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Composition of Bacterial Communities Associated with Natural and Laboratory Populations of *Asobara tabida* Infected with *Wolbachia*▽†

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***Asobara tabida* wasps are fly endoparasitoids that naturally harbor three *Wolbachia* strains, which induce cytoplasmic incompatibility and control oogenesis. To investigate whether other bacteria play a role in wasp biology, we surveyed the bacterial communities of wild *A. tabida* populations originating from different regions of France and of laboratory colonies using PCR-denaturing gradient gel electrophoresis and culture methods. *Proteobacteria* and *Firmicutes* were found to be the main phyla represented in these populations. Among these were several cultured and uncultured representatives of the genera *Acetobacter*, *Acidomonas*, *Bacillus*, *Brevibacillus*, *Duganella*, *Herbaspirillum*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*. In addition to *Wolbachia*, wild individuals harbored *Rickettsia*, which tended to be lost when insects were reared in the laboratory. The antibiotic treatment used to generate wasp sublines singly infected with *Wolbachia* also affected the overall bacterial composition, with most fingerprint sequences being characteristic of the family *Enterobacteriaceae*. We also screened for potentially heritable endosymbionts by PCR and fluorescence in situ hybridization in stable laboratory lines, with only *Wolbachia* being consistently found in wasp ovaries.**

Bacteria associated with insects play a crucial role in host development, survival, and reproduction (13). Many insects harbor bacterial endosymbionts, which establish close relationships, like the mutualistic interaction between aphids and their primary endosymbiont of the genus *Buchnera*; the bacterium uses the host as a habitat to which it supplies essential amino acids, facilitating insect growth when the diet of plant phloem sap is insufficient (5, 23). Aphids host many other nonessential bacteria as secondary or facultative symbionts. However, aphids with secondary symbionts can gain a fitness advantage in terms of diet, regimen plant host range, heat tolerance, or resistance to pathogens and parasitoids (reviewed in references 45 and 48). Multiple infections are costly to hosts and are perhaps maintained because of the benefits they confer. Recently, *Wolbachia* has been shown to protect the host *Drosophila melanogaster* from viral damage (37). However, investigating the evolutionary significance of interspecific symbioses in bacterial communities in invertebrates is challenging in that the majority of bacteria are not yet cultivable outside host cells.

Here we analyzed the main bacterial populations of *Asobara tabida* (Hymenoptera: Braconidae), endoparasitoids of *Drosophila* species and related genera (14). Usually, members of *A. tabida* are naturally multiply infected with bacteria of the genus *Wolbachia*, obligate intracellular *Alphaproteobacteria* of the order *Rickettsiales* (2, 25, 51), found in association with numerous arthropods, mainly insects, and certain nematodes, where they are mostly vertically transmitted from mother to progeny (74). The interaction between *Wolbachia* spp. and their hosts is very complex and ranges from parasitism to mutualism. In filarial nematodes, *Wolbachia* organisms are required in the host's biology (4), but *Wolbachia* spp. are mostly parasites that affect arthropod reproduction, such as by inducing parthenogenesis in some parasitoid wasps (63), feminizing genetic males in isopods (9), and inducing male killing and cytoplasmic incompatibility in many insects (39, 64). The wasp *A. tabida* harbors three *Wolbachia* strains; strains *w*Atab1 and *w*Atab2 induce cytoplasmic incompatibility, whereas *w*Atab3 is necessary for the completion of oogenesis (19, 20, 73). The involvement of *Wolbachia* strains in wasp reproduction was discovered when wasps were treated with antibiotics to generate lines harboring subsets of *Wolbachia* or aposymbiotic lines (19, 21). Only oogenesis was affected by curing *Wolbachia* *w*Atab3; other traits, such as insect size, weight, locomotion, and behavior, were unchanged (19). While antibiotic treatment has been used to determine biological roles of symbionts in this way, their effect on the overall composition of bacterial populations has not been investigated.

To investigate the potential role of bacterial endosymbionts in the biology of *A. tabida*, we studied the bacterial communities in insect populations originating from different regions of France using culture and nonculture methods and fluorescence in situ hybridization (FISH). We also examined whether antibiotherapy to generate lines with a subset of *Wolbachia* strains altered the composition or density of the bacterial communities.

MATERIALS AND METHODS

Insect collection and maintenance. Wasps of the genus *Asobara* (Hymenoptera: Braconidae) are endoparasitoids of *Drosophila*. Female wasps lay their eggs in fly larvae, where the parasitic wasp larvae feed and develop (14). *A. tabida* individuals were collected from six sites in France—Condrieu (Co), Igé (Ig), Sainte Foy-Lès-Lyon (Sfl), Saint Laurent d'Agny (Sla), and Verpillère (Vp)—and reared in the laboratory (except those from Vp). Four lines originating from Montpellier (Mo), Pierrefeu (Pi), Sfl, and Sla that had been maintained in an insectarium for six months to four years were also used. Co, Sfl, Sla, and Vp are located in Rhône-Alpes region (southwest of France), around 40 km from the city of Lyon. Igé is situated in region of Bourgogne, 80 km from Lyon. Mo (304

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km from Lyon) and Pi (500 km from Lyon) are in Languedoc-Roussillon and Provence-Alpes Côte d'Azur, respectively, both in the south.

The wasps were reared on a *Wolbachia*-free *Drosophila melanogaster* strain, originating from Sfl, at 20°C with a 12-h–12-h light-dark cycle and 70% relative humidity. Members of *A. tabida* are usually naturally infected with three *Wolbachia* strains, named *w*Atab1, *w*Atab2, and *w*Atab3 (73). An *A. tabida* line, Pi(3), infected with only *w*Atab3 was obtained from Pi(123), a triply infected line, using a moderate antibiotic treatment (19). After antibiotherapy, sublines were reared without antibiotics for seven generations (new line) or for four years (old line) before analysis.

