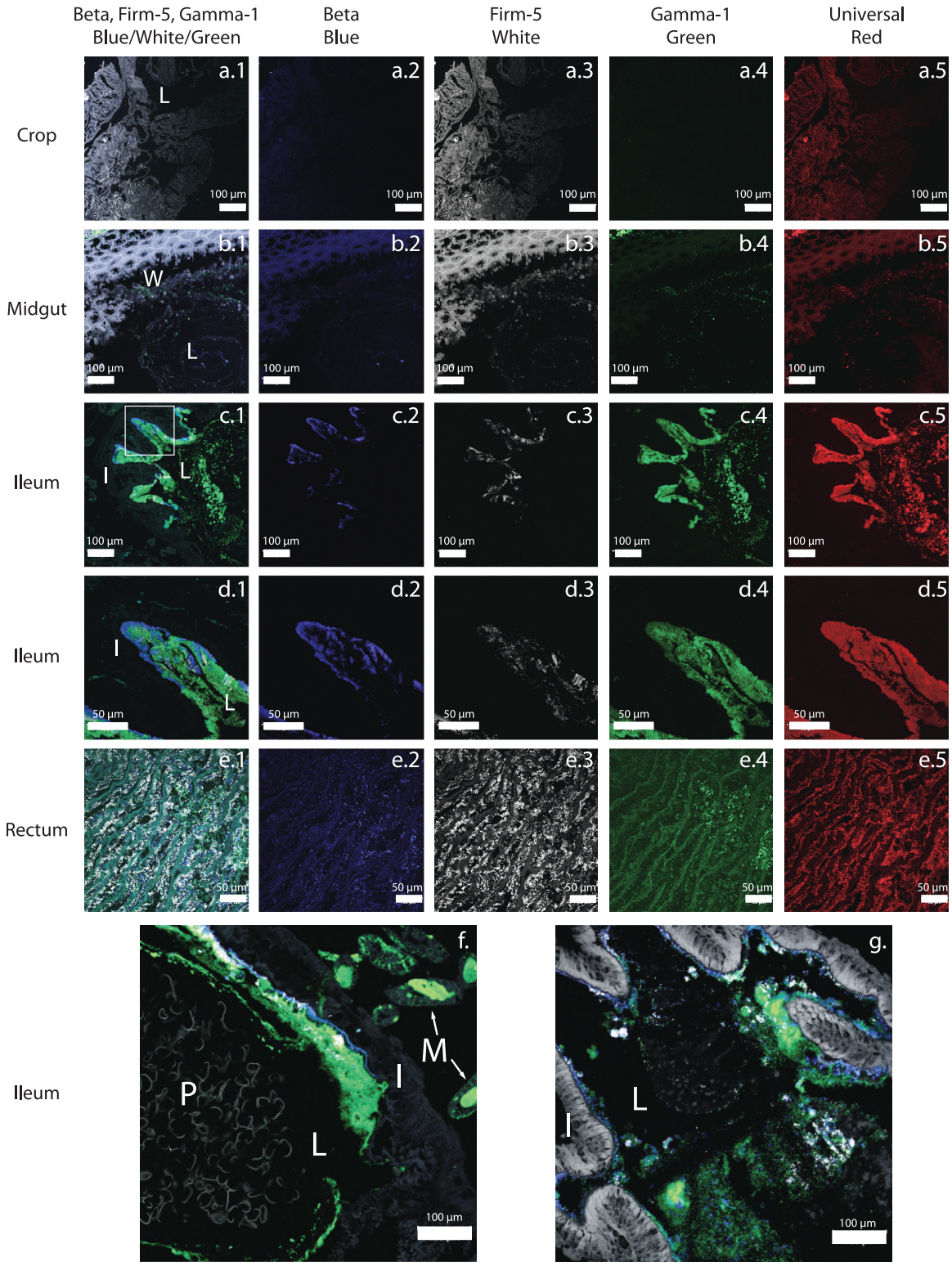

FIG 3 Localization of the Beta, Firm-5, and Gamma-1 phylotypes within the crop, midgut, ileum, and rectum of mature adult workers. Confocal microscopic images of phylotype-specific and universal bacterial FISH probes are shown, with the false coloration of specific BFG and universal bacterial probes as listed. Column 1 and frames f and g show composite images for the BFG FISH probes, columns 2 to 4 show hybridization for the individual BFG probes, and column 5 shows hybridization for the universal bacterial probe. Rows represent different gut organs; the boxed area in row c, column 1 is enlarged in row d to show the deep infoldings of the ileum filled with bacterial cells. Abbreviations: L, gut lumen; I, cuticular intima; W, midgut wall; P, partially digested pollen; and M, Malpighian tubules.

TABLE 3 Bacterial screen of larvae, NEWs, and adult nurse workers[a]

Colony (status) and life stage	Screening result (no. of bees positive/no. screened)							
	Universal Bacteria	Alpha-1	Alpha-2.1	Alpha-2.2	Beta	Firm-4	Firm-5	Gamma-1
Beltsville, MD 1 (EFB)								
3rd-instar larva	**1/5**	0/1	0/1	**1/1**	0/1	0/1	0/1	0/1
5th-instar larva	**5/5**	0/5	0/5	**5/5**	0/5	0/5	0/5	0/5
NEWs	**4/5**	0/4	0/4	**3/4**	0/4	0/4	0/4	0/4
Beltsville, MD 2 (healthy)								
3rd-instar larva	0/5	—	—	—	—	—	—	—
5th-instar larva	0/5	—	—	—	—	—	—	—
NEWs	0/5	—	—	—	—	—	—	—
Beltsville, MD 3 (healthy)								
3rd-instar larva	0/5	—	—	—	—	—	—	—
5th-instar larva	0/5	—	—	—	—	—	—	—
Tucson, AZ (healthy)								
5th-instar larva	**1/5**	0/1	0/1	**1/1**	0/1	0/1	0/1	0/1
Nurse adults	**5/5**	**2/5**	**5/5**	**2/5**	**5/5**	0/5	**5/5**	**5/5**
