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Mavragani-Tsipidou and Kostas Bourtzis
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Acetobacter tropicalis Is a Major Symbiont of the Olive Fruit Fly (*Bactrocera oleae*)[∇]

Ilias Kounatidis,^{1†} Elena Crotti,^{2†} Panagiotis Sapountzis,³ Luciano Sacchi,⁴ Aurora Rizzi,² Bessem Chouaia,⁵ Claudio Bandi,⁵ Alberto Alma,⁶ Daniele Daffonchio,² Penelope Mavragani-Tsipidou,¹ and Kostas Bourtzis^{3*}

Department of Genetics, Development and Molecular Biology, School of Biology, Faculty of Sciences, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece¹; Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, 20133 Milan, Italy²; Department of Environmental and Natural Resources Management, University of Ioannina, 30100 Agrinion, Greece³; Dipartimento di Biologia Animale, Università di Pavia, 27100 Pavia, Italy⁴; Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Università degli Studi di Milano, 20133 Milan, Italy⁵; and Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, Università degli Studi di Torino, 10095 Turin, Italy⁶

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Following cultivation-dependent and -independent techniques, we investigated the microbiota associated with *Bactrocera oleae*, one of the major agricultural pests in olive-producing countries. Bacterial 16S rRNA gene libraries and ultrastructural analyses revealed the presence of several bacterial taxa associated with this insect, among which *Acetobacter tropicalis* was predominant. The recent increased detection of acetic acid bacteria as symbionts of other insect model organisms, such as *Anopheles stephensi* (G. Favia et al., Proc. Natl. Acad. Sci. USA 104:9047–9051, 2007) or *Drosophila melanogaster* (C. R. Cox and M. S. Gilmore, Infect. Immun. 75:1565–1576, 2007), prompted us to investigate the association established between *A. tropicalis* and *B. oleae*. Using an *A. tropicalis*-specific PCR assay, the symbiont was detected in all insects tested originating from laboratory stocks or field-collected from different locations in Greece. This acetic acid bacterium was successfully established in cell-free medium, and typing analyses, carried out on a collection of isolates, revealed that different *A. tropicalis* strains are present in fly populations. The capability to colonize and lodge in the digestive system of both larvae and adults and in Malpighian tubules of adults was demonstrated by using a strain labeled with a green fluorescent protein.

Associations of insects with bacteria, protozoa, and fungi are complex and intimate, ranging from parasitism to mutualism, and may be extracellular or intracellular and may play a role in the nutrition, the physiology, or the reproduction of the insect host (10). Petri (1909 to 1910) described one of the first bacterial symbiotic associations in an insect species, the olive fly, *Bactrocera (Dacus) oleae* (31, 32).

The olive fruit fly *B. oleae* is one of the major pests of the olive tree, strongly affecting olive production worldwide, especially in the Mediterranean area, where more than 90% of the world's olive tree cultivation takes place (24, 27). Although there have been reports on the isolation of potentially effective *Bacillus thuringiensis* strains against *B. oleae*, olive fly control strategies remain almost exclusively based on insecticides, despite the awareness of a need for the use of more environmentally friendly control methods (29). Recently, new concepts are emerging, among which the symbiotic control approach is particularly noteworthy (4). This strategy includes the use of symbionts as vectors of antagonistic factors able to block the life cycle of the plant pathogen in the insect host or, alternatively, their use for the suppression of host natural populations (45). In any case, a prerequisite for developing a symbiotic control approach is the knowledge of the microbiota associated with the insect pest.

The nature of the olive fruit fly-associated microbiota is controversial. The culturable bacterium *Pseudomonas savastanoi* has been suspected to be a mutualist of *B. oleae* for more than 50 years (6, 17, 22, 25, 32, 33). In addition, traditional microbiological approaches have identified other bacteria of the genera *Bacillus*, *Erwinia*, *Lactobacillus*, *Micrococcus*, *Pseudomonas*, *Streptococcus*, *Citrobacter*, *Proteus*, *Providencia*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Serratia*, and *Xanthomonas* as associated with the olive fruit fly (3, 14, 19, 37). Recently, it was suggested that the bacterium housed within the esophageal bulb and the midgut of *B. oleae* is unculturable, and the novel name "*Candidatus* *Erwinia dacicola*" was proposed (7). The presence of "*Ca. Erwinia dacicola*" was confirmed in Italian natural populations (36).

The contradictory results obtained in previous studies prompted us to investigate the microbiota associated with both laboratory and natural populations of the olive fruit fly by employing both cultivation-independent and -dependent methods.

MATERIALS AND METHODS

Insects. Wild specimens of *B. oleae* were field collected as pupae from 10 different locations in Greece (Thessalonica, Poligiros, Sani, Lesbos, Katerini, Volos, Agrinion, Kalamata, Aigio, and Crete) in September 2006. Upon emergence, adult olive flies were surface sterilized and stored at –20°C until studied. A laboratory population of *B. oleae*, reared at the Aristotle University of Thessaloniki, was also used. Insects were kept in cages at 25 ± 1°C with a 14:10 h light-dark photoperiod. Larvae were reared on a cellulose-based artificial diet, which included yeast, soy hydrolysates, sugar, and olive oil as nutrients, Tween 80 as emulsifier, and hydrochloric acid, sodium sorbate, and nipagin as antimicrobial agents (38, 39). Adults were reared on an artificial diet, which included yeast hydrolysates, sugar, egg yolk, and streptomycin (40, 41).

* Corresponding author. Mailing address: Department of Environmental and Natural Resources Management, University of Ioannina, 2 Seferi Street, 30100 Agrinio, Greece. Phone: 30 26410 74114. Fax: 30 26410 74171. E-mail: kbourtzis@uoi.gr.

† I.K. and E.C. contributed equally to this work.

