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Long-Term Exposure to Antibiotics Has Caused Accumulation of Resistance Determinants in the Gut Microbiota of Honeybees

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ABSTRACT Antibiotic treatment can impact nontarget microbes, enriching the pool of resistance genes available to pathogens and altering community profiles of microbes beneficial to hosts. The gut microbiota of adult honeybees, a distinctive community dominated by eight bacterial species, provides an opportunity to examine evolutionary responses to long-term treatment with a single antibiotic. For decades, American beekeepers have routinely treated colonies with oxytetracycline for control of larval pathogens. Using a functional metagenomic screen of bacteria from Maryland bees, we detected a high incidence of tetracycline/oxytetracycline resistance. This resistance is attributable to known resistance loci for which nucleotide sequences and flanking mobility genes were nearly identical to those from human pathogens and from bacteria associated with farm animals. Surveys using diagnostic PCR and sequencing revealed that gut bacteria of honeybees from diverse localities in the United States harbor eight tetracycline resistance loci, including efflux pump genes (*tetB*, *tetC*, *tetD*, *tetH*, *tetL*, and *tetY*) and ribosome protection genes (*tetM* and *tetW*), often at high frequencies. Isolates of gut bacteria from Connecticut bees display high levels of tetracycline resistance. Resistance genes were ubiquitous in American samples, though rare in colonies unexposed for 25 years. In contrast, only three resistance loci, at low frequencies, occurred in samples from countries not using antibiotics in beekeeping and samples from wild bumblebees. Thus, long-term antibiotic treatment has caused the bee gut microbiota to accumulate resistance genes, drawn from a widespread pool of highly mobile loci characterized from pathogens and agricultural sites.

IMPORTANCE We found that 50 years of using antibiotics in beekeeping in the United States has resulted in extensive tetracycline resistance in the gut microbiota. These bacteria, which form a distinctive community present in healthy honeybees worldwide, may function in protecting bees from disease and in providing nutrition. In countries that do not use antibiotics in beekeeping, bee gut bacteria contained far fewer resistance genes. The tetracycline resistance that we observed in American samples reflects the capture of mobile resistance genes closely related to those known from human pathogens and agricultural sites. Thus, long-term treatment to control a specific pathogen resulted in the accumulation of a stockpile of resistance capabilities in the microbiota of a healthy gut. This stockpile can, in turn, provide a source of resistance genes for pathogens themselves. The use of novel antibiotics in beekeeping may disrupt bee health, adding to the threats faced by these pollinators.

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When antibiotics are used for controlling infections by pathogens, they also impact other microbes, including the beneficial bacteria present in healthy hosts. The selective force imposed by an antibiotic can cause the accumulation of resistance determinants, which are often encoded on mobile genetic elements that are readily transferred among community members. The impact of antibiotics on the gut microbiota of animals is a particular concern, since gut communities may act as reservoirs for resistance genes that can be transferred to pathogens (1–3) and also since perturbation of gut microbiota by antibiotic treatments could disrupt functions beneficial to hosts (4).

The honeybee (*Apis mellifera*), a highly social insect and important agricultural pollinator, is associated with eight characteristic bacterial species that together comprise over 95% of the gut bacteria in adult worker bees (5–12). A single bee or colony can

possess multiple strains of each of these species (11, 13), and several of these species (or close relatives) are also found in bumblebees (*Bombus* species) (6, 9, 14). Some of these species can be grown in axenic culture and have recently been formally named (taxonomic nomenclature) (15). Increasingly, studies are revealing beneficial functions of gut bacteria for animal hosts (e.g., see references 16 and 17). In the case of bees, the distinctive gut bacteria shared by honeybees and bumblebees have been implicated in defense against trypanosome parasites (18) and in digestive roles (13).

