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ORIGINAL ARTICLE

Assembly of the *Caenorhabditis elegans* gut microbiota from diverse soil microbial environments

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It is now well accepted that the gut microbiota contributes to our health. However, what determines the microbiota composition is still unclear. Whereas it might be expected that the intestinal niche would be dominant in shaping the microbiota, studies in vertebrates have repeatedly demonstrated dominant effects of external factors such as host diet and environmental microbial diversity. Hypothesizing that genetic variation may interfere with discerning contributions of host factors, we turned to *Caenorhabditis elegans* as a new model, offering the ability to work with genetically homogenous populations. Deep sequencing of 16S rDNA was used to characterize the (previously unknown) worm gut microbiota as assembled from diverse produce-enriched soil environments under laboratory conditions. Comparisons of worm microbiotas with those in their soil environment revealed that worm microbiotas resembled each other even when assembled from different microbial environments, and enabled defining a shared core gut microbiota. Community analyses indicated that species assortment in the worm gut was non-random and that assembly rules differed from those in their soil habitat, pointing at the importance of competitive interactions between gut-residing taxa. The data presented fills a gap in *C. elegans* biology. Furthermore, our results demonstrate a dominant contribution of the host niche in shaping the gut microbiota.
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Introduction

Studies of host–microbiota interactions in diverse animals demonstrate the importance of the gut microbiota to host health (reviewed in Clemente *et al.*, 2012; Erkosar and Leulier, 2014). Microbiota members have been shown to provide developmental cues to their host, aid in resource utilization and enhance immune protection (Xu *et al.*, 2003; Bates *et al.*, 2006; Tokuda and Watanabe, 2007; Ivanov *et al.*, 2009; Atarashi *et al.*, 2011; Chung *et al.*, 2012). Disturbances or shifts in microbiota composition are associated with disease states, including opportunistic infections and obesity (Bartlett, 2002; Ley *et al.*, 2005; Turnbaugh *et al.*, 2006). Given its importance to the host, it seems likely that the core microbiota of healthy individuals should be primarily composed of coevolved beneficial microbes, rather than randomly assorted ones. While shared core microbiotas were described for bees, termites and the simple chordate, *Ciona intestinalis* (Vojvodic *et al.*, 2013; Cariveau *et al.*, 2014; Dishaw *et al.*, 2014; Otani *et al.*, 2014), work in *Drosophila*, and especially in humans, highlighted significant inter-individual and interpopulation variability, undermining the idea of a phylogenetically defined core gut microbiota (Hamady and Knight, 2009, Consortium HMP, 2012; Wong *et al.*, 2013).

Substantial work has been carried out to characterize the factors that shape the gut microbiota. Studies of the human gut microbiota have shown effects of geographical location, diet and host genetics (De Filippo *et al.*, 2010; Wu *et al.*, 2011; Yatsunenko *et al.*, 2012; Tims *et al.*, 2013; David *et al.*, 2014; Goodrich *et al.*, 2014). However, the relative contribution of such factors in shaping the gut microbiota remains unclear, probably because of the large interindividual variation. Studies using inbred strains of mice or *Drosophila* could provide better experimental control; nevertheless, different reports using either one of the two organisms differ, ascribing dominance to either diet or host genetics (McKnite *et al.*, 2012; Chaston *et al.*, 2014; Carmody *et al.*, 2015). Thus, much of the multifaceted relationship between the environment, the host and the gut microbiota remains to be elucidated.

The nematode *Caenorhabditis elegans* offers a convenient model to characterize the contributions of the environment and/or the host to microbiota

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composition: it is a bacterivore, directly sampling the environmental microbial community; it has a simple body plan with the intestine as the major body cavity open to the outside world; it can be made germ-free by bleaching eggs; diverse gut microbiotas can be established by exposing germ-free hatchlings to different microbial environments; and it is essentially self-fertilizing, providing genetically homogenous populations. Although *C. elegans* is a well-characterized model organism, surprisingly little is known about its natural history, especially its interactions with microbes (Petersen *et al.*, 2015). This is beginning to change, through the isolation of new natural variants with a wide geographical distribution, exploration of genetic and phenotypic variability in natural *C. elegans* populations, identification of natural pathogens and characterization of its interactions with non-pathogenic bacteria, including food bacteria or species associated with worms in their habitat (Avery and Shtonda, 2003; Sivasundar and Hey, 2005; Troemel *et al.*, 2008; Coolon *et al.*, 2009; Kiontke *et al.*, 2011; Felix *et al.*, 2013; Gusarov *et al.*, 2013; Hodgkin *et al.*, 2013; MacNeil *et al.*, 2013; Franz *et al.*, 2014; Choi *et al.*, 2016). However, to date, the *C. elegans* internal microbiota remains uncharacterized.

Although *C. elegans* has long been considered a soil nematode, it is rarely isolated in its reproductive stage from soil alone. Instead, it is strongly associated with decaying vegetal matter, found in orchard soils, compost heaps and fallen rotting fruit (Grewal, 1991; Kiontke *et al.*, 2011; Felix *et al.*, 2013). Emulating such natural habitats, we have previously established natural-like minienvironments in the lab, with *C. elegans* populations grown in vials containing local soils and rotting fruit, to isolate microbiota members and characterize their contributions to the worm (Montalvo-Katz *et al.*, 2013). In the current study, we use these minienvironments for a 16S rDNA-based metagenomic analysis examining the assembly of the *C. elegans* gut microbiota from diverse microbial environments. We take advantage of the availability of genetically homogenous worm populations to reduce noise and average out interindividual variation, and thus better discern shared features of the *C. elegans* microbiota. Comparisons of worm microbiotas with microbiotas in their soil habitats reveal that the assembly of the worm gut microbiota is essentially a deterministic process, such that similar worm microbiotas can be formed from different soil communities. Our results demonstrate a dominant contribution of the host to microbiota composition, and further suggest a role for negative interactions between microbiota members.