Isolation of bacteria from insect ovaries. Emerging *A. tabida* adults were anesthetized at 4°C, surface sterilized in 70% ethanol for 5 min, and rinsed five times in sterile water. Ovaries from anesthetized females were dissected with needles in SPG buffer (218 mM sucrose, 7.2 mM K_2HPO_4, 3.8 mM KH_2PO_4, 4.9 mM L-glutamate; pH 7.2) under a binocular microscope and were surface sterilized as described above. A total of 100 ovaries were crushed in 50 μl IPL41 medium (Gibco, Invitrogen, France). Directly plating the homogenate gave rise to only a few very small colonies that did not grow further either aerobically or anaerobically. To enrich the homogenate, it was diluted and inoculated (final optical density at 540 nm of 0.01) into three Venoject tubes containing 3 ml of different media: IPL41 (Gibco), modified Luria Bertani (10 g $liter^{-1}$ Bactotryptone, 5 g $liter^{-1}$ yeast extract, 5 g $liter^{-1}$ NaCl), and PYC (5 g $liter^{-1}$ peptone, 3 g $liter^{-1}$ yeast extract, 6 mM $CaCl_2 \cdot 2H_2O$; pH 7.0). After five days' incubation at 26°C without agitation, cultures were reinoculated in both 5-ml liquid cultures and on agar plates of the respective medium. Single colonies were streaked out to check for purity and then screened using standard bacteriological techniques to determine the dilution factor and colony morphology (size, shape, margin, color, opacity, and consistency). Purified isolates were cultured in liquid medium and stored in 25% glycerol at −80°C until use.

Genomic and plasmid DNA extractions. Genomic DNA was isolated using a DNeasy tissue kit (Qiagen, Courtaboeuf, France) with the following modifications. Pools of 5 to 15 emerging adults or 100 dissected ovaries, prepared and disinfected as described above, were homogenized in 180 μl of DNeasy ATL buffer. The mixture was treated with 2 mg ml^{-1} lysozyme (Eurogentec, Angers, France) for 3 h at 37°C and then with proteinase K (Qiagen) for 12 h at 56°C. The mixture was centrifuged twice at 12,000 × *g* for 1 min. The supernatant was transferred to a new tube and treated with RNase A (final concentration, 1 mg ml^{-1}) for 2 min at room temperature. Then 200 μl of DNeasy AL buffer was added and incubated for 10 min at 70°C. After the addition of 200 μl absolute ethanol, the sample was vortexed and pipetted into the DNeasy mini-spin column. DNA was eluted with 30 μl of TE buffer (10 mM Tris [pH 7.5], 0.1 mM EDTA [pH 8]), quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc.), and stored at −20°C until use.

Genomic and plasmid DNA (recombinant vector [TOPO 2.1] containing cloned DNA fragments for sequencing) were extracted from bacteria using Nucleospin tissue or plasmid kits (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions.

Diagnostic and quantitative PCR. General PCR conditions are given in Table 1. A PCR specific for *Wolbachia* using primers (Table 1) targeting 16S rRNA genes and *wsp* loci was as previously reported (44). A LightCycler LC480 apparatus (Roche) was used for real-time quantitative PCR. The 20-μl reaction mixture contained 1× LightCycler DNA master SYBR green I (Roche), each primer at 300 nM, and 20 ng of template DNA. Amplification conditions were 10 min at 95°C; 40 cycles of 15 s at 95°C and 1 min at 60°C or 63°C for *wsp* and *rrs*, respectively; and 72°C for 30 s. Standard curves were drawn for amplification from a DNA plasmid containing *wsp* and 16S rRNA gene fragments, respectively (Table 1).

To analyze the total bacterial populations while excluding *Wolbachia* amplicons, we first targeted the 16S-23S region and then 16S alone, as all *Wolbachia* strains identified so far harbor unlinked 16S and 23S genes (7) (Fig. 1). This strategy also allowed us to disregard other bacteria, such as *Buchnera*, having a similar split rRNA gene organization (58). For this, 16S and 23S regions were amplified with standard universal primers (Table 1) as follows: 95°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 40 s, and 72°C for 3 min; and 72°C for 10 min. The 16S rRNA gene was amplified with the same conditions except that the final elongation step was for 1 min 30 s. Amplification reaction mixtures (25 μl) contained the genomic DNA template (60 ng) in 1× polymerase reaction buffer (Clontech), 200 μM each deoxynucleoside triphosphate, 200 nM each primer, and 1 U Titanium *Taq* polymerase (Clontech) and used a T-gradient thermocycler (Biometra). PCR products were electrophoresed on 0.8% agarose gel, stained with ethidium bromide, and photographed under UV with the Fisher Bioblock scientific system (Fisher, Ilkirch, France).

For DGGE, 16S rRNA gene V3 regions were amplified in 50-μl reaction mixtures containing 1 μl of *rrs* amplicons in 1× polymerase reaction buffer (Invitrogen), 1.5 mM $MgCl_2$, 250 μM each deoxynucleoside triphosphate, 0.5 μM each primer, and 0.4 U of *Taq* polymerase (Invitrogen). Amplification conditions were 94°C for 2 min; 7 cycles of 94°C for 30 S, 55°C for 30 s, and 72°C for 1 min; 21 cycles of 92°C for 30 S, 55°C for 30 s, and 72°C for 1.21 min; and 72°C for 10 min.

DGGE. Bio-Rad Dcode or Ingeny PhorU (Apollo Instruments, Compiègne, France) systems were used for DGGE analysis of the V3 PCR products. The 6% acrylamide gels contained a linear chemical gradient of urea and formamide from 35% to 65%. PCR products (5 μg per well) were run in TAE buffer (40 mM Tris [pH 8.0], 20 mM acetic acid, 1 mM EDTA) at 60°C for 16 h at 75 V (Dcode) or for 17 h at 100 V (Ingeny PhorU). After electrophoresis, gels were immersed in SYBR green for 30 min at 4°C, rinsed in water, and photographed. PCR-DGGE replicates made with the same DNA sample generated identical patterns, indicating that the amplification was reproducible. When required, bands were excised, transferred to Eppendorf tubes, and washed five times with sterilized water. After all trace of liquid had been removed, 30 μl of water was added to tubes that were heated at 60°C for 30 min and kept overnight at 4°C; 2 μl of solution was used for reamplification, and products were cloned and sequenced.

Cloning and sequencing. PCR products were purified using a QIAquick PCR purification kit (Qiagen) and cloned into chemically competent *Escherichia coli* cells (DH5α or TOP10) according to the TOPO TA 2.1 cloning kit protocol (Invitrogen). Transformants containing DNA inserts were selected for sequencing by Genoscreen (Lille, France). Sequences were analyzed with the NCBI Blastn program (http://www.ncbi.nlm.nih.gov/).