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Initial investigation of *B. oleae* microbiota. Total DNA was individually extracted from third-instar larvae and late pupae (10 to 11 days old) or adults of the laboratory strain by using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Amplification of the 16S rRNA gene was performed using two different sets of universal bacterial primers. The first pair, which amplified a 1,324-bp fragment, consisted of 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (23). The second pair, generating a 1,472-bp amplicon, consisted of fD1 (5'-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G-3') and rP2 (5'-CCC GGG ATC CAA GCT TAC GGA TAC CTT GTT ACG ACT T-3') (43). Both pairs of primers were used under the following PCR conditions: 3 min at 94°C, followed by 35 cycles of 3 min at 94°C, 1 min at 50°C, and 1 min at 70°C, and then a final extension of 10 min at 72°C using *Taq* DNA polymerase (Promega, Madison, MI). PCR products were purified with a QIAquick Gel Extraction Kit (Qiagen) and sequenced, and sequences were compared to the National Center for Biotechnology Information (NCBI) databases using BLASTn (1).

16S rRNA gene libraries. One male and one female from the laboratory strain were randomly chosen for the establishment of 16S rRNA gene libraries. DNA from the insect and its associated microorganisms was extracted using a QIAamp DNA minikit (Qiagen) and used as a template in PCRs with the universal bacterial 16S rRNA primers 63F and 1387R. 16S rRNA gene products were purified with a QIAquick gel extraction kit (Qiagen) and then cloned into the pGEM-T Easy Vector (Promega, Madison, MI) and transformed into JM109 competent cells (Promega). More than 60 16S rRNA gene clones from each library were fully sequenced and analyzed.

TEM analysis. Adult insects were dissected with sterile scalpels and small forceps in a sterile saline solution and fixed in 0.1 M cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde for 3 h at 4°C. The samples were then washed and fixed in 1% OsO₄ in 0.1 M cacodylate buffer (pH 7.2) for 1.5 h at 4°C. All samples were successively dehydrated in ethanol and embedded in Epon 812. Semithin sections were stained with 0.05% toluidine blue in 1% sodium tetraborate and examined by optical microscopy. Thin sections (80 nm) were stained with uranyl acetate and lead citrate and examined under a Zeiss EM900 transmission electron microscope (TEM) at 80 kV.

***A. tropicalis*-specific PCR assay.** One microliter of the insect DNA was used as a template in PCRs with *A. tropicalis*-specific primers AcetF3 (5'-AAT GAC TGG GCG TAA AGG G-3') and AcetR3 (5'-GGA CAC AGC CTA CAC ATA CA-3'). The PCRs were performed in a final volume of 20 µl using 20 pmol of each primer, a 0.125 µM concentration of the deoxynucleoside triphosphates, 1.5 mM MgCl₂, and 1 U of *Taq* polymerase using the company-supplied buffer (HyTest Ltd., Turku, Finland). Reactions were performed as follows: an initial denaturation step of 4 min at 94°C, followed by 35 cycles consisting of 45 s at 94°C, annealing for 45 s at 62°C, and elongation for 45 s at 72°C. The final extension step was 7 min at 72°C. *Wolbachia*-infected *Drosophila* DNA was used as a negative control. At least four PCR clones from each population of *B. oleae* were sequenced.

Isolation of *A. tropicalis*. *A. tropicalis* strains were repeatedly isolated from laboratory-reared insects (adults and pupae). At least two insects were used for each isolation attempt. Enrichment medium 1 was used as described previously (21). Pupae were surface sterilized in 1% sodium hypochlorite for 2 min, washed three times with sterile 0.9% NaCl, and homogenized by grinding in 200 µl of 0.9% NaCl. Adults were washed three times by rinsing with sterile 0.9% NaCl, followed by homogenization in 200 µl of 0.9% NaCl. The homogenate was inoculated into enrichment medium 1 (21) and incubated at 30°C for 3 days with shaking. When microbial growth was detected, microorganisms were streaked on CaCO₃ agar plates, pH 6.8, as previously described (21). After 3 days of incubation, circular nonpigmented colonies capable of clearing CaCO₃ were chosen and streaked on fresh medium. All isolates were tested for the capability to grow at 42°C on GLY medium (2.5% glycerol and 1% yeast extract, pH 5).

16S rRNA gene-based identification and fingerprinting analyses of *A. tropicalis* isolates. Chromosomal DNA was extracted from the isolates through treatment with proteinase K and sodium dodecyl sulfate, as previously described (13). The 16S rRNA gene was amplified with universal bacterial 16S rRNA gene primers 27F (5'-TCG ACA TCG TTT ACG GCG TG-3') and 1495R (5'-CTA CGG CTA CCT TGT TAC GA-3'). The PCR products were sequenced, and the sequences were compared to the databases at the NCBI using BLASTn and aligned with their closest type strain relatives using Clustal W (<http://align.genome.jp/>). Phylogenetic analyses were performed using Jukes and Cantor distance estimation with the TREECON software, version 1.3b. A 50% majority rule bootstrap consensus tree (2,000 replicates) was generated. Gaps were treated as a fifth base.

Internally transcribed spacer (ITS)-PCR fingerprinting was performed with primers ITSF (5'-GCC AAG GCA TCC AAC-3') and ITSr (5'-GTC GTA

ACA AGG TAG CCG TA-3'), as previously described (9). tRNA-PCR fingerprinting was performed with primer T3A (5'-GGG GGT TCG AAT TCC CGC CGG CCC CA-3') according to standard protocols (5). BOX-PCR was performed with primer BOX-A1 (5'-CTA CGG CAA GGC GAC GCT GAC-3') as previously described (42). Randomly amplified polymorphic DNA (RAPD)-PCR was performed with primer Opa4 (5'-ATT CGG GCT G-3'), according to standard protocols (9).