Materials and methods

Strains

C. elegans of the N2 wild-type strain were used in all experiments. *egl-26(ku228)* mutants were included in Experiment 6, initially to test for potential differences in microbiota composition compared with wild-type animals; however, with no difference detected, data from *egl-26* mutants was pooled together with that of wild-type animals (for more details see Supplementary Methods). Both strains were acquired from the *Caenorhabditis* Genetic Center.

Soils

Soils obtained from different sources differ in availability of organic matter and in microbial composition. However, none of the soils tested (those reported and others) was sufficient on its own to support *C. elegans* development, and larvae arrested as dauers, in agreement with dauers being the predominant form isolated from soil (Barriere and Felix, 2014). Therefore, soils were supplemented with produce (chopped), which allowed development to adulthood. Overripe to partially rotting produce was added to the soil in an approximate ratio (v: v) of 1:2 and left to decompose for 4 days to 2 weeks before use. Addition of produce increased microbial diversity: in Experiment 3 (see below), where this was evaluated by deep sequencing, addition of produce increased the number of identified soil genera from 80 to 588. Produce used included bananas (rich in fiber and simple sugars), potatoes (high starch and iron) and oranges, or strawberries (mostly simple sugars) (Supplementary Table S1). Enriched soils (5 g in a 25 ml glass vial) were cured of native nematodes by autoclaving, and original microbiotas restored by the addition of a microbial extract from the respective enriched soil batch 24 h before addition of worms. For more details see Supplementary Methods.

Worm growth and harvesting

Eggs were obtained from gravid worms by bleaching, and were allowed to hatch on nematode growth media plates without any food, arresting as L1 larvae. In a basic experiment, three independent populations (biological replicates) were established in beakers containing the same soil. Each was initiated with 200-400 synchronized germ-free L1 larvae, which were allowed to develop at 25 °C for 3 days (Figure 1a). In experiments examining effects of lower temperatures, worms were grown for prolonged durations—4 days at 20 °C or 5 days at 15 °C. Six independent experiments were performed overall (1, 3–7). Experiments 1, 6 and 7 were more elaborate composite experiments (further described in the relevant sections); Experiment 3 included three different soils, each with one biological replicate. One to two hundred adult gravid worms were harvested from each replicate using a Baermann funnel lined with tissue paper (Barriere and Felix, 2014), washed extensively, surface sterilized (1 h on nematode growth media plates containing 100 μg ml^{-1} gentamycin), washed once more, frozen

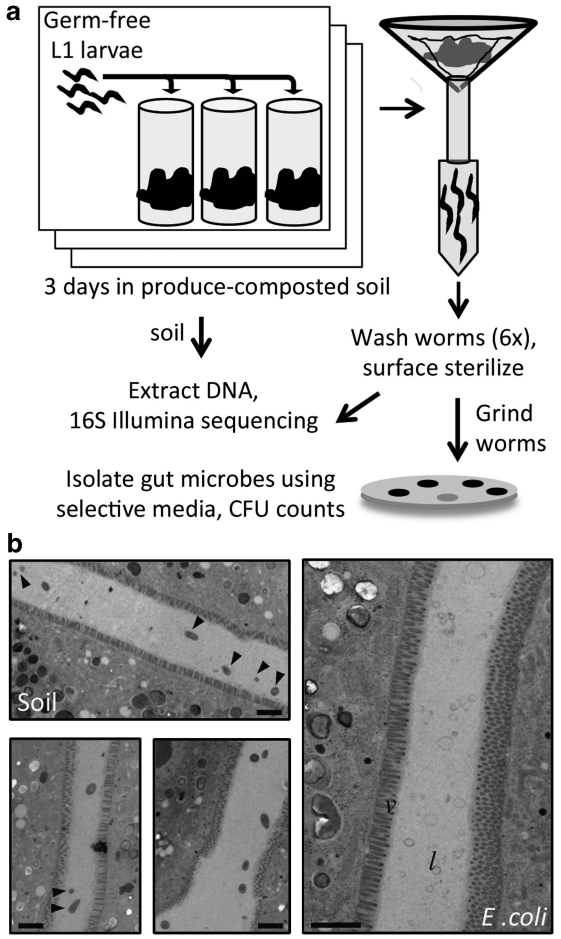

Figure 1 Characterization of the worm gut microbiota. (**a**) Analysis pipeline. (**b**) Representative electron micrographs showing longitudinal sections through the intestine of washed worms harvested from enriched soil (left, of 40 images in total from seven worms) or from *E. coli* plates (right, of 17 images in total from four worms). Note intact intestinal bacteria (arrowheads) in worms from soil, and lysed cells in worm from *E. coli*. l, lumen; v, villi. Scale bars = 2 μm.

and kept at −80 °C until use (Figure 1a). For more details see Supplementary Methods.

Colony-forming unit counts (see Supplementary Methods) and electron microscopy demonstrated efficiency of the washing procedure in removing all bacteria from the outside of worms (Supplementary Figure S1).

Electron microscopy

Young adult worms were prepared by high-pressure freezing and freeze substitution, as described previously, and imaged on a Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR, USA) (McDonald and Webb, 2011). For more details see Supplementary Methods.

Isolation and identification of culturable bacteria

Bacteria were isolated from worms grown in soils similar to those used for metagenomic analysis, and harvested as above. Washed and surface-sterilized worms were ground using a motorized pestle in 100 μl of M9, pelleted, and bacteria from supernatant grown on plates with *Enterobacteriaceae*-selective medium (Violet Red Bile Glucose Agar, 25 °C, 2 days). Isolates were identified by sequencing the full-length 16S rRNA gene, and amplified using primers 27f and 1492r; *Enterobacteriaceae* species of interest were further characterized through multilocus sequencing of hsp60, gyrB, rpoB and fusA (Supplementary Table S2).

DNA isolation

DNA was extracted from enriched soil (1 g per sample) or from worms (~100 worms per sample) using MO BIO PowerSoil DNA Isolation Kit (no. 12888; MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions, and resuspended in a volume of 50 μl. While DNA extraction from soil yielded up to 2 mg DNA, worms yielded as little as 50 ng.