Since the 1950s, the antibiotic oxytetracycline has been widely applied to colonies of bees in the United States to control larval foulbrood diseases caused by the bacteria *Melissococcus pluton* and *Paenibacillus larvae*; oxytetracycline was the only antibiotic approved for use in beekeeping until 2005 (19–22). Antibiotic treat-

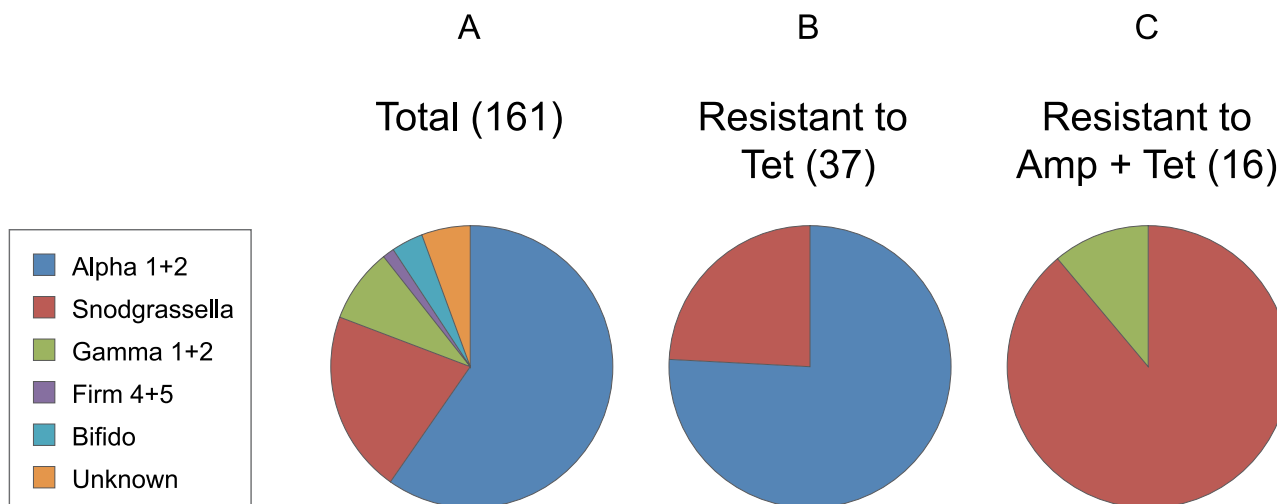


FIG 1 Species assignments of fosmid inserts used in metagenomic functional screens based on end sequencing for a random set of inserts (A), inserts exhibiting tetracycline (Tet) resistance (B), and inserts exhibiting ampicillin (Amp)/tetracycline resistance (C). The numbers of inserts are in parentheses. Taxonomic categories refer to bee gut-associated taxa (7, 8, 10, 13, 15), except that related pairs of taxa are pooled as follows: “Alpha1” plus “Alpha2” (Alpha 1 + 2), *Gilliamella apicola* plus “Gamma2” (Gamma 1 + 2), and “Firm4” plus “Firm5” (Firm 4 + 5).

ment potentially impacts the microbiota typical of healthy hosts (3, 4, 23, 24). Compared to the gut microbiota of humans and other mammals, the honeybee gut microbiota provides a distinctive and relatively simple bacterial community exposed to a known antibiotic, and the differences in treatment history between honeybees in different localities provide an opportunity to observe the impact of selection pressure by application of a single antibiotic over several decades.

In this paper, we investigate antibiotic resistance in the honeybee gut microbiota using a variety of functional and sequence-based assays on bees from colonies in the United States and from countries in which antibiotics have not been used in beekeeping. We report an accumulation of resistance genes specifically in the gut microbiota of honeybees within the United States, where oxytetracycline has been used in beekeeping.

RESULTS

We constructed a large-insert clone library (average insert size of 34 kb with total coverage of >3 Gb) using bacterial cells from the guts of worker bees from a Maryland (MD) colony (see Table S1 in the supplemental material). Analysis of end sequences of randomly selected clones confirmed that the inserts were derived from the genomes of the eight bacterial species known to dominate the honeybee gut microbiota, primarily the Gram-negative members of this community (Fig. 1A). Functional screens of the clone library for antibiotic resistance revealed clear instances of resistance only for tetracycline/oxytetracycline and carbenicillin/ampicillin. These instances of resistance occurred at a frequency of ~0.1% for fosmid inserts, corresponding to an average frequency among gut bacteria of ~10% for tetracycline resistance. An approximately 10-fold-lower frequency was observed for carbenicillin/ampicillin resistance. There was complete cross-resistance for tetracycline and oxytetracycline and for carbenicillin and ampicillin. Furthermore, 15 of 16 ampicillin-resistant clones also grew on plates containing tetracycline, indicating coselection of genes underlying tetracycline and ampicillin resistance. No clone grew on plates containing ceftazidime, gentamicin, or rifampin.