West Haven, CT (healthy)								
5th-instar larva	**5/5**	**1/5**	0/5	**5/5**	0/5	0/5	0/5	0/5

[a] Boldface numbers indicate positive reactions. EFB, European foulbrood. A dash indicates that reactions were not performed because the universal bacterial primer gave a negative result.

fluoresce to some degree under most wavelengths used to excite the FISH probes, making it difficult to observe the small bacterial cells. Nonetheless, the large bacterial population within the rectum greatly outnumbers the pollen and can be easily identified in images (Fig. 3e). Firm-5 is the most abundant phylotype in the rectum, while small amounts of Beta and Gamma-1 phylotypes are visible. Additionally, the rectum has many bacteria that were labeled only with the universal probe (eub339) and not with a specific probe, suggesting that much of the rectal community does not correspond to the Beta, Firm-5, or Gamma-1 phylotype. We note that the Firm-5 probe is specific and does not hybridize with the Firm-4 phylotype, another phylotype of *Lactobacillus* that may be abundant in the rectum.

Diagnostic PCR screen for bacteria in larvae. PCR screens for bacteria were performed on DNA samples from whole larvae, from healthy colonies from three locations, and from a colony exhibiting symptoms of infection with European foulbrood. All samples had the positive amplification of the *A. mellifera* ef1α gene, indicating the successful extraction of PCR-quality DNA. Only 6 of the 35 healthy individuals (3rd-instar larva, 5th-instar larva, or NEW) yielded a product with the universal bacterial 16S rRNA gene primers (Table 3).