FISH. Ovaries and oocytes were fixed for 20 min in freshly prepared 4% formaldehyde in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 [pH 7.4]). Samples were hybridized overnight at 37°C in hybridization buffer [50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 200 g $liter^{-1}$ dextran sulfate, 250 μg ml^{-1} poly(A), 250 μg ml^{-1} salmon sperm DNA, 250 μg ml^{-1} tRNA, 0.1 M dithiothreitol (DTT), 0.5× Denhardt's solution] containing 200 ng of each probe. Fluor-labeled oligonucleotide probes (Invitrogen) were used: two *Wolbachia* probes, W2 (5′-CTTCTGT GAGTACCGTCATTATC-3′) (36) and Wol3 (5′-TCCTCTATCCTCTTTCAA TC-3′) (60), 5′-end labeled with rhodamine; two *Pseudomonas* probes, PSEUD (5′-ACCTAGGCTCATCTGATAGCGCAAGG-3′) (59) and PSEUG (5′-GAT CCGGACTACGATCGGTTT-3′) (34), 5′-end labeled with Alexa 488; and two *Brevibacillus* probes, Brevigenus Brbac2 (5′-CATCTCCCAGTGACAGCCGA-3′) and Breviagri Brbac6 (FITC-CCCAGTGATAGCGAAAAGC) (M. Kyselková, J. Kofecky, M. Frapolli, G. Défago, M. Ságová-Marécková, G. Grundmann, and Y. Moënne-Loccoz, unpublished data), 5′-end labeled with FITC. Samples were washed twice in 1× SSC, 10 mM DTT and twice in 0.5× SSC, 10 mM DTT at 55°C for 15 min, rinsed in PBS, mounted in glycerol on a glass slide, and viewed with fluorescent (Axio Imager.Z1; Zeiss) and confocal (LSM510; Zeiss) microscopes. Fixed samples were stained with 1 μg ml^{-1} DAPI in glycerol.

Nucleotide sequence accession numbers. Sequences determined in this study were deposited in the GenBank database and are listed in Table 2.

RESULTS

Whole-insect bacterial profiling by PCR-DGGE. To explore the bacterial community of *A. tabida*, PCR-DGGE fingerprints of samples from whole insects were produced using primers (Table 1) specific to *rrs* genes and the corresponding hypervariable V3 regions, which has been shown to be useful for analyzing bacterial communities (15, 78). The genomic DNA was extracted from insects collected from different sites in France and from individuals reared in the laboratory. For each population, three pools of five individuals were tested independently. Whole-insect-body samples from females caught in the field had similar DGGE profiles (Fig. 2A), except for those from Vp. There were three intense and some faint bands in the profiles of all individuals, whereas another two bands were found only in individuals from Vp. Bands were excised from the gel, and some were cloned and sequenced (Table 2). The two major bands in the middle of the gel were considered to be from the genus *Wolbachia*, as they were present in the control

TABLE 1. Primers used in this study

Sample	Gene	Primer	Primer sequence (5′–3′)	Amplicon size (bp) and T_m (°C)	Reference
Eubacteria	*rrs* + IG[a]	REUB	5′ GCCAAGGCATCCACC 3′	Variable; 55	29
	rrs	pA	5′ AGAGTTTGATCCTGGCTCAG 3′	1,500; 55	12
		pH	5′ AAGGAGGTGATCCAGCCGCA 3′		
	V3 region *rrs*	16S (V3) 338F	5′GCCGCCCGCCGCGCGCGGCGGGCGGGGCGGG GGCACGGGGGGACTCCTACGGGAGGCA GCAG 3′	Variable; 55	49
		16S (V3) 520R	5′ ATTACCGCGGCTGCTGG 3′		
Wolbachia	*rrs*	99F	5′ TTGTAGCCTGCTATGGTATAACT 3′	864; 52	51
		994R	5′ GAATAGGTATGATTTTCATGT 3′		
	wsp	81F	5′ TGGTCCAATAAGTGTATGAAGAAAC 3′	600; 55	10
		165F	5′ AAAGGGGACTGATGATGT 3′	526; 52	79
		172F	5′ TGGTCCAATAAGTGATGAAGAAAC 3′	519; 52	79
		Aso3F	5′ AAAAATTAAACGCTACTCCA 3′	515; 52	73
		691R	5′ AAAGGGGACTGATGATGT 3′		79
Bacillus	*rrs*	BK-1F	5′ TCACAAGGCRACGATGG 3′	1,100; 58	77
		BK-1R	5′ CGTATTCACCGCGGCATG 3′		
Brevibacillus	*rrs*	F-1	5′ GCYTAAYACATGCAAGTCGARCG 3′	318; 68	32
		R-1	5′ ACTGCTGCCTCCCGTAGGAGT 3′		
Pseudomonas	*rrs*	Ps For	5′ GGTCTGAGAGGATGATCAGT 3′	990; 52	76
		Ps Rev	5′ TTAGCTCCACCTCGCGGC 3′		
Rickettsia	*rrs*	Rb-F	5′ GCTCAGAACGAACGCTATC 3′	963; 58	33
		Rb-R	5′ GAAGGAAAGCATCTCTGC 3′		
Staphylococcus	*rrs*	16S3 up	5′ ATGCAAGTCGAGCGAAC 3′	265; 57	61
		16S3 down	5′ TGTCTCAGTTCCAGTGTGGC 3′		
TOPO 2.1		M13F	5′ GTAAAACGACGGCCAG 3′	Variable; variable	
		M13R	5′ CAGGAAACAGCTATGAC 3′		
pQuantAlb	*wsp* A group	QAtdir1	5′ GRGTTGATRTTGAAGGRS 3′	264; 60	This study
		QArev2	5′ CACCAGCTTTTACTTGACC 3′		68
pQuantAlb16S	*rrs* fragment	519F	5′ CAGCMGCCGCGGTAANWC 3′	407; 63	65
		907R	5′ CCGTCAATTCMTTTRAGTT 3′		

[a] IG, intergenic gene between *rrs* and the 23S gene.