Transformation of *A. tropicalis* BA1.3 with the plasmid pHM2-GFP. Plasmid pHM2-GFP (where GFP is green fluorescent protein) (13) was electroporated into *A. tropicalis* strain BA1.3. Electrocompetent cells were prepared as previously described with slight modifications (28). Briefly, late-exponential phase cells grown in GLY medium were washed twice with cold 1 mM HEPES, pH 7, and once with cold 10% (vol/vol) glycerol. Finally, the cells were resuspended in 10% (vol/vol) cold glycerol to give 150-times-concentrated competent cells. Aliquots were stored at -80°C. A total of 750 ng of plasmid DNA was mixed with 65 µl of competent cells, transferred to a cold 0.1-cm-diameter cuvette, and pulsed at 2,000 V with an Electroporator 2510 (Eppendorf, Milan, Italy). Immediately after the pulse, 1 ml of GLY medium was added to the cells, followed by incubation at 30°C for 4 h. Transformed cells were chosen by plating on GLY agar medium containing 100 µg ml⁻¹ kanamycin, 40 µg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and 0.5 mM isopropyl-β-D-thiogalactopyranoside for the detection of a Lac⁺ phenotype. After 48 h of incubation at 30°C, blue transformants were chosen. GFP expression and the identity of strain BA1.3 transformants were verified by fluorescence microscopy and 16S rRNA gene sequencing, respectively.

Evaluation of *A. tropicalis* BA1.3(GFP) plasmid stability. The generation time of strain BA1.3(GFP) was estimated by monitoring its growth in GLY medium. The stability of plasmid pHM2-GFP in strain BA1.3(GFP) was evaluated as previously described (15). *A. tropicalis* BA1.3(GFP) was grown in GLY medium with 100 µg ml⁻¹ kanamycin and diluted the next day into GLY medium without antibiotic. The culture was grown to an optical density at 600 nm (OD₆₀₀) of 1 before dilution. This was done three consecutive times over the course of the experiment. The proportion of kanamycin-resistant bacterial cells was determined by plating on GLY plates containing 100 µg ml⁻¹ kanamycin every time the culture reached an OD₆₀₀ of 1. Total cell number was determined by plating on GLY plates without antibiotic.

Colonization experiments of *B. oleae* with *A. tropicalis* BA1.3(GFP). *A. tropicalis* BA1.3(GFP) was grown in GLY medium containing 100 µg ml⁻¹ kanamycin to a concentration of 10⁸ cells ml⁻¹. After being harvested, the cells were washed and resuspended in sterile water to a final concentration of 2 × 10⁸ cells ml⁻¹ or 1 × 10⁹ cells ml⁻¹ for the colonization experiments of olive fruit fly larvae or adults, respectively.

For the colonization experiments, three sterile petri dishes were prepared containing 2 g of sterile food and 10 larvae (4 days old) each. To the first dish, 500 µl of the bacterial suspension containing 200 µg ml⁻¹ kanamycin was added, while to the second and third dishes, 200 µl ml⁻¹ kanamycin in 500 µl of sterile water and just 500 µl of sterile water were added, respectively. The colonization experiments described above were performed in duplicate. The larvae were allowed to feed for 72 h. Every 24 h, 200 µg ml⁻¹ kanamycin was added to the petri dishes. Every 24 h after feeding, insect organs and tissues were dissected in Ringer solution (6.5 g liter⁻¹ NaCl, 0.14 g liter⁻¹ KCl, 0.2 g liter⁻¹ NaHCO₃, 0.12 g liter⁻¹ CaCl₂ · 2H₂O, 0.01 g liter⁻¹ NaH₂PO₄ · 2H₂O, pH 6.8). Organs and tissues were fixed in 4% paraformaldehyde at 4°C for 10 min and mounted in glycerol for analysis by fluorescence microscopy (Eclipse 80i; Nikon, Japan) and confocal laser scanning microscopy (CLSM) (Eclipse 80i; Nikon, Japan).

As a further control of *B. oleae* larva colonization by *A. tropicalis*, cocolonization experiments with both *A. tropicalis* BA1.3(GFP) and *Escherichia coli* DH5α pKan(DsRed) were carried out. *A. tropicalis* BA1.3(GFP) and *E. coli* DH5α pKan(DsRed) were grown in GLY and LB medium, respectively, both supplemented with 100 µg ml⁻¹ kanamycin. Cells were harvested by centrifugation, washed, and resuspended in sterile water to a final concentration of 2 × 10⁸ cell ml⁻¹. A total of 500 µl of each *A. tropicalis* BA1.3(GFP) and *E. coli* DH5α pKan(DsRed) cell suspension was mixed, supplemented with 100 µg ml⁻¹ kanamycin, and administered to 10 larvae placed, in duplicate, in a petri dish. Four control experiments with the administration of 100 µg ml⁻¹ kanamycin or *A. tropicalis* BA1.3(GFP) or *E. coli* DH5α pKan(DsRed) or water were performed.

Colonization experiments of adults were performed by placing 20 adults (1 day old), 10 females and 10 males, in a small cage. A 100-µl bacterial suspension (10⁷ or 10⁸ cells) was added to 0.5 g of adult sterile food containing 200 µg ml⁻¹ kanamycin, and small drops of the obtained mixture were placed inside the cage on parafilm-covered glass slides. Appropriate controls without the addition of bacteria were done; these included a cage with 20 insects fed with the sterile food without the addition of the transformed bacteria or kanamycin and a second cage

TABLE 1. Bacterial species found in 16S rRNA gene libraries from *B. oleae*

Phylum	Order	Most similar known species (% 16S rRNA gene similarity)	% Clones detected in ^a :	
			Males	Females
Alphaproteobacteria	Rhodospirillales	<i>A. tropicalis</i> (99)	43.8	16.9
Firmicutes	Lactobacillales	<i>E. faecalis</i> (98)	39.0	5.6
Firmicutes	Bacillales	<i>P. glucanolyticus</i> (99)	17.2	77.5

^a The percentages reported are calculated on a total of 64 clones for the male and 71 clones for the female individual.

with 20 insects fed with the sterile food containing 200 $\mu\text{g ml}^{-1}$ kanamycin. The insects were allowed to feed for 72 h. Every 24 h, 200 $\mu\text{g ml}^{-1}$ kanamycin was added to the adult food. Every 24 h postfeeding, organs and tissues were dissected in Ringer solution and were fixed and mounted in glycerol for fluorescence microscopy and CLSM analyses.