After duplicate clones were eliminated (determined on the basis of matching end sequences), we retrieved 20 unique inserts with tetracycline resistance and nine more with ampicillin/tetracycline resistance (see Table S2 in the supplemental material). Many (51%) resistant clones were unique, suggesting that additional resistance loci could be retrieved from the library if more clones were sequenced. Screening the resistant inserts by diagnostic PCR assays and sequencing revealed that inserts contained known resistance loci: *tetB* (13 clones), *tetC* (11 clones), *tetD* (1 clone), or *tetL* (1 clone) (Table S2). Previous designations for the species of bee gut bacteria are “Alpha1” and “Alpha2” from the Alphaproteobacteria, *Snodgrassella alvi* from the Betaproteobacteria, *Gilliamella apicola* and “Gamma2” from the Gammaproteobacteria, “Firm4” and “Firm5” from the Firmicutes, “Bifido” from the Bifidobacteriaceae. (7, 8, 10, 15). Using these designations, taxonomic assignments based on end sequences indicated that 68% of tetracycline-resistant clones are from *S. alvi* (*Betaproteobacteria*) and 24% are from “Alpha1” (*Alphaproteobacteria*) (Fig. 1B). Our initial retrieval of only *tetB*, *tetC*, *tetD*, and *tetL* by screening fosmids may reflect incompatibilities of some loci with expression in *Escherichia coli* hosts, as well as low representation of some species, particularly the Gram-positive species, in the library.

Ampicillin-resistant clones yielded a product for which the inferred amino acid sequence was 100% identical to that of a known ampicillin resistance gene corresponding to *bla*_{TEM-1}, an extended-spectrum beta-lactamase of *E. coli* (GenBank accession no. CAJ13634). Most ampicillin-resistant clones also encoded TetB, except for one that encoded TetD. Of the ampicillin/tetracycline-resistant clones, 13 of 16 were from *S. alvi* (*Betaproteobacteria*), and three were from the related *Gilliamella apicola* or “Gamma2”, based on assignment of end sequences (Fig. 1C).

To determine the tetracycline resistance gene content within the honeybee gut microbiota, we screened for 21 known tetracycline resistance genes using diagnostic PCR in a panel of samples from different American localities, including MD, Florida (FL), Arizona (AZ), Washington (WA), Connecticut (CT), and Utah