V3 fragments amplified from *Wolbachia* strain *w*Ri purified from infected *Drosophila simulans*. One faint band gave rise to two distinct sequences characteristic of the uncultivable genera *Duganella* and *Rickettsia*. The intense and lowest band also corresponded to *Rickettsia*. Amplification with specific *rrs* primers (Table 1) and sequencing of the 967-bp PCR products confirmed the band was from a *Rickettsia* sp., closely related (99% identity) to *Rickettsia* strain Brugge (Table 2). The upper bands exclusively found in individuals from Vp generated three sequences closely related to sequences from *Herbaspirillum*, *Pseudomonas*, and an unknown uncultivable bacterium. In some experiments, *Duganella*, *Herbaspirillum*, and *Pseudomonas* have been reported as contaminants (66). However, clonable PCR products were not generated with V3 primers in controls without a DNA template under the conditions used here, suggesting that there was no contamination.

Of the natural *A. tabida* populations, only lines from Co, Ig, Sfl, and Sla could be maintained in an insectarium. F_2 females and males of these recent laboratory-reared lines mostly had DGGE patterns similar to those of their F_0 parents, except for an additional band in Sfl F_2 insects and the absence of fragments corresponding to *Duganella* and *Rickettsia* in Co F_2 insects (see Fig. S1 in the supplemental material). In contrast, the long-term laboratory colonies (Sfl, Sla, Mo, and Pi) gave identical patterns (Fig. 2B) that differed from those of both natural and laboratory F_2 populations. Although the two bands corresponding to *Wolbachia* were detected, bands indicative of *Duganella* and *Rickettsia* were absent. Three new bands found were indicative of the family *Enterobacteriaceae* and the genera *Acetobacter* and *Acidomonas* (Table 2). As laboratory rearing clearly influences the composition of the bacterial community, we examined whether the bacteria detected in the wasps could be acquired from fly larvae during parasitic development. DGGE fingerprints (Fig. 2C) of the nursery *Wolbachia*-free *D. melanogaster* had many bands, which were clearly distinct from those found in *A. tabida*. DGGE profiles were similar in adult insects at emergence and at one week after emergence.

DGGE fingerprints of antibiotic-treated individuals. Usually, antibiotics are applied to host tissues to eliminate *Wolbachia* in order to analyze a particular phenotype ascribed to this symbiont. To investigate whether antibiotic treatment affects the bacterial composition of *A. tabida*, we used the Pi(3) line,

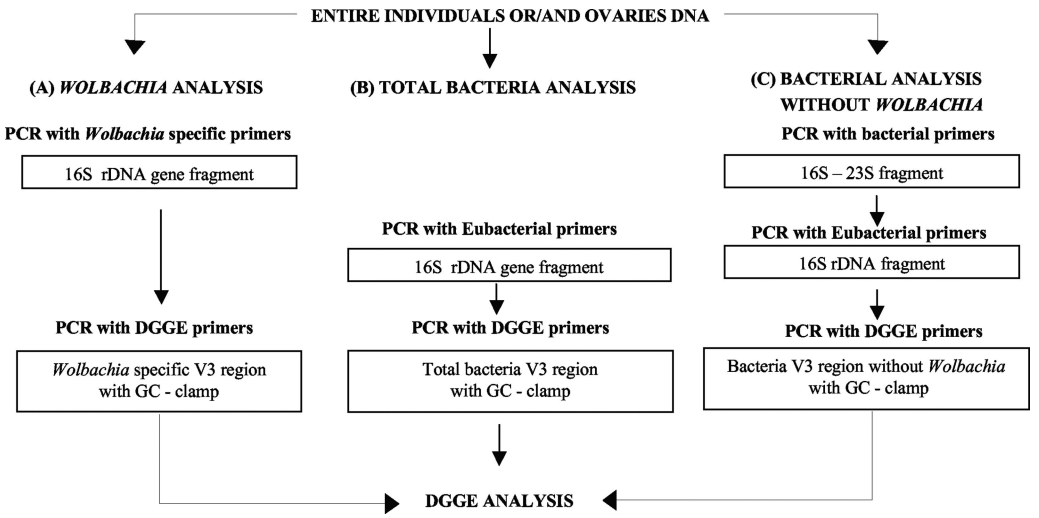

FIG. 1. Schematic view of PCR-DGGE strategies used to analyze the composition of bacterial communities in whole individuals and ovaries of *A. tabida*. (A and B) Two-step nested PCR strategy for amplification of the *rrs* V3 region from *Wolbachia* (A) and the total bacterial community (B). (C) Three-step nested-PCR strategy to amplify the *rrs* V3 region from the bacterial community while excluding *Wolbachia* amplicons.

generated from the triply infected line Pi(123) after moderate tetracycline treatment (19), and individuals were stably maintained in the laboratory. Pi(3) is singly infected with *Wolbachia* strain *w*Atab3, which is required for wasp oogenesis. The absence or presence of *w*Atab1 and *w*Atab2 was confirmed by PCR specific for *wsp* alleles (data not shown). PCR-DGGE amplification of Pi(3) yielded one intense band corresponding to *Wolbachia* (Fig. 3A, lanes 3 to 6), compared to the V3 fragments from Pi(123) (lanes 1 and 2) and *Wolbachia* strain *w*Ri, used as a control. Additional faint bands were consistently seen in both Pi(3) males and females. Moreover, the DGGE profiles of Pi(3) individuals newly generated by antibiotic treatment (Fig. 3A, lanes 3 and 4) showed one of the bands attributed to *Acidomonas* in Pi(123), whereas individuals reared for a long period in the laboratory had lost this band (Fig. 3A, lanes 5 and 6).

To determine whether the additional bands observed in Pi(3) are a genuine consequence of tetracycline treatment or an artifact where the high density of *Wolbachia* sequences in Pi(123) affects the detection of other bacterial sequences, a fingerprinting strategy was used that excluded *Wolbachia rrs* gene sequences from the profile (see Materials and Methods) (Fig. 1). Fingerprints were obtained for both old and new generations of Pi(3) and of Pi(123) in this way. A positive control containing the *rrs* gene of *Wolbachia* strain *w*Ri as the template was included. All the DGGE profiles (Fig. 3B) lacked the *Wolbachia* bands, as expected. Pi(123) female (lane 1) and male (lane 2) samples had only the two major fragments previously attributed to *Acetobacter* and *Acidomonas*, suggesting that the multiple *rrs* genes of the three *Wolbachia* strains did not impair amplification of the V3 regions of potential coinfecting bacteria in triply infected individuals. Again, Pi(3) had the most bands (Fig. 3B, lanes 3 to 6). Except for the absence of *Wolbachia* bands, the DGGE patterns were mostly similar to that of Pi(3) *Wolbachia* sequences (Fig. 3A), albeit with some bands being more intense. Several of these V3 fragments amplified from Pi(3) were cloned. All 16 cloned fragments (8 from males and 8 from females) were sequences characteristic of members of the *Enterobacteriaceae* (Table 2). Overall, these results indicate that experimental exclusion of *Wolbachia* V3 fragments did not significantly affect the detection of coinfecting bacteria. Therefore, tetracycline treatment affected the composition of the bacterial community of *A. tabida*, probably by eliminating other tetracycline-sensitive bacteria and/or allowing tetracycline-resistant bacteria to occupy free niches.