RESULTS AND DISCUSSION

Bacterial diversity in *B. oleae* and isolation of *A. tropicalis*.

DNA extracted from laboratory as well as from field-collected olive fruit flies was used for 16S rRNA gene PCR amplification with two different sets of universal bacterial primers. Male and female adults, third-instar larvae, and late pupae (10 to 11 days

old) were analyzed. Surprisingly, direct sequencing of the obtained PCR products revealed the presence, in all tested samples, of a single bacterial species, the acetic acid bacterium *A. tropicalis* with a nucleotide identity of 99.7% to *A. tropicalis* strain A77 (DQ523494). This indicates either that the chromosomes of this bacterium by far outnumbered those of other bacteria or that the PCR primers used were biased.

Two 16S rRNA gene libraries were established, one from a male and the other from a female (1-day-old adults were used). Sequence analysis confirmed the presence of *A. tropicalis* as well as the presence of *Enterococcus faecalis* and *Paenibacillus glucanolyticus* (Table 1). The three bacterial species were detected both in male and female insects. *A. tropicalis* and *E. faecalis* were more abundant in the male while *P. glucanolyticus* was more abundant in the female.

The bacterial diversity associated with the insect was also studied by ultrastructural analysis. Midguts of male and female *B. oleae* insects were shown to harbor a polymorphic bacterial community. However, electron microscopy analysis alone cannot establish whether this association was transient or permanent. Four different morphotypes of gram-negative bacteria were recognized, as shown in Fig. 1. The only morphotype consistent with the predominant bacteria identified in the

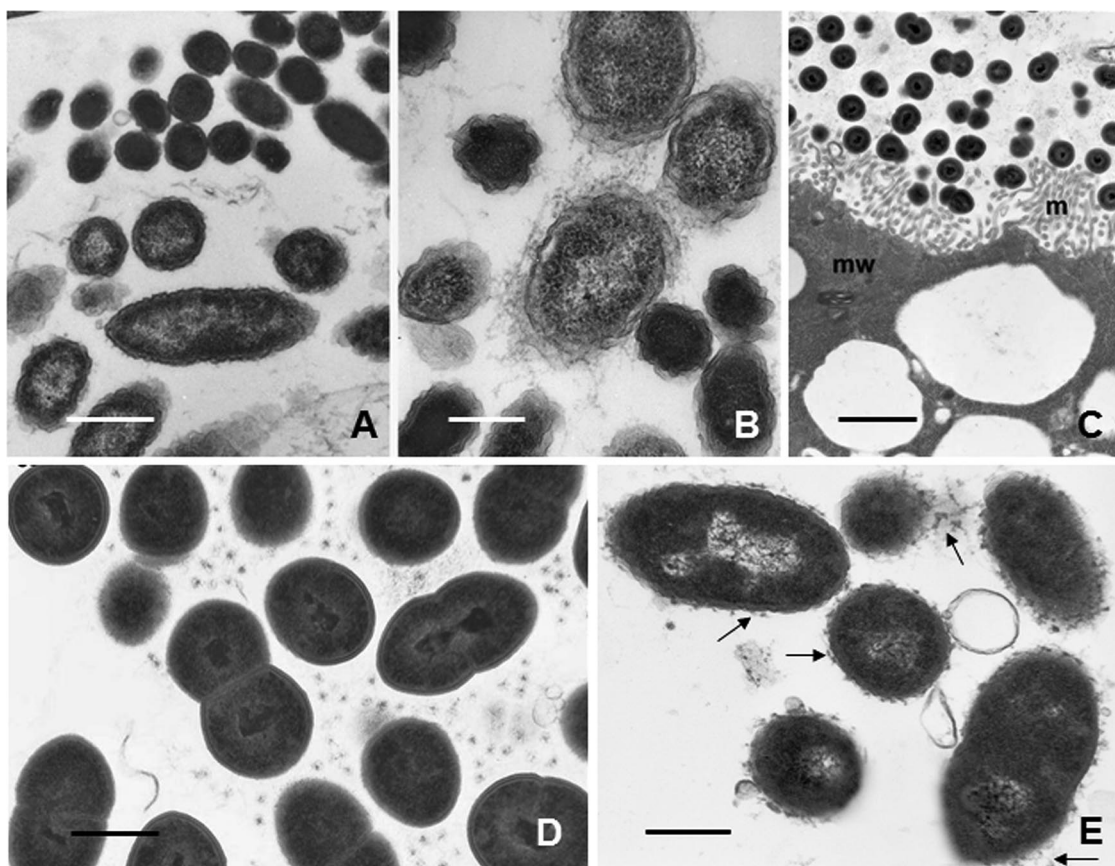


FIG. 1. TEM images of the midgut of *B. oleae* adults showing four different morphotypes of gram-negative bacteria. (A) Cluster of small bacteria with another group of larger bacteria. (B) Details of the bacterial cells. (C) Clusters of bacteria were observable in the midgut lumen. m, microvilli; mw, midgut wall. (D) Detail at a higher magnification, showing the diplococcal structure of bacteria, possibly belonging to the genus *Neisseria*. (E) Bacterial cells with a brightly appearing nucleoid region and traces of an extracellular fibrous matrix (arrows) can be recognized in the gut (Fig. 4J). Bars, 0.6 μm (A), 0.2 μm (B), 1.9 μm (C), 0.5 μm (D), and 0.15 μm (E).