Sequencing library preparation

Owing to the lower DNA concentrations from worm samples, nested PCR was used to obtain sufficient material for sequencing, first using primers 27f and 1492r to amplify the full-length 16S rRNA gene (94 °C for 45 s, 50 °C for 60 s, 68 °C for 90 s, 20 cycles), followed by amplification of the 16S V4 region using the 515f primer and barcoded versions of 806r (Supplementary Table S2) (94 °C for 45 s, 50 °C for 45 s, 68 °C for 45 s, 20 cycles) (Caporaso *et al*., 2012; Delgado *et al*., 2013; Tremblay *et al*., 2015). PCR reactions were carried out using 1 μl DNA template (containing 1 ng DNA at the minimum), 0.2 μM of each primer and the Invitrogen HiFi Platinum Taq Kit (no. 11304-011; Invitrogen, Carlsbad, CA, USA) in a total volume of 25 μl. V4 region PCR was carried out in triplicate, combined and gel purified using Qiagen Gel Extraction Kit (no. 28704; Qiagen Inc., Valencia, CA, USA). The library was prepared by combining equimolar ratios of each barcoded sample, and submitted for 150-bp, paired-end sequencing using Illumina HiSeq2500 (Illumina, San Diego, CA, USA) at the Coates Genomics Sequence Laboratory at UC Berkeley.

Metagenomic analysis

Sequence reads processing. Sequencing was performed on three separate occasions. Following each round, V4 16S rDNA reads were quality-filtered using QIIME (v.1.8.0) with default parameters (Caporaso *et al*., 2010). In total, 90% of reads passed quality filtering, with an average of 849 326 reads per sample (Supplementary Table S3). Filtered reads were clustered into operational taxonomic units (OTUs) at a 97% similarity cutoff using *uclust* (Edgar, 2010), and the taxonomy of each OTU was assigned based on similarity to reference sequences

in Greengenes release 13_5, as described previously (Yatsunenko *et al*., 2012).

Assessing microbial diversity. Before diversity calculations, communities were corrected for uneven sampling by rarefying all samples at 71 298 sequences. Beyond this value, analysis of additional sequences does not increase proportionally the number of identified OTUs (Gihring *et al*., 2012). Phylogenetic diversity metrics were calculated using the Greengenes reference tree. *Alpha diversity*: Faith's phylogenetic and Shannon's diversity indices were calculated to assess community diversity of both soil and worm gut microbiotas (Faith, 1994; Shannon, 1997). Shannon's diversity index is a composite measure of richness (number of OTUs present) and evenness (relative abundance of OTUs), whereas Faith's phylogenetic diversity does not take abundance into account. *Beta diversity*: Weighted and unweighted UniFrac distances were calculated from the OTU abundance table, and used in principal coordinates analysis with the R package *phyloseq* (Lozupone and Knight, 2005; McMurdie and Holmes, 2013). Both UniFrac distances incorporate the phylogenetic relationships between OTUs, but the weighted metric also takes relative abundance into account. Weighted distances were those used throughout the paper.

Microbiota clustering
Microbiotas were clustered using UPGMA (Unweighted Pair Group Method with Arithmetic Mean), an agglomerative hierarchical clustering method (Lozupone and Knight, 2005), using weighted UniFrac distances. To test cluster stability, Jackknife analysis (data set resampling, 100 permutations) was carried out using either 50% of the normally used sequences (~35 000 per sample), only one of the three repeats for each experiment, or two repeats of each experiment, or removing a whole experiment in random in each permutation. Each iteration was followed by recalculation of unweighted distances and UPGMA clustering.

Indicator species analysis
Species characteristic of soil or worm microbiotas were identified using the R package *indicspecies*, which assesses the strength of the relationship between species abundance and groups of sites by comparing species prevalence in microbiotas of one group to their prevalence in microbiotas of other groups (Caceres and Legendre, 2009). Taxa of interest were those with a statistically significant association with a particular subset of the microbiotas ($P<0.05$, Sidak corrected). Enrichment values were calculated for each indicator taxa, as a log-transformed ratio of the abundance in worms to the abundance in soil, and presented together with abundance values following hierarchical clustering (Eisen *et al*., 1998; de Hoon *et al*., 2004).

Cooccurrence analyses
Checkerboard scores (C-scores) were calculated using the R package *bipartite*, and compared with a distribution of C-scores generated from 5000 permutations of the same data set (Stone and Roberts, 1990; Dormann *et al*., 2008). The C-score is a measure of the proportion of OTU pairs that are mutually exclusive (indicating negative interactions), and allows testing rules of community assembly, with random species assortment as the null hypothesis. Additional comparisons of species interactions between worm and soil microbiotas were based on OTU pairs that demonstrated negative interactions, and were present in at least one soil and one worm microbiota. A negative interaction was defined as either OTU pair members that never cooccurred or a pair with negatively correlated prevalence, both identified using the Cytoscape plugin CoNet 1.0b6 ($P<0.05$, false discovery rate correction, 1000 permutations) (Faust *et al*., 2012). Interaction networks were constructed with Cytoscape 3.1.1 using both significant positive and negative interactions identified using CoNet (Shannon *et al*., 2003).

Statistical evaluation
UniFrac tests were used to compare specific microbiotas, and PERMANOVA, or *t*-test, for group comparisons. $N=10$ soil microbiotas (not including technical replicates), and 27 worm microbiotas for all tests. For presentation purposes (principal coordinates analysis), technical replicates were included.

Method validation
Before using our analysis pipeline to characterize the worm microbiota, we verified that methods employed allowed focusing on live gut bacteria, finding negligible or no contributions from contaminating bacteria or from partially digested food bacteria (Supplementary Figures S1 and S2). We additionally found no observable bias introduced by DNA amplification (Supplementary Figure S3). Full details are provided in Supplementary Methods.