***Wolbachia* and total bacterial density.** As antibiotic treatment influences the composition of the bacterial community, we counted *Wolbachia* bacteria and all bacteria in whole bodies of untreated Pi(123) and treated Pi(3) females. There were significantly more *Wolbachia* organisms per wasp in triply infected ($12 \times 10^3 \pm 0.02 \times 10^3$) than in singly infected ($2 \times 10^3 \pm 0.001 \times 10^3$) individuals (Kruskal-Wallis test; $P < 0.0003$), with cell numbers being similar to those previously reported (47). Pi(3) individuals also harbored fewer bacteria, $5 \times 10^5 \pm 0.001 \times 10^5$ in total, compared to $8.4 \pm 0.009 \times 10^5$ in Pi(123). Overall, these findings demonstrate that when tetracycline was applied to generate singly infected Pi(3), it greatly reduced *Wolbachia* density but only slightly reduced the total number of bacteria, indicating that some bacterial populations increased after antibiotic treatment.

Bacteria in *A. tabida* ovaries. To search for potential maternally transmitted endosymbionts, PCR-DGGE V3 profiles of dissected insect ovaries (DIO) from Pi(123) and Pi(3) lines were obtained, and the two major bands were exclusively assigned to *Wolbachia* (Fig. 3C). Interestingly, PCR exclusion of the *Wolbachia rrs* gene fraction in DIO allowed new bands to be detected in both Pi(123) (Fig. 3D, lane 1) and Pi(3) (lane 2). A total of five doublets were excised from the gel, cloned, and sequenced. BLAST analysis (Table 2) indicated that sequences from Pi(123) were closely related to sequences from the *Gammaproteobacteria* (a *Pseudomonas* sp. and an endosymbiont of the bivalve *Petrasma* sp.), whereas those from Pi(3) were attributed to the group of *Firmicutes* (two *Staphylococcus* strains and one *Streptococcus* strain).

TABLE 2. Phylogenetic affiliation of sequences obtained in this study

Analysis and sample type	Band(s), name of clone, or fragment	Size (bp)	Accession no.	Phylogenetic affiliation	Closest relative organism	Accession no.	No. with identity/total (%)
DGGE							
Entire individuals	1; 2; 7; 8; 12; 13; 27; 28	169	FJ481970	*Alphaproteobacteria*	*Wolbachia* endosymbiont of *D. melanogaster* wMelPop	AB360385.1	169/169 (100)
	3a	171	FJ562342	*Alphaproteobacteria*	*Rickettsia* sp. strain Brugge	AF322443.1	170/171 (99)
	3b	193	FJ562343	*Betaproteobacteria*	Uncultured *Duganella* sp.	AM946212	186/193 (96)
	4	171	FJ562342	*Alphaproteobacteria*	*Rickettsia* sp. strain Brugge	AF322443.1	170/171 (99)
	5a	175	FJ562344		Uncultured bacterium, clone COL[lowem]aai14h01	EU460112.1	166/175 (94)
	5b	194	FJ562345	*Gammaproteobacteria*	*Pseudomonas* sp.	EU308476	194/194 (100)
	6	194	FJ562346	*Betaproteobacteria*	*Herbaspirillum* sp. strain IEH 4430	FJ267649	190/194 (97)
	9; 16; 22–26	194	FJ481973	*Gammaproteobacteria*	*Enterobacteriaceae*	AM940408.1	191/194 (98)
	10–14-17	169	FJ481971	*Alphaproteobacteria*	*Acetobacter pasteurianus* bh12	FJ227313.1	168/169 (99)
	11–15-18	169	FJ481972	*Alphaproteobacteria*	*Acidomonas methanolica*	AF127398.1	168/169 (99)
	19–21	195	FJ481974	*Gammaproteobacteria*	*Enterobacteriaceae*	AM940408.1	193/195 (98)
Ovaries	29–30	194	FJ481975	*Gammaproteobacteria*	*Pseudomonas* sp. R-35722 strain Z57b	AM886087.1	194/194 (100)
	31–32	194	FJ481969	*Gammaproteobacteria*	Endosymbiont of *Petrasma* sp.	FM213449.1	194/194 (100)
	33	194	FJ481976	*Firmicutes*	Uncultured *Staphylococcus* sp., clone WLB24	FJ405254.1	194/194 (100)
	34	195	FJ481977	*Firmicutes*	*Streptococcus* sp. strain F1	FJ405281.1	194/194 (100)
	35	194	FJ481978	*Firmicutes*	*Staphylococcus pasteuri* NJ-1	FJ392804.1	193/194 (99)
Bacterial isolates from ovaries							
Tri-infected *A. tabida*	KZ17a	1528	FJ481959	*Firmicutes*	*Brevibacillus agri* NCHU 1002	AY319301.1	1516/1530 (99)
Mono-infected *A. tabida*	KZ17b	1528	FJ481959	*Firmicutes*	*Brevibacillus agri* NCHU 1002	AY319301.1	1516/1530 (99)
	KZ2	1542	FJ481960	*Firmicutes*	*Staphylococcus epidermis* strain SR1, clone step.1051c07	AF270147.1	1539/1543 (99)
	KZ3	1543	FJ481961	*Firmicutes*	*Bacillus pumilus* SAFR-032	CP000813.1	1537/1544 (99)
Genus specific amplification							
Tri-infected *A. tabida*	*Brevibacillus*	321	FJ481962	*Firmicutes*	*Bacillus* sp. OS1	EF428970.1	320/321 (99)
	Pseudomonas	990	FJ481964	*Gammaproteobacteria*	Uncultured bacterium, clone nbt05e04	EU535862.1	990/990 (100)
	Staphylococcus	282	FJ481965	*Firmicutes*	Uncultured *Staphylococcus* sp., clone IS034B20	AY807427.1	280/283 (98)
	Staphylococcus	282	FJ481966	*Firmicutes*	*Staphylococcus pasteuri* ELA-9	FJ195007.1	271/282 (96)
	Rickettsia	967	FJ603467	*Alphaproteobacteria*	*Rickettsia* sp. strain Brugge	AF322443.1	955/967 (99)
Mono-infected *A. tabida*	*Brevibacillus*	299	FJ481963	*Firmicutes*	*Bacillus pumilus* Sua-BAC003	EU870500.1	298/299 (99)
	Pseudomonas	990	FJ481964	*Gammaproteobacteria*	Uncultured bacterium, clone nbt05e04	EU535862.1	990/990 (100)
	Staphylococcus	282	FJ481967	*Firmicutes*	Uncultured bacterium, clone AGB17	FM211024.1	281/282 (99)
	Staphylococcus	282	FJ481968	*Firmicutes*	Uncultured bacterium, clone nbt221d06	EU536773.1	278/282 (98)