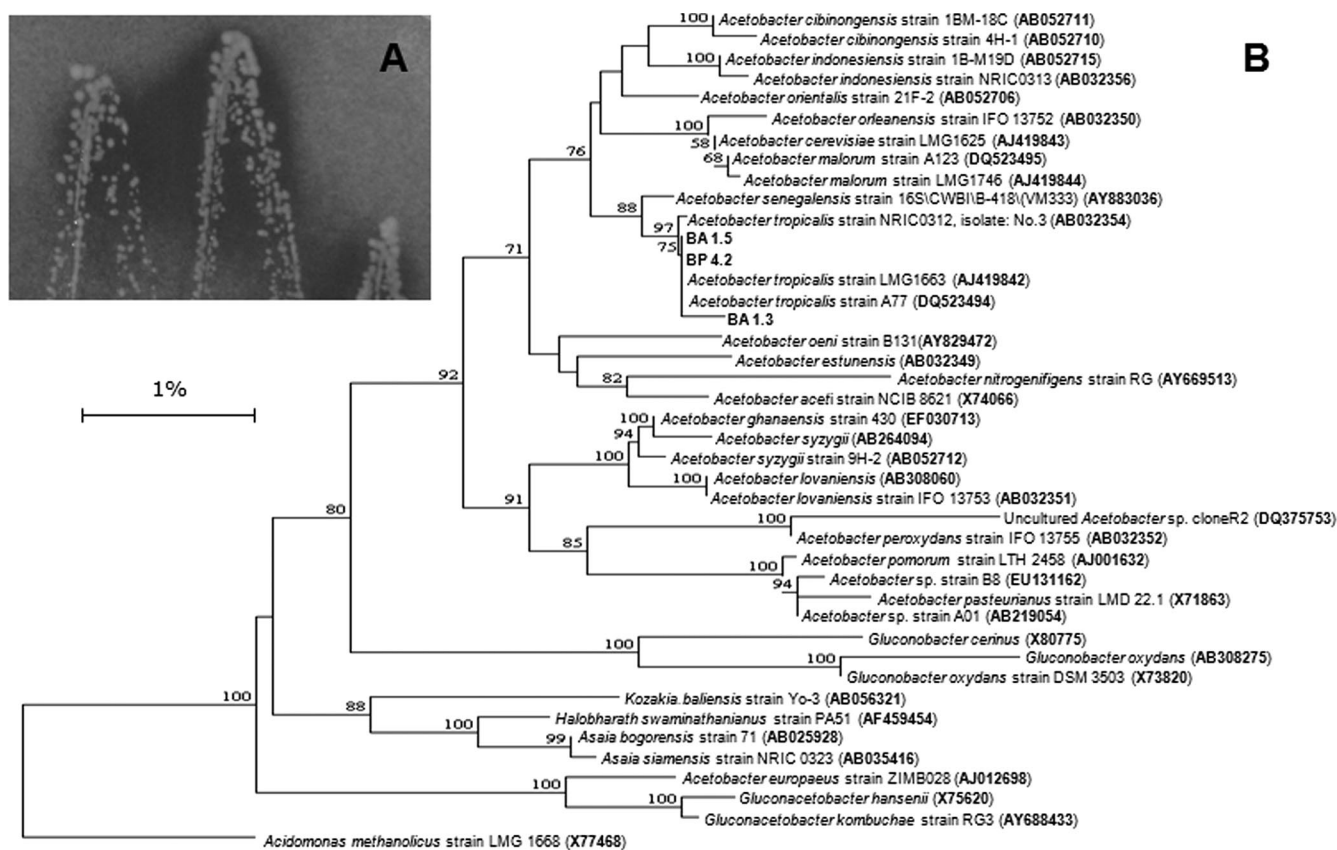


FIG. 2. The culturable acetic acid bacterium associated with the olive fruit fly *B. oleae* is *A. tropicalis*. (A) *A. tropicalis* strain capable of dissolving the carbonate in the medium, as observed by the formation of haloes that surround the colonies. (B) Phylogenetic position of strains BP4.2, BA1.3, and BA1.5 based on 16S rRNA gene sequences. Numbers at each node represent percentages of bootstrap replications calculated from 2,000 replicate trees. The scale bar represents sequence divergence.

clone libraries was one with features typical of acetic acid bacteria: bacterial cells lined by an extracellular fibrous matrix and characterized by a peripheral electron-dense cytoplasm and a nucleoplasm appearing as a filamentous bright structure (Fig. 1E). Such features would be expected for *A. tropicalis*.

Our isolation attempts were mainly based on use of enrichment medium 1, which has been specifically designed for the isolation of bacteria of the family *Acetobacteraceae* (21). With the use of this approach, it was possible to obtain in culture *A. tropicalis* isolates from *B. oleae*, as shown in Fig. 2A. Twenty-four circular nonpigmented colonies capable of clearing CaCO_3 in the agar medium were chosen as putative acetic acid bacteria. All strains were tested successfully for growth at 42°C, a feature of *A. tropicalis* (30). The isolation of *A. tropicalis* was confirmed by partial sequencing (about 700 bp) of the 16S rRNA gene of all the isolates. All 24 sequences showed more than 98% identity with the 16S rRNA gene sequence of *A. tropicalis* A77 (DQ523494), forming a distinct phylogenetic clade (Fig. 2B); whether this clade corresponds to a distinct species demands further investigation. The isolation of *A. tropicalis* was performed repeatedly from both laboratory and natural populations in different seasons over a period of 2 years (2006 to 2008), suggesting that *A. tropicalis* is stably associated with the olive fly.