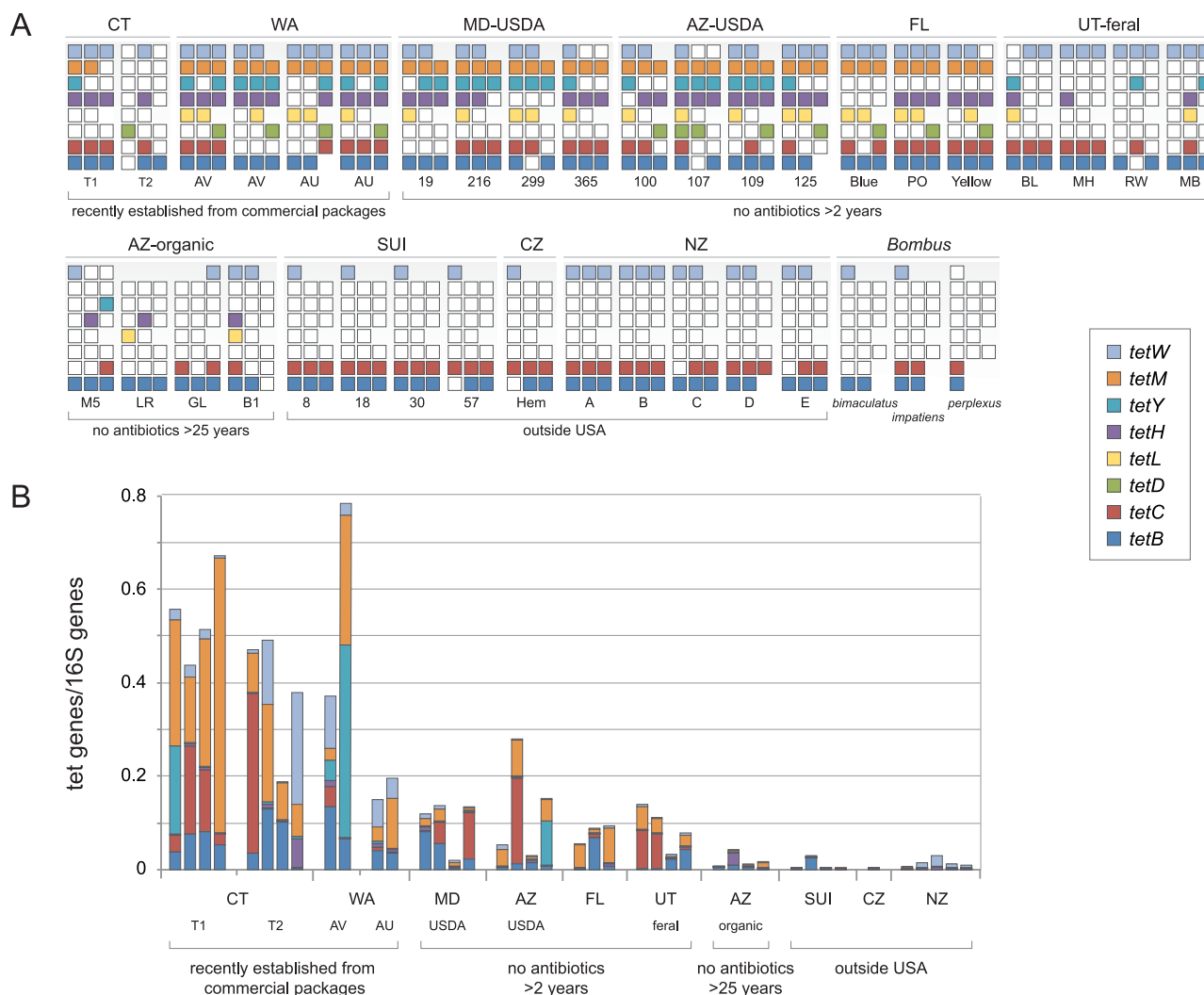


FIG 2 Presence of tetracycline resistance genes in gut microbiota of honeybees and bumblebees. (A) Occurrence of eight loci in individual bees from different sources (13 other loci were screened but not detected). Filled and empty boxes indicate positive and negative results, respectively, for the tetracycline resistance genes in the assays; the absence of a box indicates that the gene was not assayed. (B) Numbers of copies of tetracycline resistance loci relative to 16S rRNA copies in the microbiota of honeybees sampled from several locations. Numbers are based on absolute quantification results using quantitative PCR with diagnostic primers for each gene. Seven genes (excluding *tetL*) were screened.

(UT), and representing different colony histories with respect to recent antibiotic treatments. We repeatedly detected the same eight tetracycline resistance genes, including genes encoding tetracycline efflux pumps (*tetB*, *tetC*, *tetD*, *tetH*, *tetL*, and *tetY*) and ribosomal protection proteins (*tetM* and *tetW*) (Fig. 2A). Although some individual bees failed to amplify for a particular locus, the eight loci were typically present in bees from every colony, with few exceptions such as the absence of *tetY* from all FL bees and of *tetD* from all MD bees. Further pooled DNA samples from 150 individual bees from each of eight MD colonies yielded the same five tetracycline efflux pump genes detected in amplifications from samples of individual bees: *tetB*, *tetC*, and *tetH* from all MD colonies and *tetD* and *tetY* from four and five of the eight colonies (see Fig. S1 in the supplemental material). The *tetL* gene was present in most MD bees and in two of four AZ colonies but absent from most WA individual bees (Fig. 2A). Overall, PCR screening results indicate that these eight tetracycline resistance

genes are widespread in American honeybee colonies but that their presence can vary among colonies or locations.

Sharply contrasting results were obtained in screens of honeybees from Switzerland (SUI), the Czech Republic (CZ), and New Zealand (NZ), countries where antibiotics have not been permitted in beekeeping (18). The microbiotas of SUI, CZ, and NZ bees sometimes had *tetB*, *tetC*, or *tetW* but lacked *tetD*, *tetH*, *tetY*, *tetM*, and *tetL* (Fig. 2A). Results for wild Connecticut bumblebees resembled those for SUI, CZ, and NZ honeybees, with detection of only the *tetB*, *tetC*, or *tetW* gene, depending on the sample (Fig. 2). Thus, the use of antibiotics in American beekeeping is associated with the widespread occurrence of five additional tetracycline resistance loci in bee gut bacteria.