Results

Characterization of the C. elegans gut microbiota
Initially germ-free L1 wild-type larvae were grown in natural-like minienvironments of soil and rotting produce (Figure 1a) (Montalvo-Katz *et al*., 2013). Under these conditions, worms developed to adulthood at a rate similar to worms grown on agar plates with *Escherichia coli*, but unlike worms on *E. coli*, those from soil showed intact gut bacteria, potentially part of the worm microbiota, that persisted through the 2-h long washing, avoiding digestion (Figure 1b).

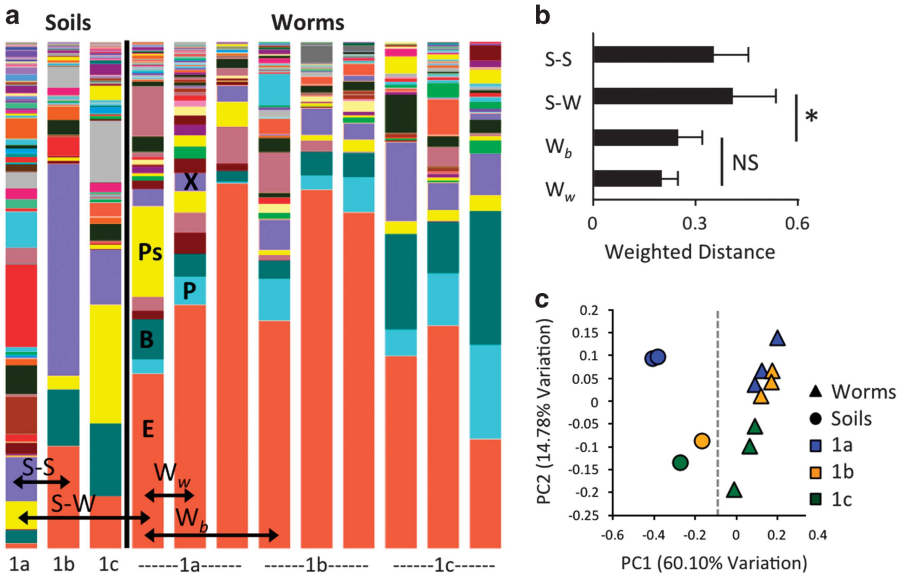

Figure 2 Worms assemble similar microbiotas from diverse soils. (**a**) Soil and worm microbiota composition. Bars, each representing a microbiota from a worm population (>100 worms) or from their respective soil environments (1 g), as labeled, showing relative abundance of taxa (family-level, color-labeled). Major groups are highlighted: *Enterobacteriaceae* (E), *Burkholderiaceae* (B), *Propionibacteriaceae* (P), *Xanthomonadaceae* (X) and *Pseudomonadaceae* (Ps). (**b**) Similarity between microbiotas. Weighted UniFrac distances between soil microbiotas (S–S, as demonstrated in panel a), between microbiotas of worms grown in the same soil (W_w), between microbiotas of worms grown in different soils (W_b), or between respective soil and worm microbiotas (S–W); shown are averages ± s.d.s for all possible pairwise comparisons; *$P = 0.007$ (Student's *t*-test with 1000 Monte Carlo simulations). (**c**) Principal coordinates analysis (PCoA) of worm and soil microbiotas (designated) using weighted UniFrac distances. Data for Soil 1a includes one technical replicate. Dashed line highlights the statistically significant separation between soil and worm microbiotas ($P = 0.002$, PERMANOVA with 1000 permutations). Axes represent the principal coordinates accounting for most of the observed variation.

The overall composition of the worm microbiota may be shaped through neutral assortment of species from the diverse microbial environment, or by a directional process leading to a gut microbiota that is distinct from the environment. An experiment aimed at discerning between these two modes was performed by dividing one batch of soil into three parts, and supplementing each with a different type of produce to foster divergent microbial communities. Three independent populations of wild-type worms were grown in each of these three soils. Deep sequencing of bacterial 16S rDNA was used to characterize bacterial composition of worm microbiotas (nine samples), as well as the microbiotas in their soil habitat (three samples). Around a million high-quality sequences originating from live gut bacteria (Supplementary Figure S2 and Supplementary Methods) were analyzed for each microbiota, clustered at a 97% sequence identity threshold and sorted to 4310 OTUs. Raw data can be downloaded from http://metagenomics.anl.gov/ (ref. no.: 13213).

Comparative analysis of worm and soil microbiotas

Comparison of soil and worm microbiotas showed that, while microbiota composition differed substantially between the three soils, microbiotas of their worm inhabitants did not resemble any of the soil microbiotas, and showed a significantly greater similarity among themselves. Similarity was seen not only within the three replicates grown in the same soil but also between worms grown in different soils (Figures 2a and b). Prominent members of worm gut microbiotas included members of five families: *Enterobacteriaceae* (relative abundance of 48.9 ± 17.4%), *Burkholderiaceae* (9.26 ± 8.17%), *Propionibacteriaceae* (6.7 ± 5.4%), *Xanthomonadaceae* (6.1 ± 4%) and *Pseudomonadaceae* (4.3 ± 5.2%) (Figure 2a). Shared abundance in worms was confirmed for members of the *Enterobacteriaceae* and *Pseudomonadaceae* families by quantitative PCR with taxa-specific primers (Supplementary Figure S4 and Supplementary Methods). Principal coordinates analysis showed a pronounced separation between soil and worm microbiotas along the primary principal coordinate, and a weak association between the composition of soil microbiotas and microbiotas of their worm inhabitants, as demonstrated by the distribution along the secondary principal coordinate (Figure 2c). These results supported the notion that worms can use different available environmental communities to reproducibly assemble similar microbiotas that are distinct from their environment, and suggests that assembly of the worm gut microbiota is a host-dependent and deterministic process.