The prevalence of *A. tropicalis* in field-collected olive fruit

flies from 10 different areas in Greece was evaluated with an *A. tropicalis*-specific PCR. All 220 flies tested (10 males and 10 females from each of the 10 natural and the single laboratory populations studied) were positive for the presence of *A. tropicalis*. Several of these PCR products were sequenced and showed higher than 99% nucleotide identity with *A. tropicalis*.

It is noteworthy that the bacterium "*Ca. Erwinia dacicola*," which was recently proposed to be one of the major symbionts of the olive fruit fly (7), was never detected in the laboratory colony of *B. oleae*; however, it was present in all field-collected olive fruit flies (data not shown). *A. tropicalis*, however, was consistently detected by all methods used in both laboratory and natural populations studied.

Phylogenetic analysis and molecular typing of *A. tropicalis* isolates. Almost the entire 16S rRNA gene of three *A. tropicalis* isolates (BP4.2, BA1.3, and BA1.5) was sequenced and used for phylogenetic analysis. As shown in Fig. 2B, the isolates BP4.2, BA1.3, and BA1.5 clustered in the same tree branch with known *A. tropicalis* strains.

The intraspecific diversity of the *A. tropicalis* symbionts was assayed with different fingerprinting approaches, such as ITS-PCR, tRNA-PCR, BOX-PCR, and RAPD-PCR (9). The 24 *A. tropicalis* isolates from the laboratory olive fruit fly population exhibited the same ITS-PCR and tRNA-PCR fingerprinting profiles, presenting a single band of about 850 and 1,000 bp,

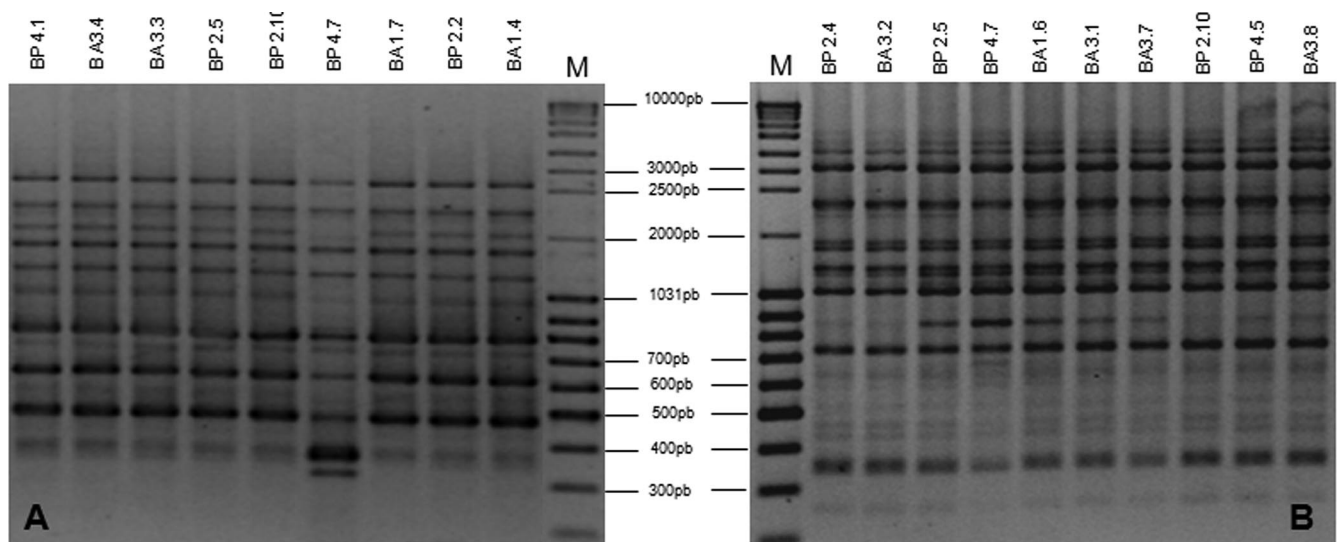


FIG. 3. Molecular typing of *A. tropicalis* isolates. (A) BOX-PCR profiles of several *A. tropicalis* isolates from *B. oleae*. Two profiles (the first profile is represented by strains BP4.1, BA3.4, BA3.3, BP2.5, BP2.10, BA1.7, BP2.2, and BA1.4; the second profile is represented by strain BP4.7) differing by the presence or absence of bands of 2,000 and 400 bp can be distinguished. Each lane represents an *A. tropicalis* isolate. M, MassRuler DNA Ladder Mix (Fermentas). (B) RAPD-PCR profiles performed on *A. tropicalis* strains isolated from *B. oleae*. With respect to a main profile (strains BP2.5, BA1.6, BA3.1, BA3.7, BP4.5, and BA3.8), two other profiles were characterized by the absence of a band of around 2,500 bp (strain BP4.7) and by the absence of a band of around 900 bp (strains BP2.4, BA3.2, and BP2.10). Each lane represents an *A. tropicalis* isolate. M, MassRuler DNA Ladder Mix.

respectively (data not shown). BOX-PCR (Fig. 3A) and RAPD-PCR (Fig. 3B) were able to distinguish several profiles among the strains examined. BOX-PCR discriminated two band pattern types with more than 10 bands in the range between 300 and 3,000 bp. One profile was represented in 23 out of 24 strains examined. RAPD-PCR unraveled three genotypes, which were characterized by complex band patterns between 300 and 4,000 bp. Eighteen strains showed the same pattern type, while the other two patterns were both represented by three strains. This typing survey of the molecular diversity of *A. tropicalis* associated to *B. oleae* showed that a single insect can be colonized by different symbiotic strains. Similar multiple infection phenomena have been reported in insects associated with *Wolbachia* (44).