To determine the prevalence of these or other tetracycline resistance genes, resistance gene sequences were used to query scaffolds of a metagenomic sequence data set derived from the guts of bees from AZ (USDA) (13). Nine scaffolds were found to contain

TABLE 1 Tetracycline resistance loci present in the honeybee gut microbiota

Resistance locus	Fosmid insert ID ^a	Metagenomic scaffold ID	Cultured isolate ID	Associated mobile element type ^b	Bacterial source ^c
<i>tetB</i>	A3_16, T3_21	NODE_608118	wkB1, PEB0162	Transposon; conjugative plasmid	<i>G. apicola</i> , ^{d,f} <i>S. alvi</i> , ^d Alpha1 ^d
<i>tetC</i>	T4, TA1, TA7, T3_2, T3_18	NODE_563228	wkB2, wkB4, wkB5, wkB9	Transposon	<i>S. alvi</i> , ^{d,e,f} Alpha1 ^d
<i>tetD</i>	A3_15	NODE_168019, NODE_133356		Transposon	<i>S. alvi</i> ^d
<i>tetH</i>		NODE_214129		Plasmid	<i>G. apicola</i> ^f
<i>tetL</i>	T3_7	NODE_20898	B10I28		Alpha1, ^d <i>P. larvae</i> ^f
<i>tetY</i>		NODE_531442			
<i>tetM</i>		NODE_594695		Transposon	Firm ^e
<i>tetW</i>		NODE_40887	wkB3		Bifido ^{e,f}

^a ID, identification.

^b Mobile element type inferred on the basis of sequence homology to previously studied elements.

^c Taxonomic assignment based on fosmid inserts, metagenomic scaffolds, and 16S rRNA sequences from isolates. Some scaffolds could not be confidently binned.

^d Taxonomic assignment based on fosmid inserts.

^e Taxonomic assignment based on metagenomic scaffolds.

^f Taxonomic assignment based on 16S rRNA sequences from isolates.

the same set of eight tetracycline resistance loci (see Table S4 in the supplemental material). These metagenomic data represent a more complete sampling of bee gut communities, since they circumvent potential biases in cloning and resistance gene expression. In addition, other genes encoding potential efflux pump proteins were detected in the AZ metagenomic data set by querying with sequences of known efflux pump proteins. However, none of these additional efflux pump genes clustered closely with known tetracycline resistance proteins (see Fig. S2 in the supplemental material), and none was retrieved from our functional assays.

Estimates of the abundances of the tetracycline resistance loci in gut bacteria revealed extensive variation among colonies and localities in the frequencies of particular loci (Fig. 2B). These quantitative results are broadly consistent with the results of diagnostic PCR screens and show that the gut microbiotas of SUI, CZ, and NZ honeybees have very low copy numbers of resistance genes, even for those few loci detected. Among American bee colonies, relative numbers of different resistance loci varied extensively.

The variation in tetracycline resistance determinants observed among American honeybee colonies may reflect different recent histories of oxytetracycline treatment for individual colonies. Most of our samples had unknown histories of antibiotic treatment, largely due to their origin from mixing other colonies or from commercial bee packages. To determine whether resistance loci decline when antibiotic exposure is terminated, we obtained samples from four managed colonies in southern Arizona that were unusual in having not been treated directly or mixed with outside bees for over 25 years and samples from long-established feral colonies in Utah, also expected to have no recent exposure. These samples showed markedly lower copy numbers of resistance loci compared to other American samples (Fig. 2). The FL, MD, and AZ (USDA) colonies, which had no antibiotic treatment for at least 2 years prior to sampling, showed intermediate levels of resistance loci. The highest frequencies were observed for colonies