To examine the reproducibility of worm microbiota assembly, we expanded on the initial experiment by performing five additional ones, each carried out with a different soil/produce combination, and together providing a greater diversity of soil

microbiotas. Three independent worm populations were grown in each soil, and worm microbiotas were compared with their respective soil microbiotas. The number of sequences analyzed for each of the microbiotas was similar to that from the first experiment; raw data can be downloaded as described above. Of the 4445 OTUs identified overall, 2656 were found in worms, representing 830 genera, 311 families and 26 phyla (Supplementary Table S4). Similar to the first experiment, average-weighted distances between independently formed worm microbiotas in each of the five additional experiments was significantly smaller than the average distance between worm microbiotas and their respective soil microbiota (Supplementary Figure S5). From the expanded space of microbial diversity defined by all examined soils, worm microbiotas occupied a significantly distinct, limited subspace (Figure 3a). In agreement with this, microbial diversity (α-diversity) was significantly reduced in worm microbiotas compared with that of soils; this was observed both with the phylogenetic diversity metric and with the Shannon index, and was prominent regardless of the depth of analysis (Figure 3b and Supplementary Figure S6).

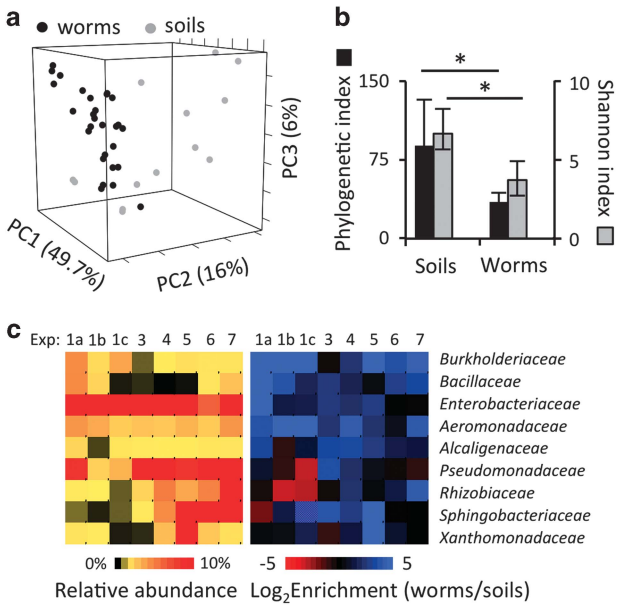

Figure 3 The worm gut core microbiota. (**a**) Principal coordinates analysis (PCoA) of soil and worm microbiotas, based on weighted UniFrac distances, expanding the diversity described in Figure 2c; soil and worm microbiotas are significantly distinct ($P=0.002$, PERMANOVA with 1000 permutations). (**b**) Worm microbiotas demonstrate a decrease in microbial diversity compared with soil. Two indices of α-diversity are shown, with averages ± s.d.s for 10 soil microbiotas and 27 worm microbiotas. *$P<0.01$ (Student's *t*-test). (**c**) The worm gut core microbiota. Heat maps present either relative abundance (left) or enrichment in worms compared with soil (right) of indicator genera that were pooled into the family level; enrichment of taxa not detected in the respective soil but present in worms is shown as patterned. Only the most abundant taxa (>0.1% in any microbiota) are shown; each value is an average of triplicate measurements (or six repeats in the case of Experiment 6) (see Supplementary Table S4 for full list of taxa, and Table S5 for core families abundance).

The C. elegans core microbiota

To identify taxa that were associated with worms, and distinguished worm microbiotas from those in soil, we used indicator species analysis (Caceres and Legendre, 2009). This identified members of nine bacterial families that were shared among all worm microbiotas. These were defined as the worm core gut microbiota, accounting for 35–85% of the sequences obtained from worms, and enriched up to 6.5-fold in worms compared with their soil environment (Figure 3c and Supplementary Table S5). Included in this group were members of families that were prominent in the first experiment, such as *Enterobacteriaceae* and *Pseudomonadaceae*, as well as members of families that were less prevalent, but more enriched (for example, *Burkholderiaceae* and *Bacillaceae*).

As partial 16S rDNA sequencing does not allow reliable species-level identification of OTUs, metagenomic analysis was complemented by isolation of gut microbiota members from similar soil-grown worms. Focusing on members of the most dominant core family, *Enterobacteriaceae*, 23 isolates were characterized using full-length 16S rRNA gene sequencing. Ten were identified as *E. coli* (resembling various environmental strains, but not lab strains), 1 was unequivocally identified as a *Serratia* or *Yersinia* species and the remaining 12 were identified as *Enterobacter* or *Pantoea* species. Multilocus sequencing identified 9 out of the 10 *Enterobacter* isolates tested as different strains of *Enterobacter cloacae.* While biases introduced by culturing may distort the real prevalence of *E. cloacae* in the worm gut, it can still be inferred that this species is a representative of the worm gut *Enterobacteriaceae*.

Worm microbiota types

Whereas the core microbiota defined a common denominator for all worm microbiotas, cluster analysis using weighted UniFrac distances further distinguished between two clusters of worm microbiotas, W_1 (including microbiotas from Experiments 1 and 3) and W_2 (including microbiotas from Experiments 4 to 7) (Figure 4a). The distinction between the two clusters remained intact even when only subsections of the data were used as the basis for clustering (see Materials and methods), attesting to independence of sampling biases. Furthermore, intracluster pairwise distances were significantly smaller than intercluster distances, supporting their separation (Figure 4b). Clustering using unweighted UniFrac distances recapitulated the distinction between the two clusters of worm microbiota, with the exception of Experiment 3, which switched cluster identity when clustering with unweighted