In contrast, most (10/14) of the bees from a colony previously determined to be infected with European foulbrood yielded a band with the universal 16S rRNA gene primers. Of these, 3rd-instar larvae mostly lacked bacteria (1/5 positive reactions), 5th-instar larvae all contained bacteria (5/5), and most NEW individuals contained bacteria (4/5). Nearly all phylotype-specific PCR screens were negative; however, some individuals were positive for the Alpha-2.2 phylotype in both healthy colonies and in the European foulbrood colony (Table 3). Limited sequencing indicated that the positive universal 16S rRNA gene products in the European foulbrood colony represented multiple bacterial species, including the common secondary invader *Enterococcus faecalis* (15).

Assessing the sources of the characteristic microbiota in NEWs. All DNA samples had the positive amplification of the *A. mellifera* ef1α gene, indicating that the DNA was of good quality. For bees in cup cages provided only bee bread as a potential source of microbiota, several samples (3/12) yielded fungal amplicons, while bacteria were completely absent (0/12) (see Table S1, cups 1.1 and 1.2, in the supplemental material). Bee bread from the hive contained both bacteria and fungi (2/2 samples); however, the characteristic microbiota phylotypes were nearly absent (see Table S1). The only characteristic phylotype present was Alpha-2.2 (see Table S1).

All NEWs reared in cup cages with nurse workers were positive for bacteria and negative for fungi (see Table S1 in the supplemental material). Nurses were positive for nearly all phylotypes except Alpha-1. NEWs exposed to these nurses contained several of the characteristic phylotypes but were not as consistently colonized as the nurses (see Table S1). NEWs exposed to nurse gut homogenate were positive for many of the characteristic *A. mellifera* phylotypes (see Table S1). NEWs reared in a frame cage acquired several phylotypes but colonization was patchy among individuals, whether or not they were exposed to nurses (see Table S1).

Community composition of gut organs based on deep sequencing of 16S rRNA genes. Each sample had a minimum of 2,291 good sequences for data analysis (see Table S3 in the supplemental material). In addition to bacterial 16S rRNA genes, the 926f and 1492r primer set amplified the *A. mellifera* 18S rRNA gene, which represented a large proportion of the ileum and midgut samples before they were removed for analyses (see Table S4). Blastn identified multiple 97%-sequence-similarity OTUs for each phylotype, but manual chimera checking reduced that to 1 to 2 OTUs (see Table S4). Each organ sample was dominated by a subset of 4 abundant OTUs, which correspond to the Beta, Gamma-1, Gamma-2, and Firm-5 phylotypes (Fig. 4). Alpha diversity metrics for each sample are listed in Table S3. Using the weighted-UniFrac metric, principal component analysis (PCA) and un-