Because DGGE fingerprinting revealed the presence of bacterial genera that have potentially cultivable relatives, a culture-dependent analysis was performed. Liquid cultures of DIO homogenates were plated on rich media. Two colony types were recovered from Pi(123) and three from Pi(3). For each colony type, two clones were selected for complete sequencing of the *rrs* gene. The *rrs* gene sequences (Table 2) of two isolates from Pi(123) and one isolate from Pi(3) corresponded to *Brevibacillus agri*. Two other isolates from Pi(3) were identified as *Bacillus pumilus* and *Staphylococcus epidermidis*. For all isolates, sequence similarities were up to 99% with respect to the *rrs* sequences of the type strains reported in databases.

To further investigate the occurrence of the *Firmicutes* and the *Gammaproteobacteria* in *A. tabida* ovaries, available genus-specific primers targeting *Brevibacillus*, *Staphylococcus*, and *Pseudomonas* were used in PCR on Pi(123) and Pi(3) DIO samples. In all cases, a PCR product of the expected size was obtained (data not shown). Cloning and sequencing the fragments confirmed the identity of the targeted genus (Table 2) and hence its occurrence in wasp ovaries.

Visualization of oocyte-inhabiting bacteria by FISH. To localize potential vertically transmitted bacterial symbionts, we used FISH for the first time in *A. tabida* using genus-specific fluor-labeled oligonucleotide probes on fixed oocytes from Pi(3) and Pi(123). DAPI staining showed both cell nuclei and dots which correspond to bacteria (Fig. 4A). Specific signals for *Wolbachia* were detected in the cytoplasm of posterior oocytes (Fig. 4B and C). The signals were more intense in Pi(123) oocytes (Fig. 4B), extending to the subpole area, than in Pi(3) oocytes (Fig. 4C), and the location was usual for *Wolbachia* strains in these lines (19, 57). No fluorescent signals

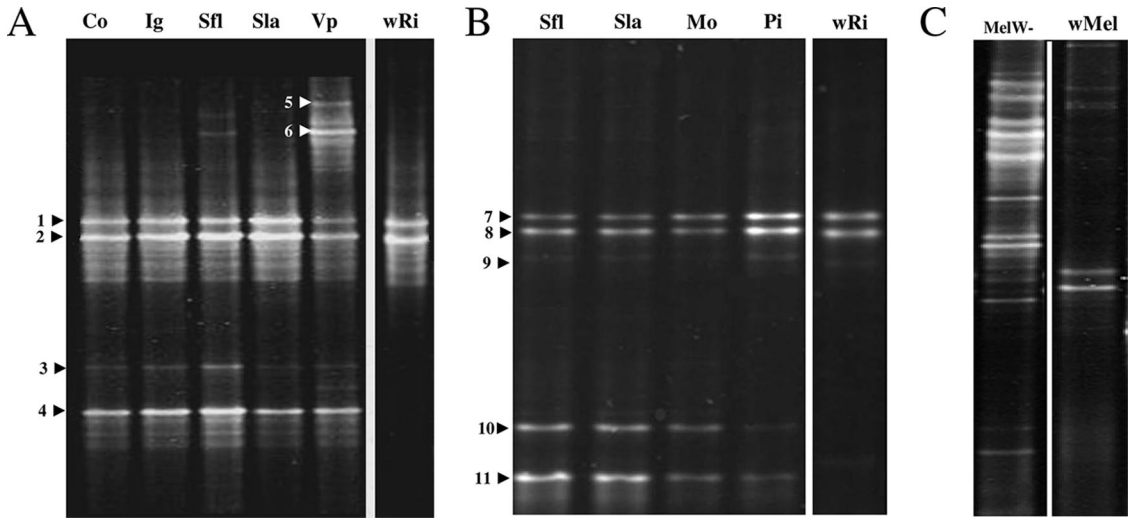

FIG. 2. DGGE profiles of hypervariable *rrs* V3 segments of *A. tabida* in native (A) and laboratory (B) populations. wRi, *Wolbachia* strain purified from *D. simulans* Riverside (44). Numbers correspond to cloned and sequenced bands (Table 2). (C) Profile of *D. melanogaster* used to rear *A. tabida* lines. wMel, *Wolbachia* strain purified from infected *D. melanogaster*.

were detected in ovaries with probes targeting the genera *Brevibacillus* and *Pseudomonas*, although fluorescent dots were observed in cultures from both bacteria (not shown).

DISCUSSION

Bacterial endosymbionts of the wasp *A. tabida* were investigated by culture and nonculture methods. Members of the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Firmicutes* were found, with some variation in the species recorded, depending on the method used. These results were in accordance with previous reports of microbes associated with insects, including *Drosophila* (17), mosquitoes (56), and termites (27). Although the main bacterial phyla are shared with many other arthropod lineages, we found that native *A. tabida* populations harbor a microbial community dominated by the genera *Wolbachia* and *Rickettsia*, in the order *Rickettsiales*. Two genera, *Duganella* and *Herbaspirillum*, were also found in some native *A. tabida* populations.

The multiple infection of *Asobara* species by *Wolbachia* is well documented (20, 47, 73); these bacteria are transmitted transovarially, and two strains induce cytoplasmic incompatibility, whereas one strain controls oogenesis through a cell death process (21, 52, 73). Before this study, no *Asobara* species had been found to be associated with *Rickettsia*. The latter genus is usually described as the causative agent of rickettsiosis in vertebrates transmitted by blood-feeding arthropods (3). However, in recent years, members of *Rickettsia* have been found in association with nonhematophagous arthropods, in which they induce some changes in reproduction, as does the related genus *Wolbachia* (54). The *Rickettsia* strain found here was similar to the obligate symbiont necessary for the development of oocytes and egg laying in parthenogenetic booklice (55). Its role in *A. tabida* is yet to be established.