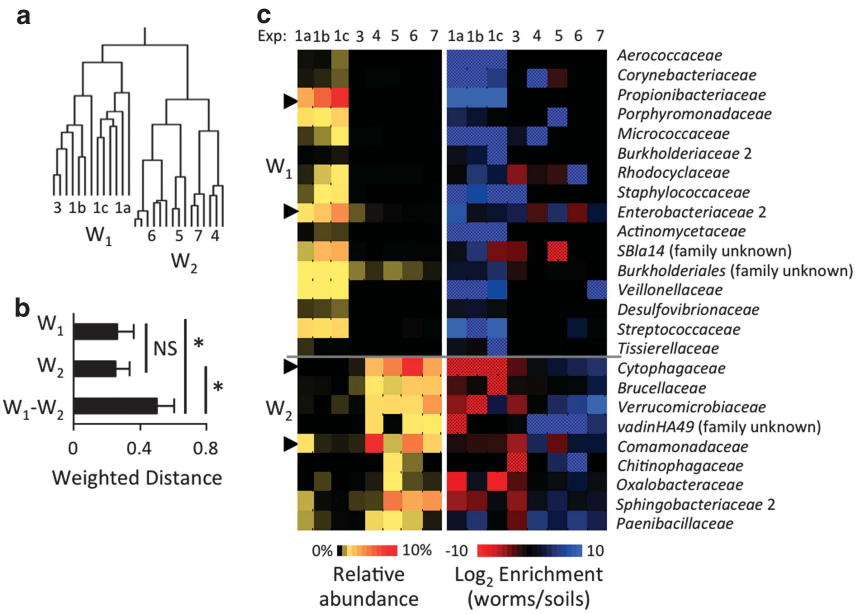

Figure 4 Worm microbiotas can be divided to two distinct types. (**a**) UPGMA clustering distinguishes between two worm microbiotas ($P=0.001$, PERMANOVA with 1000 permutations); Jackknife analysis (see Materials and methods) confirmed the stability of identified clusters. (**b**) Weighted distances within and between members of the two worm microbiota clusters (W_1, W_2); averages ± s.d.s for pairwise comparisons; $*P=0.001$ (Student's *t*-test with 1000 Monte Carlo simulations). (**c**) Heat map of abundance (left) and enrichment (right) of taxa distinguishing between microbiotas of the two worm clusters, with indicator genera pooled into the family level, and presented as described under Figure 2 (see Supplementary Table S5 for a full list of indicator genera). Enrichment of taxa not detected in soil but present in respective worm microbiotas is shown as patterned blue, and taxa not detected in worms but present in soil, as patterned red. Arrowheads mark prominent indicator families.

distances (Supplementary Figure S7). Overall, the identification of two stable clusters of worm microbiotas suggested that there were at least two distinct types of worm microbiotas.

To investigate further, indicator species analysis was used to identify taxa that were associated with either one of the two types. Taxa identified represented more than 10 families for each type (Supplementary Table S5). Among those, members of the *Propionibacteriaceae* family and of a subgroup of *Enterobacteriaeceae* were particularly prevalent in W_1 microbiotas, and members of *Cytophagaceae* and *Comamonadaceae* were prevalent in W_2 (Figure 4c, arrowheads). This suggested that the separation between the two worm clusters might be driven by a small number of auxiliary taxa. However, reclustering of worm microbiotas following removal of these four prominent taxa failed to dissolve the clustering structure. Indicator (type-specific) taxa were enriched in their respective worm microbiotas compared with soil, and in addition, indicator taxa of the W_2 type were depleted in W_1 microbiotas, suggesting negative effects exerted by W_1 taxa (Figure 4c). Furthermore, differences between the two clusters were not solely attributed to unique indicator species but also to differences in the prevalence of core taxa. *Enterobacteriaceae* members were significantly more abundant in W_1 microbiotas than in W_2 microbiotas, and *Pseudomonadaceae* and *Sphingobacteriaceae* members were significantly less abundant (Figure 3c and Table 1). These observations suggested that the distinction between the two worm clusters is attributed both to inclusion of indicator taxa and to changes in the prevalence of core taxa.

Table 1 Abundance of core microbiota members in worm enterotypes

Core family	W_1[a]	W_2[b]	P-*value*
Burkholderiaceae	7 ± 8[c]	0.6 ± 0.3	0.006
Bacillaceae	1.44 ± 2	0.6 ± 0.8	0.2
Enterobacteriaceae	55 ± 20	13 ± 8	<0.0001
Aeromonadaceae	2 ± 3	2 ± 3	0.84
Alcaligenaceae	0.4 ± 0.5	0.38 ± 0.3	0.85
Pseudomonadaceae	8 ± 9	17 ± 7	0.004
Rhizobiaceae	0.5 ± 0.5	5 ± 3	<0.0001
Sphingobacteriaceae	0.2 ± 0.3	17 ± 10	<0.0001
Xanthomonadaceae	4.5 ± 4	11 ± 11	0.06

Abbreviation: UPGMA, Unweighted Pair Group Method with Arithmetic Mean.
Worm enterotypes were defined using UPGMA.
[a]Average of 13 worm microbiotas.
[b]Average of 14 worm microbiotas.
[c]Relative abundance, averages ± s.d. (%).

Community analysis

Community ecology theory offers a framework to address questions related to the assembly and structure of communities (Koenig *et al.*, 2011; Costello *et al.*, 2012). While indicator species

analysis pointed at the preferred inclusion of particular taxa in worm microbiotas, additional tools can be used to assess community assembly rules. In such analyses, the null hypothesis assumes neutral species assortment. One tool is the C-score, which is the average number of instances of mutual exclusion in a set of communities. A C-score was calculated for worm microbiotas, and compared with a distribution of scores generated through random permutations from the same data set. This comparison rejected the null hypothesis, indicating that worm microbiotas are not neutrally assembled (Figure 5a). Analysis performed at the genus level lead to a similar conclusion (Supplementary Figure S8).

Cooccurrence analysis performed at the OTU and genus level in soil microbiotas also rejected neutral species assortment as the mode shaping soil microbiota structure (Supplementary Figure S8). Thus, it was possible that this non-neutral assortment in soils dictated the non-neutral species assortment demonstrated in worms. However, comparisons of species pairs with negative interactions (representing mutual exclusion) in worms and in soils showed negligible to non-existing overlap between interactions in soil and in worms (Figure 5b). This suggested that different rules affected microbiota assembly in soils and in worms. Corroborating this, interaction networks drawn for soil and worm microbiotas showed that negative interactions were far more prevalent in worms than in the soil, and suggested that competition between microbes may be a common feature in the worm's gut.