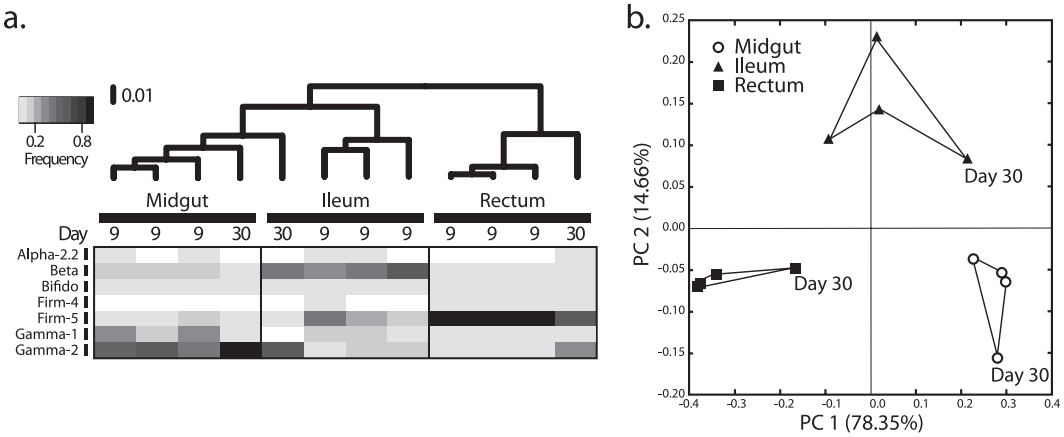

FIG 4 Bacterial community profiles in midgut, ileum, and rectum samples from four individual *A. mellifera* workers (3 day 9 workers and 1 day 30 worker) characterized using abundances of 16S rRNA gene sequences in 454 Pyrotag data. (a) Phylotype frequencies. The dendrogram shows UPGMA clustering of bacterial communities based on the weighted-UniFrac metric; all nodes have >90% bootstrap support. Each phylotype was assigned sequence clusters having top Blastn hits to members of that phylotype in GenBank. No clusters comprising >0.05% of any sample had a top Blastn hit different from that of the characteristic *A. mellifera* phylotypes (Alpha-2.2, Beta, Bifido, Firm-4, Firm-5, Gamma-1, and Gamma-2), and these low-frequency clusters are not shown. (b) Principal components analysis of the *A. mellifera* gut organ bacterial communities (day 30 worker samples are labeled). Pyrotag data were analyzed in QIIME (4) using the weighted-UniFrac metric.

weighted pair-group method using average linkages (UPGMA) analyses separated the organ's communities into distinct clusters. Day 30 samples were more loosely clustered with the day 9 samples, falling outside the day 9 clades on the UPGMA tree, and were more distant from day 9 samples on the PCA plots (Fig. 4). Pyrotag data sets are available through the NCBI Sequence Read Archive (accession number SRP008053).

"*Candidatus* Snodgrassella alvi" and "*Candidatus* Gilliamella apicola." We propose the following candidate names for two of the organisms that were the focus of our study.

"*Candidatus* Snodgrassella alvi," new lineage. The phylogenetic analysis of 16S rRNA gene sequences indicates that the Beta phylotype represents a unique clade of *Neisseriaceae* that is related to the genera *Simonsiella* and *Alysiella* (Fig. 5a) (34). The corresponding 16S rRNA gene sequences have been found only within the guts of several *Apis* and *Bombus* species and have >5% sequence divergence from *Alysiella*, *Simonsiella*, and other *Neisseriaceae* genera (30, 34). Distinguishing attributes include the existence within the alimentary canal of corbiculate bees and the unique 16S rRNA gene sequence TTAACCGTCT GCGCTCGCTT (positions 572 to 592 according to *Escherichia coli* scheme); GenBank accession number AY370189 is a representative sequence. The lineage is named in reference to the entomologist Robert E. Snodgrass (1875 to 1962), who made important contributions to insect anatomy, morphology, evolution, and *A. mellifera* biology with his 1910 publication of *The Anatomy of the Honey Bee* (51). The epithet, alvi, is derived from the Latin word *alvus*, meaning beehive or digestive organs, and refers to the presence of "*Ca.* Snodgrassella" in the bee gut.

"*Candidatus* Gilliamella apicola," new lineage. The phylogenetic analysis of 16S rRNA gene sequences indicates that the Gamma-1 phylotype resides in a clade closely related to *Pasteurellaceae* and *Enterobacteriaceae* but distinct from either (Fig. 5b) (34). Sequences representing the Gamma-1 phylotype have been exclusively identified within the guts of several *Apis* and *Bombus* species and have ~6% sequence divergence from *Orbus hercynius* and >10% sequence divergence from members of the *Pasteurellaceae* or *Enterobacteriaceae* (30, 34). Distinguishing attributes include existence within the alimentary canal of corbiculate bees and the unique 16S rRNA gene sequence CGAGGTCGCCTCCCTT TGTA (positions 1246 to 1266 in the *E. coli* scheme); GenBank accession number AY370191 is a representative sequence. The lineage is named in reference to the entomologist Martha Gilliam, who pioneered the study of the microbial associates of *A. mellifera* during 3 decades of research. The epithet, apicola, is derived from the Latin word *apis*, meaning bee, and the Latin suffix *cola*, meaning inhabitant of, and it refers to the presence of "*Ca.* Gilliamella" in the bee gut.