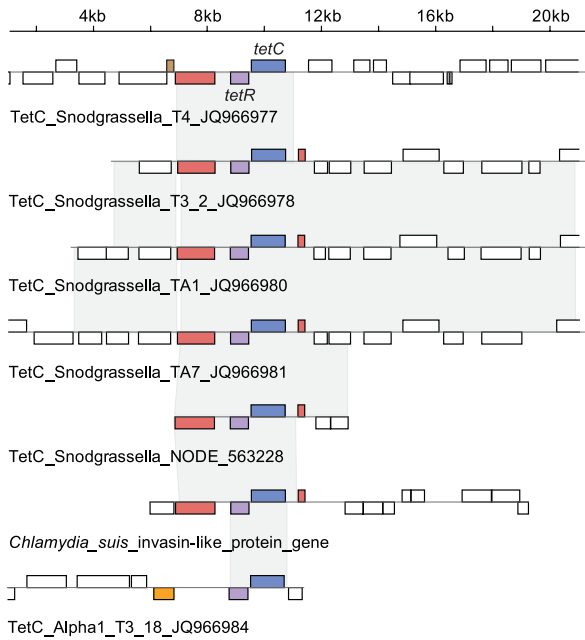
in CT and WA established from package bees purchased from commercial bee suppliers 0 to 12 months before sampling.

To confirm results from diagnostic PCR and to link tetracycline resistance gene types with their source genomes, sequences of full-length open reading frames were recovered from PCR amplification using DNA from worker bee guts from a MD colony, fosmids derived from a MD colony, and cultured bacterial isolates from the guts of CT bees (Table 1). Isolate identities, based on 16S rRNA sequences, were confirmed for *G. apicola*, *S. alvi*, “Alpha1”, “Firm5”, and “Bifido” species using established designations for these bee-associated species (7–10, 15). Strains corresponding to several of the characteristic gut species possessed resistance genes (Table 1). For known tetracycline resistance loci, nucleotide sequences from different colonies shared 99 to 100% identity with one another and with published sequences from other sources (Fig. 3; see Fig. S2 in the supplemental material), indicating that the various tetracycline resistance loci in the guts of American honeybees have been transferred recently among taxonomically and ecologically distinct bacteria.

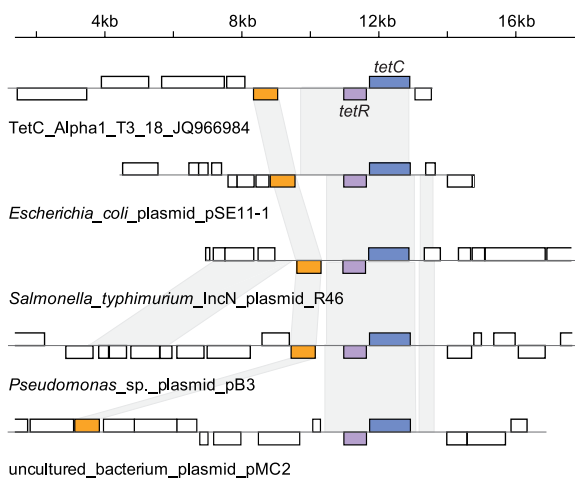
We examined cultured isolates of the constituent species of the bee gut microbiota for tetracycline resistance and for the presence of resistance genes. When isolates from CT bee colonies (13) were plated on medium with 12 µg/ml oxytetracycline, resistant strains were readily recovered despite the absence of selection for resistance in the initial isolation procedure. For *G. apicola*, 77% (10/13 isolates) were resistant, and 100% (all 11 isolates) of *S. alvi* isolates were resistant. Resistant members of Alpha1 (3/14 isolates), Bifido, and Firm5 species were also recovered (see Table S5 in the supplemental material).

The observed resistance of many isolates was attributable to known tetracycline resistance genes, often associated with large increases in the tetracycline MICs of isolates from the bee gut microbiota (see Table S5 in the supplemental material). For strains carrying *tetB* (*G. apicola* wkB1, PEB0162), *tetC* (*S. alvi* wkB2, wkB4, wkB5, and wkB9), or *tetW* (Bifido wkB3), tetracy-