Colonization experiments of *B. oleae* with *A. tropicalis* BA1.3(GFP). Strain BA1.3, representing the most abundant genotype, was chosen for labeling with a plasmid carrying the GFP cassette and for colonization experiments of both larva and adult flies. Due to the rapid loss of this plasmid, the colonization experiments were carried out under antibiotic selection by administering 200 $\mu\text{g ml}^{-1}$ kanamycin in the insect food every day. The sensitivity of *A. tropicalis* BA1.3(GFP) to 200 $\mu\text{g ml}^{-1}$ kanamycin had been previously evaluated in GLY medium plates, revealing no sensitivity to the antibiotic.

Fluorescence microscopy analysis indicated the localization of the GFP-tagged *A. tropicalis* within the larval gut (Fig. 4A and B). All tested specimens (six larvae for each sampling time) showed a large number of fluorescent cells and microcolonies within the digestive system both at 48 and 72 h post-feeding compared to control larvae (Fig. 4C and D). The presence of diffused single cells within the gut was also documented by CLSM (Fig. 4G). Interestingly, the GFP-tagged *A. tropicalis* cells were restricted to a dense brown matrix, which is probably the peritrophic membrane, within the gut (Fig. 4A,

B, and C). This matrix appeared as a flexible gel in which the bacterial cells were fully entrapped (Fig. 4A). The consistency of such a gelatinous matrix could be demonstrated by pricking the surface of the intestine with a small needle. The entire gelatinous matrix leaked out through these small holes, forming small hernias over the surface of the gut (Fig. 4E). The stretched gelatinous matrix appeared concentrated in the hernias, where all the fluorescence could be observed, and absent from the underlying gut content (Fig. 4F). As shown in Fig. 4J, TEM observation of the gut content showed a massive presence of cells with a bright nucleoid region surrounded by a matrix, two aspects typical for acetic acid bacteria (13). The acetic acid bacterium *Asaia* exhibits the same features and is furthermore surrounded by a dense matrix, presumably of polysaccharidic nature, as visible in Fig. 4K (13). *Acetobacter* species are well known, especially in the food industry, for the capacity of producing extracellular polysaccharides: they are either useful in traditional vinegar preparation as main components of the so-called “vinegar mother” (a slimy matrix used as vinegar inoculum) or disadvantageous in industrial production since they affect the product filterability (16).

The ability and specificity of the colonization of *B. oleae* by *A. tropicalis* were further assessed by cofeeding olive fruit fly larvae with a DsRed-labeled *E. coli* and the GFP-labeled strain BA1.3(GFP). Fluorescence and CLSM examinations showed that in all experiments performed, the larval gut was always colonized by green fluorescent *A. tropicalis* but never by red fluorescent *E. coli* cells (data not shown), indicating that *A. tropicalis* possesses a specific capacity of colonizing the olive fruit flies. Figure 4H and I show CLSM images of the gut and Malpighian tubules of adults after colonization experiments. The organs were massively colonized by the GFP-labeled bacterium already at 48 (five adults out of five) and 72 (four adults out of four) h postfeeding.