DISCUSSION

From our results, we conclude that *A. mellifera* has a consistent pattern of colonization by the Beta, Firm-5, and Gamma-1 phylotypes that is influenced by host age and gut morphology. The abundance and community structure of the microbiota changes through the *A. mellifera* life cycle, and it varies among the organs of the adult gut. Bacteria were absent and potentially actively excluded from larvae and newly emerged workers. The microbiota is established in the adult worker gut after brood cell emergence through contact with the hive and trophallaxis between nestmates; the characteristic phylotypes are maintained throughout the worker's life, spanning diverse tasks and dietary regimens.

As observed in previous surveys (2, 7, 25, 34, 36, 37, 40, 57), all naturally reared adult *A. mellifera* workers harbored the characteristic bacterial phylotypes within their guts (Fig. 1 to 4; also see Table S1 in the supplemental material). The deep sampling provided by the Pyrotag data reaffirmed that the BFG phylotypes are consistent and dominant members of the microbiota (7, 34), accounting for ~73% of the total 16S rRNA gene sequences recovered (see Table S4 in the supplemental material). The Gamma-2 phylotype was also a major constituent, representing ~23% of the sequences. The rarer members of the gut microbiota, such as the *Bifidobacterium* phylotype, are consistently present (Fig. 4) and may play important functional roles. The proportions of individual phylotypes are somewhat different from those observed in