The coexistence of heritable *Wolbachia* and *Rickettsia* with other symbionts has been reported for the hematophagous tick *Ixodes scapularis* (8), the aphid *Cinera cedri* (31), and the whitefly *Bemisia tabaci* (33). *Wolbachia* may infect an estimated 20% to 70% of insect species (38, 75). In some natural host populations, the prevalence of *Wolbachia* within species can be high, up to 97% incidence for *Drosophila simulans* infected with strain *w*Ri (69) and almost 100% for mosquito *Aedes albopictus* infected with *w*AlbB (41). However, between 20% and 100% of *Bemisia tabaci* whiteflies from the north to the south of Israel were infected with *Rickettsia*, *Wolbachia*, and three other bacterial genera (33). Overall, these data indicated that multiple infections with members of the *Rickettsiales* occur in several invertebrate lineages.

When bacterial populations in laboratory-reared *A. tabida* were surveyed, *Wolbachia* was consistently found in all individuals, whereas *Rickettsia* was not detectable in the F_2 generation of one of the four native populations recently maintained in the insectarium. Moreover, *Rickettsia* was never found in stock lines that had been reared in the laboratory for a long period; two of these lines (Sfl and Sla) originated from regions where recently caught individuals harbored *Rickettsia*, suggesting that this bacterium could become dispensable for the wasp under laboratory conditions. Loss of *Rickettsia* was accompanied by the appearance of *Acetobacter*, *Acidomonas*, and members of the family *Enterobacteriaceae*. The evolutionary behavior governing the dynamics of such cohabitation is mostly unknown and is under investigation.

The composition of bacterial populations was consistently modified by the antibiotic treatment applied to generate a subline of *A. tabida* singly infected by *Wolbachia* strain *w*Atab3. There were more bands in the DGGE patterns of the tetracycline-treated Pi(3) line than in those of the parental triply infected Pi(123) line. It is possible that the tetracycline-sensitive fraction of the bacterial population was eliminated, allowing an increase in the number of resistant bacteria. No significant difference in the overall density of bacteria was found between treated and untreated lines. However, multi-infected individuals harbored a significantly higher proportion of *Wolbachia*, consistent with previous data confirming that the strain

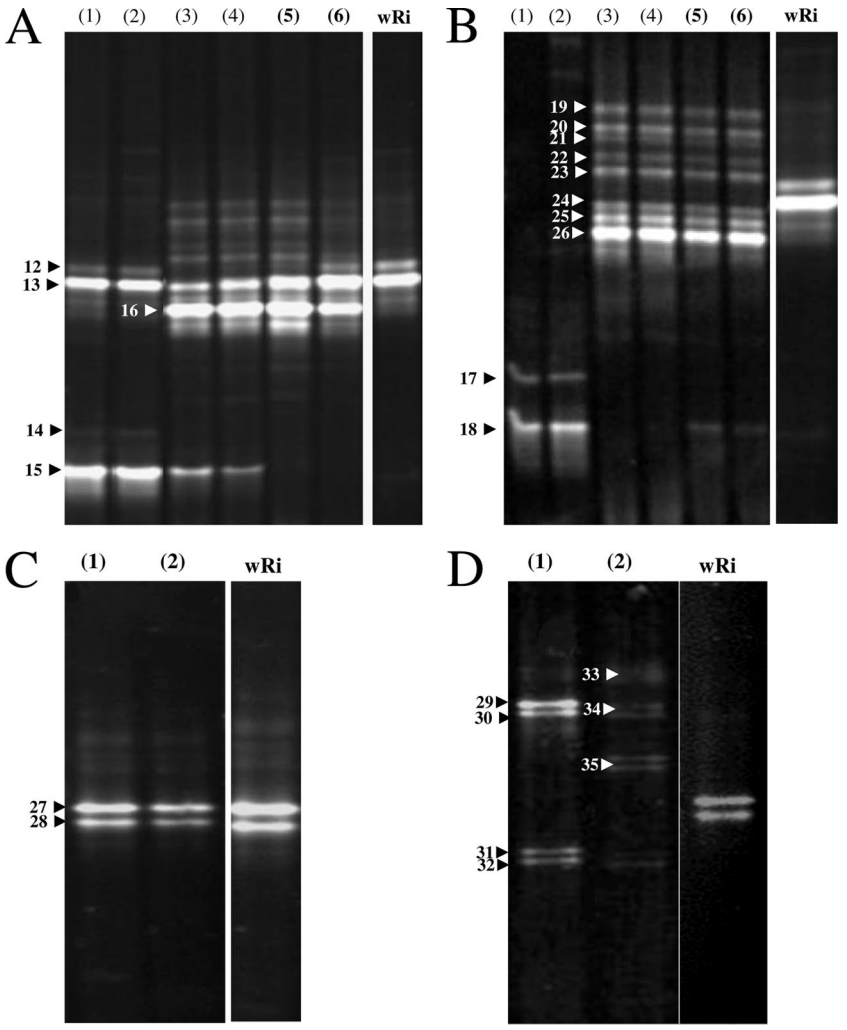

FIG. 3. Examples of *rrs* V3 DGGE profiles from untreated Pi(123) and tetracycline-treated Pi(3) *A. tabida*. Amplification with (A and C) and without (B and D) *Wolbachia* sequences is depicted in Fig. 1. (A and B) Whole triply infected female (lane 1) and male (lane 2) and whole singly infected female (lanes 3 and 5) and male (lanes 4 and 6). (C and D) Ovaries from triply infected (lane 1) and singly infected (lane 2) females. wRi, *Wolbachia* strain purified from *D. simulans* Riverside. Numbers correspond to cloned and sequenced bands (Table 2).