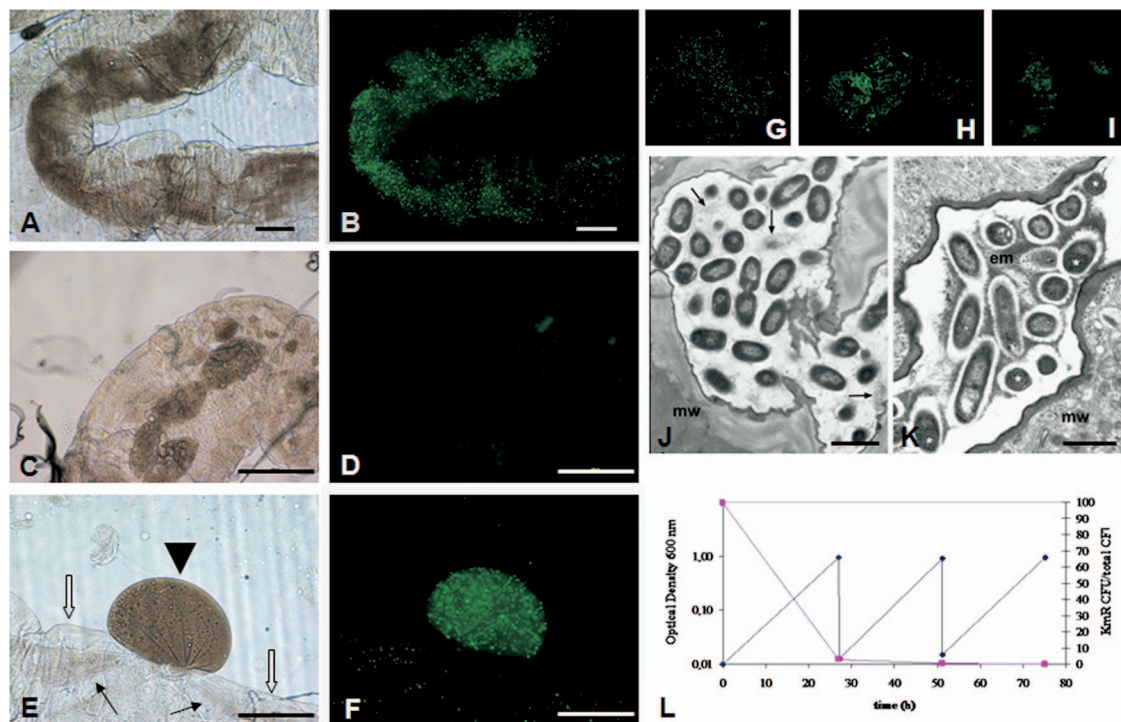


FIG. 4. Recolonization of the *B. oleae* organs by *A. tropicalis*. Colonization of *B. oleae* larvae (A to G) and adults (H and I) with *A. tropicalis* BA1.3(GFP). Phase-contrast (A and C) and fluorescence (B and D) microscope images of larval gut of insects fed (A and B) or not (C and D) with GFP-labeled bacteria. Comparison of pictures A and B indicates that fluorescent cells do not occupy the whole gut content but are probably restricted to the peritrophic membrane. Phase-contrast (E) and fluorescence (F) microscopy images of a small hernia (arrowhead) of an insect exposed to *A. tropicalis* labeled with GFP are also shown. The gut is indicated by open arrows (E), while black arrows indicate the flow of the gelatinous matrix entering the hernia. Bar, 100 μm (A to F). CLSM images of larval gut (G), adult gut (H), and Malpighian tubules (I) of insects exposed to the GFP-labeled bacteria are shown. TEM images of the midgut of adult male of *B. oleae* (J) and adult female of *Anopheles stephensi* (K) are also shown. Clusters of bacteria with morphological signatures typical of acetic acid bacteria (bright nucleoid region and extracellular matrix), probably belonging to genus *Acetobacter*, are visible in panel J (arrows, remnants of the bacterial extracellular matrix; mw, midgut wall; bar, 1.6 μm) while in panel K bacteria of the genus *Asaia* (asterisks) are shown (em, extracellular matrix; mw, midgut wall; bar, 1.2 μm). (L) Maintenance of plasmid pHM2-GFP in *A. tropicalis* BA1.3(GFP) in the absence of selection. The blue line indicates the OD₆₀₀ of the cultures during the experimental time course. The pink line represents the plasmid maintenance of the recombinant strain without antibiotic selection. It is indicated as the ratio between the number of kanamycin resistance bacteria (Km^r CFU) to the total number of bacteria (total CFU).

It has been suggested that the peritrophic membrane of *B. oleae* and closely related insect species may work as a mesh that prevents the passage of bacteria from intestinal lumen to the surface of the epithelium (26). This hypothesis may explain our observations that the majority of *A. tropicalis* cells were detected within the peritrophic membrane, which was already formed in 4-day-old larvae. In agreement with this hypothesis, it has also been reported that, in contrast to other *Tephritinae*, there are no bacteria external to the peritrophic membrane of *B. oleae* (26). However, the recolonization experiments showed the presence of *A. tropicalis* in both the gut and Malpighian tubules of the adult flies (Fig. 4), suggesting that these bacteria can be localized outside the gut (and the peritrophic membrane). In addition, these experiments (Fig. 4) provided detailed information on how *A. tropicalis* organisms colonize the intestinal lumen internal to the peritrophic membrane: (i) bacterial cells can grow in the gut lumen since fluorescent bacterial microcolonies are visible (microcolonies are the consequence of bacterial growth, and (ii) bacterial cells are concentrated at the peritrophic membrane, suggesting that they establish an interaction with this structure.

Recent studies have shown that acetic acid bacteria are

commonly associated not only with plants (18) but also with various insect species. For example, *Acetobacter* has been reported in *Drosophila* (8, 34, 35), *Gluconacetobacter* in *Apis* (2), and *Asaia* in *Anopheles* (13). All these arthropods live on sugar-based diets, suggesting that the presence of acetic acid bacteria could be linked to the insect sugar metabolism. The properties of the *B. oleae* gut environment are not described in the literature. Given that both *Drosophila* and *Bactrocera* flies are taxonomically closely related (Acalypratae group, a subsection of Schizophora), the conditions in the *B. oleae* gut may be similar to those of *Drosophila melanogaster*. The gastrointestinal tract of *Drosophila* is composed of an acidic crop, a highly alkaline ventriculus, and a neutral to acidic hindgut (8), where *Acetobacteraceae* can find a suitable environment to thrive. It is known that *Acetobacteraceae* acidify the medium during growth and likely create microniches with an acidic pH in the neutral and alkaline portions of the insect host's gut. This may explain why *Drosophila* and *Bactrocera*, both dipteran members of Acalypratae, are inhabited by *Acetobacteraceae*.

B. oleae is known to be associated with different bacterial species. However, this is the first time, to our knowledge, that bacteria belonging to the genus *Acetobacter* have been re-

ported as symbionts of these flies (12, 20); the presence of another acetic acid bacterium, *Asaia* sp., in association with *B. oleae* was recently reported (36). The detection of *A. tropicalis* in all olive fruit fly insects of both laboratory and natural populations suggests that its presence is not due to accidental contaminations and that it cannot be considered an opportunistic or occasional phenomenon. The specificity of this association was investigated with insect recolonization experiments: GFP-labeled *A. tropicalis* cells were localized in the digestive systems of the larvae and in the digestive systems and Malpighian tubules of adults in contrast to *E. coli* DH5 α pKan(DsRed), which did not result in any bacterial establishment in the body of the olive fruit fly. It is noteworthy that *A. tropicalis* appeared associated to a slimy matrix within the intestine. It cannot be ruled out that this matrix was contributed by *A. tropicalis* and could play a role in maintaining the shape and the mechanical functions of the gut. Many acetic acid bacteria produce polysaccharides that can be important in food products and in industrial production (18).

There is scant knowledge about the role, the distribution, and the transmission route of acetic acid bacterial symbionts of insect hosts. For example, the precise role of *Asaia* in the mosquito *Anopheles stephensi* has not yet been established (13); however, the multiplicity of *Asaia* transmission routes to the progeny in *Anopheles* (environmental, maternal, paternal, and venereal) suggests that this bacterium plays an important role in the biology of the host (11). The capacity of acetic acid bacteria to grow on different sugars and alcohols suggests that these bacteria affect the nutrition and the physiology of the host through their digestive processing of the ingested food. It has also been suggested that the presence of acetic acid bacteria in *Drosophila* contributes to gut homeostasis by balancing the ratio of different symbionts and regulating apoptosis of host cells (35).

This study demonstrated the presence of *A. tropicalis* in olive fruit flies in different seasonal periods, as well as from different geographical locations. Further studies will be necessary in order to elucidate the nature of the association between *A. tropicalis* and *B. oleae*.

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