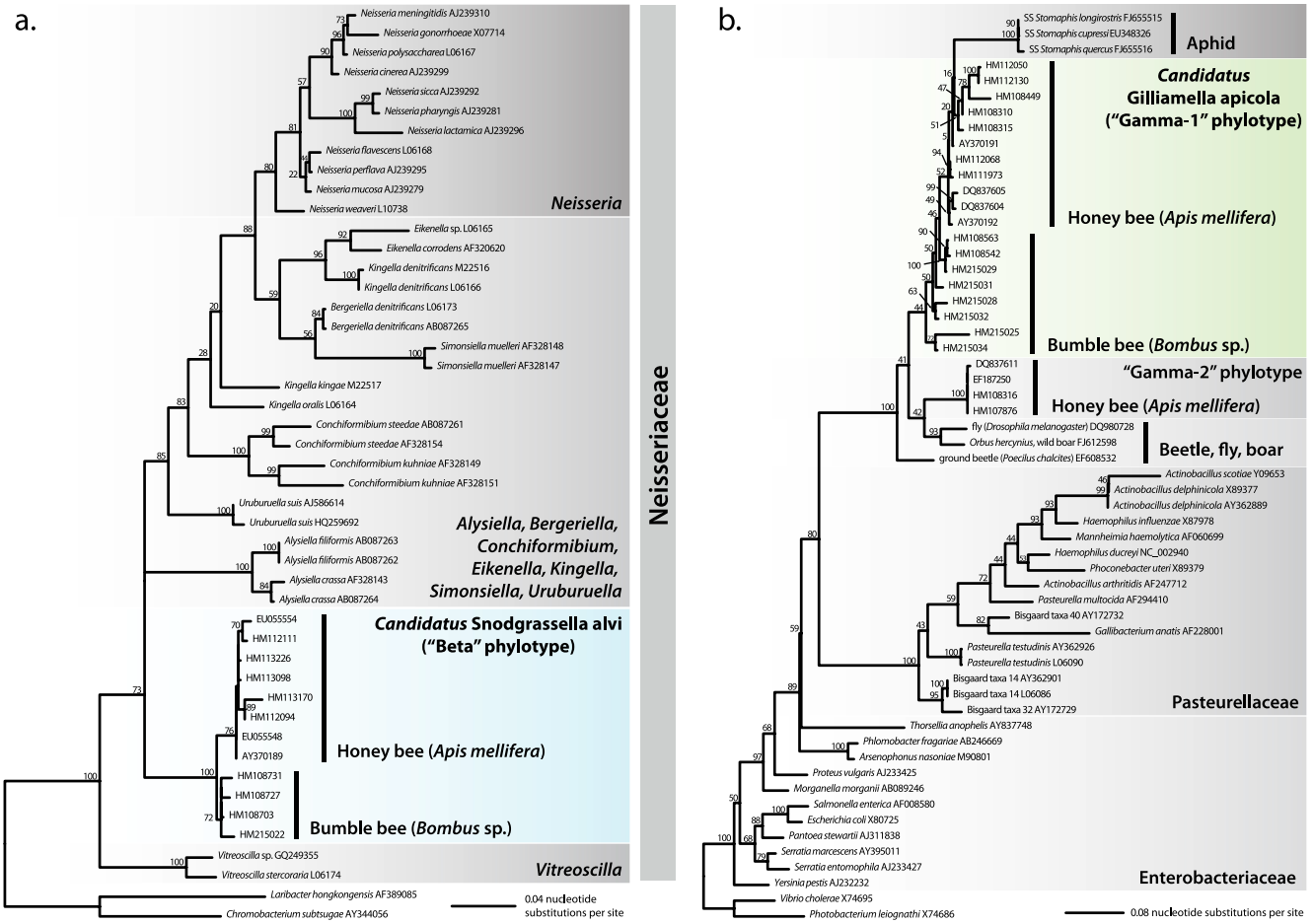

FIG 5 Phylogenetic placement of (a) "*Candidatus* Snodgrassella alvi" (Beta phylotype) within the *Neisseriaceae* and (b) "*Candidatus* Gilliamella apicola" (Gamma-1 phylotype) within the *Gammaproteobacteria*. Numbers on branches represent bootstrap support (RAxML with 100 bootstrap replicates).

some other studies (7, 34), potentially reflecting differences in DNA extraction protocols or biological differences among colonies.

Adult workers. As adult workers age, their progression of tasks requires more time outside the hive and more exposure to different foods and sources of microorganisms (21). Overall, the adult microbiota is fairly constant as the individual worker transitions from feeding brood within the hive to foraging in the extrahive environment. Our results suggest that the microbiota of older workers (day 30) is larger (Fig. 1a) and shifts toward higher proportions of the Gamma-1 and Gamma-2 phylotypes (day 30 samples cluster outside the day 9 samples) (Fig. 4).

The crop is a muscle-lined organ of the gut that is capable of distending to accommodate nectar collected by foraging workers (44, 51). Even though the crop often contains nutrient-rich nectar that could be used as an energy source for microbes, it contains very few bacteria (Fig. 1c, 2, and 3a). The frequent filling and emptying of the crop as nectar is collected and transferred to the hive for honey production could perturb the microbial community and prevent bacterial colonization. Alternatively, the enzymes added during nectar processing in the crop (35, 62) could actively deter bacteria; these enzymes have been hypothesized to be responsible for antimicrobial properties of honey (59, 60).

Within the adult midgut, the principal site of digestion, epithelial cells secrete enzymes that digest food so that it can be absorbed through specialized midgut cells and the hindgut epithelium (26, 54). Unlike the rest of the gut, the midgut wall lacks a thin layer of cuticle called the intima (51). Instead, the midgut epithelium produces the peritrophic membrane, a loose film that aids in digestion, protects the epithelial cells from abrasive food particles (e.g., pollen exine), and acts as a barrier to pathogens (53, 63). This membrane is continually produced by the midgut epithelium and then shed as the meal passes, which inhibits microbial attachment (53). The presence of digestive enzymes and the peritrophic membrane could explain the relatively depauperate midgut (1 to 4% of the total BFG microbiota; Fig. 1 and 2), even though the midgut is the largest organ in the *A. mellifera* alimentary canal (10). Further, FISH microscopy showed that most midgut bacteria were located posterior, near the pylorus, which projects into the midgut (Fig. 3b and c). This suggests that the midgut microbiota is carried over from the ileum that was dissected with the midgut.