Effects of temperature on worm microbiota assembly

Our results demonstrate that assembly of the worm microbiota followed rules distinct from those operating in their habitat, and pointed to the host niche as an important contributor to shaping of its gut microbiota. However, our initial results suggested a weak effect of the environmental microbial diversity on microbiota composition (Figure 2c). To further examine this possibility, we followed the assembly of worm microbiotas from the same soil at different temperatures. We expected temperature to modulate environmental bacterial prevalence, which in turn may affect the composition of the worm microbiota. Temperatures at the range of 15–25 °C indeed modulated bacterial prevalence, both in soil and in worms (Figure 6a). This was apparent for certain bacterial genera (for example, *Sphingobacterium* sp.), but not for others (for example, *Pseudomonas* sp.). However, temperature-dependent changes that were observed in taxa in soil communities were not reflected in worms, indicating that different temperature-dependent processes operated in soil and in worms, further pointing at host-specific processes as the driver of changes in microbiota composition (Figure 6b).

Discussion

Our characterization of the worm gut microbiota revealed a diverse community, attesting to complex interactions between worms and their commensals.

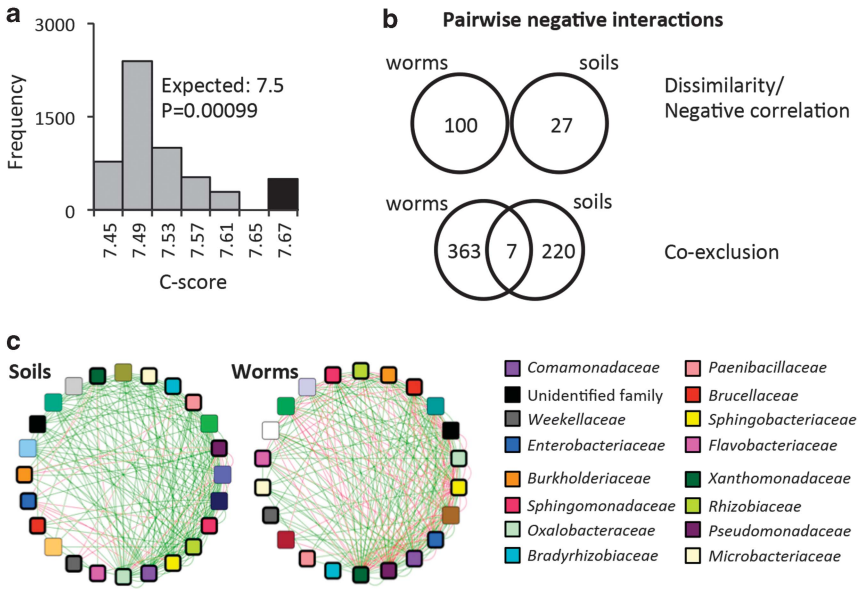

Figure 5 Worm microbiotas are assembled in a non-random fashion. (**a**) C-score for cooccurrence patterns observed among worm microbiota OTUs (black column), compared with a score distribution generated from 5000 random permutations of the same data set (gray). (**b**) Negative interactions between OTUs in soils or in worms do not overlap. Only OTUs found both in soils and in worms were included. Negative interactions were determined by dissimilarity measures and negative correlation based on abundance (top), or based on absence/presence and coexclusion (bottom) (see Materials and methods). (**c**) Interaction networks between OTUs in soils or in worms, as designated, using abundance data for OTUs present in both worm and soil microbiotas. Interactions were calculated at the OTU level and pooled at the family level (legend shown on the right). Green lines represent cooccurrences (positive interactions); red lines represent coexclusion (negative interactions).

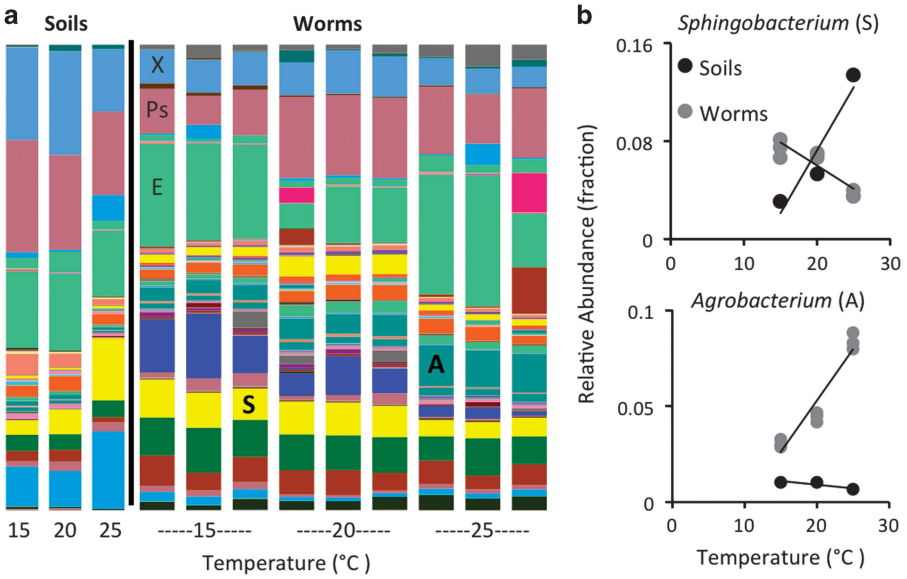

Figure 6 Temperature modulates worm microbiotas through a host-dependent process. (**a**) Bar plots showing relative prevalence of different taxa (genus level (to increase resolution), color-labeled) in worm and soil microbiotas incubated at designated temperatures. Three biological replicates for each temperature were analyzed. Soils were incubated at the same temperatures and for the same duration as worms. Prominent genera from core taxa are designated in gray: *Enterobacteriaceae*, genus unknown (E), *Pseudomonas* (Ps) and *Xanthomondaceae*, genus unknown (X); see Supplementary Table S4 for a full list of taxa. (**b**) Temperature affects certain genera differently in soil or in worms (highlighted in bold in panel **a**).