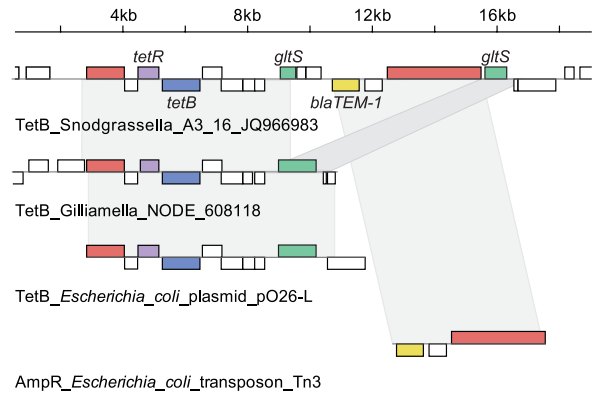
(A) TetC



(B) TetC



(C) TetB



(D) TetL

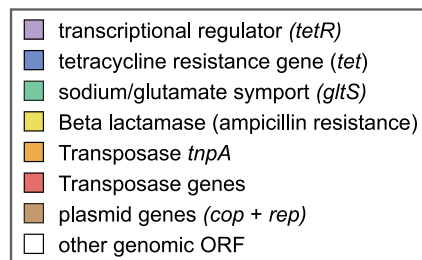
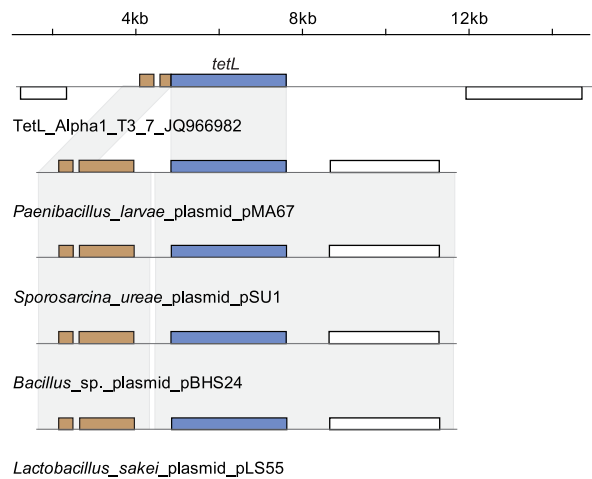


FIG 3 Genetic organizations of fosmid inserts and metagenomic scaffolds containing tetracycline resistance genes within the honeybee gut microbiota compared to chromosomal regions containing homologous genes from other bacteria. Gray shading indicates regions sharing >99% nucleotide sequence identity. Comparisons for regions containing *tetC* (A and B), *tetB* (C), and *tetL* (D) are shown. See supplemental Text S1 for a detailed description of individual resistance loci in the bee gut microbiota.

cline MICs were $\geq 12 \mu\text{g/ml}$; in contrast, MICs were $< 0.5 \mu\text{g/ml}$ for isolates of these species lacking resistance genes (*G. apicola* wkB7, bumblebee *G. apicola* wkB11, and bumblebee *S. alvi* wkB12).

Information on the chromosomal context of resistance genes, from sequenced fosmids and metagenomic scaffolds containing

resistance loci and from PCR screens spanning resistance genes and mobility genes, shows that tetracycline resistance genes are consistently associated with mobile elements, such as transposons and plasmids (Fig. 3 and Table 1; see Tables S3, S4, and S6 in the supplemental material), implying that most resistance determinants in the bee gut microbiota are newly acquired and not native

elements present in the genomes of the ancestral bee gut microbiota. In most cases, sequences of chromosomal fragments spanning resistance loci and associated mobility elements show >99% sequence identity to genes previously characterized from human pathogens or from domesticated animals, such as pigs and chickens (Fig. 3).

DISCUSSION

The gut microbiota of honeybees in the United States provides an unusual example of a clearly defined microbial community subjected to a single broad-spectrum antibiotic for a prolonged period (19, 20, 22). These gut bacterial communities have accumulated an abundant and diverse set of tetracycline resistance genes, encompassing eight resistance loci that are found in diverse geographic localities. Bee colonies are transported widely within the United States, both by suppliers who provide bees to beekeepers and by migratory pollination services, so there is ample opportunity for rapid dissemination of the loci among colonies and localities.