The *A. mellifera* ileum is a relatively small organ between the midgut and the rectum that has deep infoldings that provide surface area for the absorption of nutrients not collected in the midgut (45, 54). Despite the midgut being much larger than the ileum, the BFG population was nearly twice as large in the ileum (5 to 10% of total BFG) (Fig. 1c, 2, and 3c to f). In comparison to the midgut, the ileum has abundant attachment sites on its intima

infoldings and access to partially digested, unabsorbed nutrients. FISH images provide further evidence that attachment is important for bacterial colonization, particularly for the Beta and Gamma-1 phylotypes (Fig. 3f and g).

The ileum community appears as a stratified biofilm relative to the gut wall (Fig. 3d, columns 1 to 4, f, and g). The biofilm is structured with the Beta phylotype abutting the host tissue, the Gamma-1 phylotype distributed in a thick mat adjacent to the Beta and the ileum wall, and the Firm-5 phylotype present in small pockets along the ileum wall. The attachment of the Beta phylotype may enable colonization and attachment by later phylotypes, such as Gamma-1. The resulting bacterial mat could create microgradients (e.g., nutrient, oxygen, and pH) which could provide separate niches for the utilization of a variety of substrates, similarly to the termite paunch community (3).

The rectum, like the crop, distends to fit more contents; this occurs continually as workers retain digested waste until they take a defecation flight to dispose of it outside the hive (49). In this relatively static environment, akin to the termite paunch, the contents of the rectum (mainly empty pollen exines) could serve as a nutrient source for bacteria, since the carbohydrates found in the exine are recalcitrant to direct digestion by *A. mellifera* (42, 58). Consistently with the stable, nutrient-rich environment, the rectum harbored the majority of the microbiota, accounting for 87 to 94% of total BFG 16S rRNA genes per bee (Fig. 1c and d and 2). The Firm-5 phylotype dominated the rectal community and was ubiquitous throughout its lumen, being interspersed with the digested pollen husks. Overall, the rectum contained the majority of the 16S rRNA gene copies for the BFG phylotypes and also contained additional bacterial cells that hybridize only with the universal eubacterial probe and not with specific BFG probes. These non-BFG bacterial cells most likely represent the remaining phylotypes from the characteristic microbiota (i.e., Alpha-1, Alpha-2.1, Alpha-2.2, Bifido, Firm-4, and Gamma-2). FISH microscopy surveys of these other phylotypes would illuminate their distributions in the bee gut.

Larvae. The presence of a gut microbiota is nearly universal among animals (33), but our non-culture-based methods revealed that healthy *A. mellifera* larvae from colonies at three geographic locations had few or no bacteria in their guts (Table 3). The scarcity or absence of gut bacteria in *A. mellifera* larvae seems especially odd in light of the well-characterized gut community of nurse workers that orally feed larvae (2, 7, 17, 25, 34). *A. mellifera* larvae have a blind gut that prevents digested substrates from being voided until just before pupation (5, 51). The absence or scarcity of bacteria in *A. mellifera* larvae has been noted on the basis of culture-based methods (18, 19). However, other culture-based studies have suggested that larvae naturally have large amounts of the characteristic phylotypes within their guts (16, 36, 40). In these studies, larvae were not surface sterilized, and contamination from the brood cell could have transferred small amounts of these phylotypes to the surface of the larvae. Alternatively, most larvae screened were concurrently infected with the pathogen *Paenibacillus larvae* (40), potentially altering the natural dynamics of the larval microbiota and allowing microbial colonization, as we observed for colonies known to be infected with European foulbrood. Using culture-independent methods, Mohr and Tebbe (36) determined 16S rRNA gene profiles but not total numbers of bacteria in adult and larval *A. mellifera*. They found that gut community profiles of adults were consistent during 3 years of sampling (and consisted of the phylotypes we report here), but that larval profiles differed from adult profiles, varied within and between sampling years, and often lacked the characteristic microbiota phylotypes (36). The irregularity of bacterial presence suggests that the larval microbiota represents the bacterial community present in their food, bee bread. In the current survey, larvae that were positive for bacteria were nearly devoid of the characteristic phylotypes except Alpha-2.2 (Table 3). Alpha-2.2 was found in raw bee bread (Table 3) and was previously found to occur in diverse bee and wasp species (34). Potentially, variation in the bacterial community of bee bread could explain why the presence of bacteria is variable between larvae.