A core microbiota was identified, accounting for a significant part of the gut microbiota, and shared among all worms in spite of the high microbial diversity within and between their soil habitats. Discerning this core microbiota was likely facilitated by the use of natural-like lab minienvironments, and the genetically homogenous worm populations grown in them, helping in averaging out interindividual variability. Starting with natural-like environments, we presumed that assembled microbiotas should be representative of the natural *C. elegans* microbiota. In support of this, *Enterobacteriaceae*, *Pseudomonadaceae* and *Bacillaceae,* bacteria, which comprise a major part of the core microbiota, were also cultured from *C. elegans* isolated from compost (Grewal, 1991). Members of the first two families were also abundant in *Rhabtidiae* nematodes (*C. elegans*' mother family) isolated from grass soil (Ladygina *et al.*, 2009). Thus, the model we established replicates *C. elegans*–microbe interactions that are likely common in nature.

Interestingly, *Rhabtidiae* worms were among various taxa extracted from the same grass soil, including other bacterivore nematodes, fungivores and predator nematodes; while members of all groups hosted *Pseudomonas* sp., only *Rhabtidiae* hosted *Enterobacteriaceae*, suggesting host specificity (Ladygina *et al.*, 2009). On the other hand, worm interactions with *Pseudomonas* appear to be common, as further indicated by its culturing also from *Pristionchus* nematodes (insect parasites) isolated from the wild (Rae *et al.*, 2008). The association between *Pseudomonas* sp. and nematodes spanning the evolutionary divide between *Caenorhabditis* and *Pristionchus* (~280–430 million years (Dieterich *et al.*, 2008)) suggests a long-standing relationship between nematodes and this core microbiota member.

Using the experimental setup established, we followed the assembly of the *C. elegans* gut microbiota. We found that the composition of worm microbiotas was reproducibly similar, but distinct from their respective environmental starting points. This suggested deterministic shaping of the worm microbiota that was largely independent of the environmental diversity. Results also demonstrated that interactions between microbiota members in the worm gut differed from interactions between the same taxa in the soil, suggesting a contribution of the host niche to shaping of its gut microbiota.

The animal gut niche is expected to be restrictive in terms of physical conditions (pH, oxygen levels, substrates for colonization) and resources (that is, diet and metabolism). Studies in vertebrates have suggested that the composition of the gut microbiota can be determined by multiple factors. A recent study in twins pointed at host genetics as a dominant factor in determining the abundance of certain bacterial families (Goodrich *et al.*, 2014). In contrast, work performed in mice with diverse genetic backgrounds highlighted the dominance of diet over host genetics in shaping the gut microbiota (Carmody *et al.*, 2015). Our results in *C. elegans* lend support to host factors, more than the environmental microbial diversity, as dominant contributors in shaping microbiota composition.

Although our results do not directly reveal which factors are at play, the assembly of worm microbiotas

in different temperatures demonstrated temperature-dependent, but host-specific effects on microbiota composition in worms (disjoined form effects in the environment). This provided further evidence that host-specific processes had a role in shaping the gut microbiota. Taken together, these lines of evidence support host contribution to shaping of its gut microbiota.

Potential host-dependent factors include feeding behavior, which impacts intake of microbes, or host metabolism and epithelial structure, which define an intestinal environment that may favor the growth of certain microbes. However, the multitude of negative interactions observed between worm microbiota members, specifically in the context of the worm intestine, suggest that host factors that regulate the gut environment are more relevant in shaping microbiota composition than feeding behavior.

The inferred negative interactions between worm microbiota members further suggest that species competition within the intestinal niche is an important factor in shaping microbiota composition. The core microbiota includes members of families, such as *Enterobacteriaceae*, *Xanthomonadaceae*, *Burkholderiaceae* and *Pseudomonadaceae*, that are known as strong competitors with flexible metabolism and fast growth, and are highlighted by the data as hubs of negative interactions (Lupp *et al.*, 2007; Compant *et al.*, 2008; Ryan *et al.*, 2009; Silby *et al.*, 2011; Staib and Fuchs, 2014). Competition between microbiota members may further have a role in promoting different types of worm microbiotas, such as the two suggested by our data. Members of the *Comamonadaceae* family are indicative of the W_2 microbiota type, and negatively interact with numerous other families, including the core family, *Enterobacteriaceae*. The abundance of the latter is significantly decreased in W_2 microbiotas compared with W_1 microbiotas (Table 1), suggesting that *Comamonadaceae* members may be able to displace other strong competitors, and shift the microbiota composition. Unlike *Comamonadaceae* bacteria in W_2 microbiotas, no W_1 indicator taxa emerges as a strong competitor. However, reduced abundance of W_2 indicators and of certain core members (that is, *Pseudomonadaceae* and *Sphingobacteriaceae*) in W_1 microbiotas to levels below their environmental abundance suggest that negative interactions may have a role in shaping W_1 microbiotas as well. Work in humans previously described distinct enterotypes, which transcended age, body mass index and sex (Arumugam *et al.*, 2011). Diet has been associated with two of the human enterotypes, but a change in diet did not cause a shift between enterotypes in adults (Wu *et al.*, 2011). The two microbiota types that we identified in *C. elegans* may represent a similar phenomenon to that observed in humans. Here, however, the main driver differentiating between the two 'enterotypes' appears to be interspecies competition. Additional sampling would be required to assess the importance of the two microbiota types in *C. elegans* biology.

This study takes the first step in establishing *C. elegans* as a model to better understand the principles that shape the gut microbiota. Our results demonstrate that assembly of the gut microbiota is essentially a deterministic process controlled both by the host and by interactions between microbiota members. Furthermore, characterization of the *C. elegans* microbiota fills a gap in our knowledge of its natural history, and provides a framework to consider the evolution of *C. elegans* interactions with microbes, mutualistic or pathogenic.

Conflict of Interest

The authors declare no conflict of interest.

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