In contrast, the gut microbiota of SUI, CZ, and NZ honeybees contain only 2 or 3 resistance loci, each in very low copy number, as was also true for bumblebees caught in the wild. Since antibiotics have not been used in beekeeping in these two European countries or in New Zealand and since bumblebees, as a wild species, are not expected to encounter artificial antibiotic applications, the resistance loci in these samples are likely to be naturally occurring. Antibiotic production and resistance mechanisms are widespread in natural microbial communities (24). Alternatively, chronic exposure to sublethal concentrations of environmental antibiotics can maintain resistance loci (25), potentially accounting for the few resistance genes identified in SUI, CZ, and NZ honeybees and in wild bumblebees. The presence of *bla*_{TEM-1} in some *S. alvi* strains is not readily explained by treatment history, since beta-lactams are not approved for use in beekeeping; *bla*_{TEM-1} is hypothesized to have hitchhiked with the associated *tetC* or *tetD* gene as part of a compound transposon (e.g., Fig. 3C).

Prolonged exposure to a single broad-spectrum antibiotic imposes strong selective pressure on a microbial community that is expected to result in loss of strain diversity. It is possible that antibiotic perturbation may shift the gut microbiota to an alternative state that is broadly similar but different in critical aspects (4, 23). These shifts could affect host health: in the case of the distinctive gut bacteria of honeybees and bumblebees, metagenomic and experimental studies suggest beneficial roles in neutralization of dietary toxins, nutrition, and in defense against pathogens (13, 18).

Following the emergence of resistance to oxytetracycline in *P. larvae* in 1996, alternative antibiotics were tested for its control (19, 26). In October 2005, Tylosin was approved by the U.S. Food and Drug Administration for use in beekeeping and was marketed to beekeepers. In 2007, accelerated losses of colonies occurred throughout the United States: the causes of these losses are not clear but appear not to be attributable to spread of a particular pathogen (27, 28). Speculatively, disruption of the gut microbiota by a novel antibiotic might contribute to the decline of colonies of bees, with such effects potentially becoming less pronounced as members of the microbiota acquire resistance capabilities.

MATERIALS AND METHODS

A condensed summary of approaches is provided here; detailed methods are provided as supporting text (Text S1) in the supplemental material. Briefly, we performed functional screens for antibiotic resistance and sequence-based assays, using samples from several localities in the United States and SUI, CZ, and NZ (see Table S1 in the supplemental material). For the functional screens, we constructed a fosmid library in *E. coli* vectors using DNA from a pooled sample of gut bacteria from 150 workers of a MD honeybee colony (USDA Bee Research Laboratory). The fosmid library had a total of $\sim 1 \times 10^5$ fosmid clones with an average insert size of 34 kb and was estimated to represent at least 3 Gb. BLASTX analysis of end reads of 161 randomly selected inserts against GenBank and against the metagenomic sequence data set from AZ (USDA) bees (13) was used to determine the representation of bacterial species within the library. We screened this library for resistance to seven antibiotics, including tetracycline and oxytetracycline. We also carried out targeted PCR screens using published or newly designed primers (Table S7) based on sequences of known tetracycline resistance genes. We screened a panel of bees from colonies from several localities in the United States and from SUI, CZ, and NZ. We also screened several wild bumblebees, collected in CT. To obtain quantitative measures of resistance gene abundance in bee gut microbiotas, we used quantitative PCR (qPCR) to estimate the absolute numbers of copies of resistance loci in a sample relative to the absolute numbers of bacteria estimated as copy numbers of 16S rRNA genes.

To determine the chromosomal contexts of the resistance genes and their possible modes of transfer, we obtained sequences of the amplified loci themselves and of selected fosmid inserts bearing resistance loci, and we searched the AZ metagenomic sequence library for scaffolds with resistance loci. Phylogenetic analyses were used to determine the relationships of the retrieved gene sequences to previously published genes for tetracycline resistance and to other efflux pump genes. These DNA sequences are available in the NCBI nucleotide database (accession nos. JQ966977 to JQ966984 for full length fosmid inserts, JQ966985 to JQ966992 for amplified tetracycline resistance genes, and JS807327 to JS807645 fosmid-insert end sequences).

Cultured isolates representing the major species in the bee gut microbiota were established from freshly collected CT bees using conditions described previously (13). Isolate identities were based on a criterion of >99% identity of 16S rRNA sequences with known sequences for these species. Using PCR, these isolates were screened for known tetracycline resistance genes. The levels of resistance of isolates to tetracycline were measured as MICs using the Etest method.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00377-12/-/DCSupplemental>.

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