Attempts at determining the protective function of probiotic bacteria have focused on introducing *Lactobacillus* species (including the Firm-5 phylotype) to larvae and subsequently infecting these larvae with a pathogen (16). However, these experiments relied on strains of the Firm-5 phylotype originating from adult workers and thus do not address the natural presence of Firm-5 in larvae.

The scarcity of bacteria in larvae suggests a growth-suppressing antimicrobial agent or strong immune response produced by the larvae and/or delivered by nurse workers during trophallaxis (14). In many social insects, larvae are only fed adult-processed foods, which could be altered to inhibit microbial growth or enriched for a certain subset of nonpathogenic/probiotic microbes, thus insulating the young from opportunistic pathogens (8, 9). This may be especially important for *A. mellifera*, since many of its most destructive diseases attack brood (fungal, bacterial, arachnid, and protozoan) (47, 50). These pathogens often infect host tissues by passing through the midgut wall after being consumed (50, 63). Potentially, antimicrobial compounds in larval guts prevent the proliferation of the phylotypes characteristic of adult guts but fail to prevent the colonization of resistant brood pathogens.

Newly emerged workers. NEWs contain few or no bacteria as anticipated, since *A. mellifera* goes through a complete metamorphosis in which the gut intima is shed (28). In contrast to NEWs, day 9 adults have a fully developed microbiota that is not significantly different from that of older bees (Fig. 1a and 2a). Because day 9 individuals are fully colonized, the microbiota is acquired within the home colony before their first foraging flights. This system blurs the line between vertical and horizontal transmission; individuals probably do not obtain their microbiota from their mother but through direct or indirect contact with their sisters or the hive contents. Therefore, transfer occurs within the colony or superorganism, as in termites and leafcutter ants (22, 61). Thus, NEWs may obtain the characteristic microbiota through the consumption of comb-stored honey and bee bread, through the trophallaxis of nectar from older workers (48, 49, 62), or through contact with the comb. Phylotypes may differ in their usual routes of transfer. In our current survey, bee bread lacked nearly all the characteristic bacterial phylotypes and did not transfer the characteristic phylotypes to NEWs (see Table S1 in the supplemental material). Further, our assays showed that colonization by certain phylotypes can occur though contact with the comb or natural emergence from the brood cell and also through exposure to (and possibly trophallaxis by) older nestmates (see Table S1). The crop is often referred to as the "social stomach" of the colony because it distributes and receives food shared among nestmates. Although the crop contains few bacteria (Fig. 1a, 2, and

3a), a few transferred cells could seed a young bee's gut and replicate into a full microbiota (27). Normal trophallaxis could homogenize the microbiota profiles of individuals within the colony. Coprophagy, the eating of feces directly or indirectly through contact with hive surfaces, is another potential transmission route.

Potential roles of the bee gut microbiota. Studies in many animals are revealing that gut bacteria routinely perform a number of specific beneficial functions in their hosts. Bumblebees, which are close relatives of honeybees, also contain both phylotypes corresponding to the Beta and the Gamma-1 phylotypes within their guts (34, 36), and a recent study provided evidence that these gut bacteria provide adult bumblebees with protection against parasitic protozoans (31). This raises the possibility that the Beta or Gamma-1 phylotype of *A. mellifera* also functions in protection against disease organisms. These potential beneficial functions are critical, as *A. mellifera* is the most important pollinator in agricultural systems and thus is a significant link in the human food supply.

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