*w*Atab3 does not proliferate to occupy the niche liberated by the other two strains (*w*Atab1 and *w*Atab2). Surprisingly, all the bands detected in the tetracycline-treated line were characteristic of the family *Enterobacteriaceae*. This may represent diverse genera, as the *rrs*-based phylogeny of *Enterobacteriaceae* is not clear (50, 62). Members of the *Enterobacteriaceae* are regularly found in invertebrates, including *Photorhabdus* and *Xenorhabdus* in *Steinernema* or *Heterorhabditis* (28), *Klebsiella* in *Drosophila* (17), *Pantoea* in mosquitoes (56), and *Rhanella* in pine beetles (71). Furthermore, several insect lineages, such as aphids, psyllids, whiteflies, weevils, and wasps, harbor members of the *Rickettsiales* together with some heritable *Enterobacteriaceae*, notably *Arsenophonus*, *Buchnera*, *Hamiltonella*, *Regiella*, *Serratia*, *Sodalis*, and *Wigglesworthia* (1, 5, 30, 35, 45, 46, 67). However, none of the sequences recorded here was unequivocally matched to these genera; this will require further taxonomic study.

To look for potential maternally inherited symbionts, nonculture methods, exclusion of *Wolbachia* sequences from DGGE analysis, and culture were used to identify members of the *Gammaproteobacteria* and *Firmicutes* in ovary homogenates. In addition to *Wolbachia*, DGGE *rrs* V3 sequences came from a *Pseudomonas* sp. (an endosymbiont of the bivalve *Petrasma* sp.), *Streptococcus*, and *Staphylococcus*. Isolated bacteria were *Bacillus pumilus*, *Brevibacillus agri*, and *Staphylococcus epidermis*, all members of the *Firmicutes*. Mostly known as being associated with vertebrates (26), members of *Firmicutes* have also been described in insects, including *D. melanogaster* (18), gypsy moths (11), and the mosquito *Culex quinquefasciatus* (57). However, only *Wolbachia* was detected in ovaries by FISH, a technique applied for the first time in *A. tabida*. The location of *Wolbachia* in oocyte poles is in agreement with published data (19, 57). Probes specific for *Brevibacillus* and *Pseudomonas* did not produce clear signals in entire ovaries, suggesting that PCR products or isolates obtained from homogenates might be dissection contaminants from other organs of the insect body, such as the gut, which is known to contain such bacteria (72).

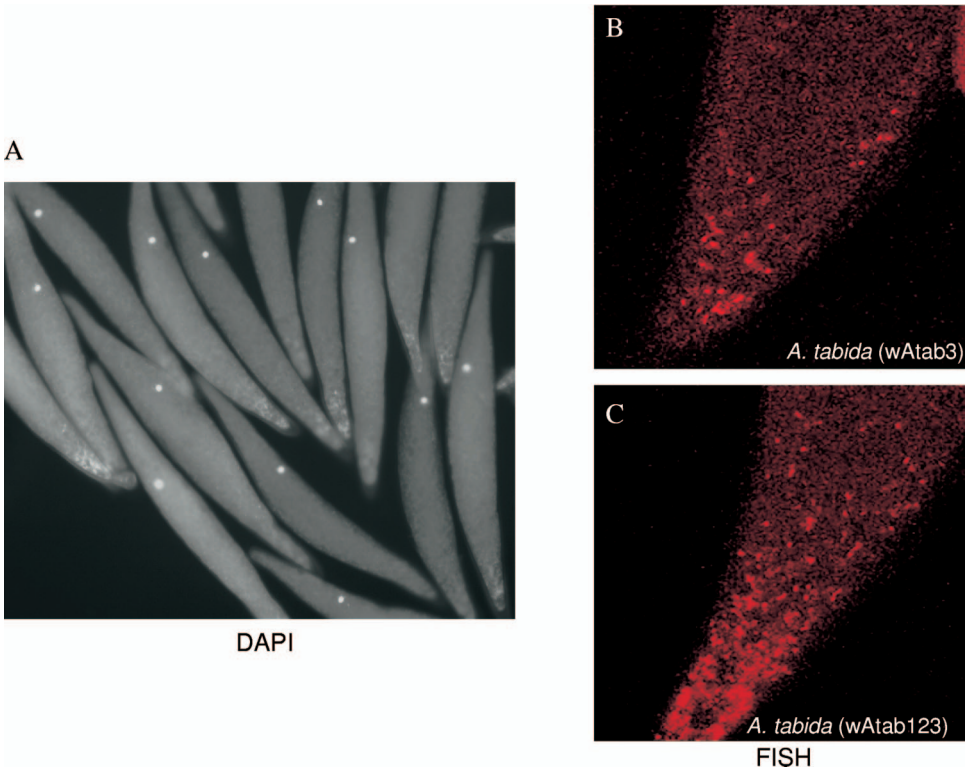

FIG. 4. Fluorescent confocal microscopy of *A. tabida* oocytes infected with *Wolbachia*. (A) DAPI staining of *A. tabida* oocytes containing *Wolbachia* strain *w*Atab3. (B and C) Specific probe for *Wolbachia* in singly (B) and triply (C) infected oocytes.

Multiple bacterial infections, commonly found in members of the Arthropoda, may create competition or cooperation between symbionts and the host immune defense (13, 16, 24, 43). To infect and persist in a host population, symbiotic bacteria usually increase host fitness or manipulate host reproduction, maximizing their transmission from one generation to the next. In *A. tabida*, three *Wolbachia* strains coexist and are maintained either by inducing cytoplasmic incompatibility or by controlling oogenesis. The biological roles of other bacteria discovered in the wasp are under investigation. Many of these bacteria are commonly found in the environment, including soil and plants. For instance, members of the *Enterobacteriaceae* such as *Herbaspirillum* can evolve as endophytes able to fix atmospheric nitrogen in planta (53). As insects grow on diets with extremely high carbon-to-nitrogen (C:N) ratios, bacterial nitrogen fixation may provide an additional nitrogen source (6). Commensal *Firmicutes* in the digestive tracts of some insects contribute to the degradation of polymers, such as chitin and cellulose, and of aromatic compounds that can be toxic to microorganisms (42). Acetic acid bacteria such as *Acetobacter* and *Acidomonas* use sugar fermentation to produce ethanol, which in turn can be oxidized into acetic acid, which acidifies their habitats, notably insect guts (40). Some bacteria detected here, assigned to known or unknown groups, are not yet cultivable and may have roles in *A. tabida* biology. When such potential symbiotic roles are studied, antibiotics should be used in the knowledge that they may affect both the targeted and nontargeted bacteria, as shown here, and thus influence the outcomes of the biological interactions, as recently found in different animal taxa (22, 70).

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