The Olive Fly Endosymbiont, “Candidatus Erwinia dacicola,” Switches from an Intracellular Existence to an Extracellular Existence during Host Insect Development†‡§

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As polyphagous, holometabolous insects, tephritid fruit flies (Diptera: Tephritidae) provide a unique habitat for endosymbiotic bacteria, especially those microbes associated with the digestive system. Here we examine the endosymbiont of the olive fly [Bactrocera oleae (Rossi) (Diptera: Tephritidae)], a tephritid of great economic importance. “Candidatus Erwinia dacicola” was found in the digestive systems of all life stages of wild olive flies from the southwestern United States. PCR and microscopy demonstrated that “Ca. Erwinia dacicola” resided intracellularly in the gastric ceca of the larval midgut but extracellularly in the lumen of the foregut and ovipositor diverticulum of adult flies. “Ca. Erwinia dacicola” is one of the few nonpathogenic endosymbionts that transitions between intracellular and extracellular lifestyles during specific stages of the host’s life cycle.

Another unique feature of the olive fly endosymbiont is that unlike obligate endosymbionts of monophagous insects, “Ca. Erwinia dacicola” has a G+C nucleotide composition similar to those of closely related plant-pathogenic and free-living bacteria. These two characteristics of “Ca. Erwinia dacicola,” the ability to transition between intracellular and extracellular lifestyles and a G+C nucleotide composition similar to those of free-living relatives, may facilitate survival in a changing environment during the development of a polyphagous, holometabolous host. We propose that insect-bacterial symbioses should be classified based on the environment that the host provides to the endosymbiont (the endosymbiont environment).

Bacteria are diverse, abundant, and ubiquitous and have greatly influenced the evolution of eukaryotic life (38, 39). Bacterial symbionts of eukaryotes are generally studied in terms of benefiting or impairing their eukaryotic hosts (3, 7, 21, 26). The constraints that the host’s life history and diet impose on their endosymbionts have yet to be considered in depth. Insect-bacterium symbioses, both mutualistic and pathogenic, are ideal for examining host constraints on endosymbionts. The diversity of insect lifestyles and diets provides a continuum of habitats for endosymbionts, exhibiting different selection pressures on insect-associated bacteria.

Tephritid fruit flies (Tephritidae: Diptera) provide a unique habitat for endosymbiotic bacteria, especially digestive-system microbes. First, most tephritids feed on fleshy fruit (such as olives) as larvae, and as adults they are optimal foragers on pollen, bird feces, phyloplane bacteria (15, 52), homopteran honeydew (44), and other food sources in the environment (15, 18). Thus, digestive-tract endosymbionts may be exposed to different nutrients during an insect’s life time. Second, Diptera are holometabolous, undergoing complete metamorphosis.

Vertically transmitted endosymbionts must survive the breakdown and rebuilding of the host tissues with which the bacteria associate (22). We predict that, in order to survive in such a changing environment, the endosymbiont would have a genome similar in size to those of free-living relatives, rather than a greatly reduced genome, as is seen in obligate nutritional mutualists, such as Buchnera aphidicola.

The interaction between the olive fly, Bactrocera oleae, and its digestive-system bacterial associates has been studied for more than a century (5, 9, 17, 28, 29, 32, 33, 36, 52, 55, 56), yet several important questions remain unanswered. Do all olive fly life stages have the same species of bacterial endosymbiont? With which organs of the digestive system do the bacteria associate? Where are the bacteria located in relation to host tissues (are they extracellular or intracellular)? How ubiquitous is the association between the olive fly and specific bacteria? Are the same species of bacteria found in olive flies from the Old World also in flies that have recently infested the United States? Is the nucleotide composition of the olive fly symbiont more similar to those of its free-living relatives or to those of obligate endosymbionts?

The purpose of this paper is to address the questions listed above and to characterize the olive fly-bacterial symbiosis more thoroughly by using microscopy and culture-dependent and -independent techniques to examine all life stages of wild olive flies from the United States. We provide a comprehensive description of the biology of the olive fly symbiosis throughout host development, and we highlight the distinctive nature of a highly variable endosymbiont environment provided by the host and its consequences for bacterial genome evolution. Better understanding of tephritid endosymbionts may provide in-
sight into control methods for these significant agricultural pest insects.

MATERIALS AND METHODS

Collection and sampling. In 2003, infected flies were collected, and a Tucson laboratory population (TLP) culture was established from emerged larvae. The TLP culture was maintained on surface-sterilized olives. Adult B. oleae flies were trapped in torula yeast-baited McPhail traps (as described in reference 8) in olive groves of the National Clonal Germplasm Repository at Wolfkill Experimental Orchard in Winters (Yolo County), CA, and in Tucson (Pima County), AZ, in 2005. Sixty adults (30 males and 30 females) were collected from the Winters site, and 40 adults (20 males and 20 females) were collected from Tucson (APHIS permit number 68318).

All olive fly life stages (eggs, third-instar larvae, and pupae) were collected from the TLP and California populations and were examined for bacterial microbiota. Life stages were surface sterilized by vortexing for 15 s in a 1% sodium hypochlorite (bleach)–0.1% Triton X mixture and then rinsing twice with distilled water. The rinse water was plated on Luria-Bertani medium (4) and used as a template for PCR with the universal prokaryote 16S rRNA primers 10F and 1507R (42). No colonies or PCR products were observed (data not shown). TLP cultures were screened with "ag-cre" primer set (16), which contains two sets of degenerate primers, one targeting a half-sphere of 2% agar wrapped in Parafilm (Pechiney Plastic Packaging, Chicago, IL) as an oviposition substrate. Eggs (24 h postoviposition) were aseptically removed from agar. Olive fly eggs were fixed in 4% paraformaldehyde for 30 min at 22°C and washed twice, daily for 10 days, for colonies. Emergent colonies were picked and streaked for single colonies on the same type of medium from which they were collected. Bacteria from single colonies were grown overnight in liquid medium and were stored in 40% glycerol at −80°C. Olive fly tissues were fixed in 4% paraformaldehyde for 30 min at 22°C and washed twice with PBS. Cells were dripped onto microscope slides (Fisherbrand Super Frost Plus; Fisher Scientific, Pittsburgh, PA) for FISH. Insect tissues fixed in parafomaldehyde had high levels of autofluorescence. Therefore, a different fixation protocol (19) was used. Flies were surface sterilized, and the esophageal bulb was dissected as described above. Esophageal bulbs were fixed in Carnoy’s fixative for 24 h, rinsed twice for 5 min with 100% ethanol, and transferred to 6% H2O2 for bleaching. A drop of Vectashield (Vector Labs, Burlingame, CA) was added. Samples were viewed under a Zeiss 510 Meta confocal microscope. Esophageal bulbs were sectioned using a cryostat and hybridization buffer. Samples were rinsed in 3 M sodium chloride and 0.3 M sodium citrate, followed by a PBS rinse. To protect the fluorescent samples from bleaching, a drop of Vectashield (Vector Labs, Burlingame, CA) was added. Samples were viewed under a Zeiss 510 Meta confocal microscope. Esophageal bulbs and cultured cells were both hybridized with a 6-carboxyfluorescein isothiocyanate-diluted labeled EUB338 (2) probe (data not shown), EdF1-Cy5, and EnF2-Cy3 and hybridization buffer. Samples were rinsed in 3 M sodium chloride and 0.3 M sodium citrate, followed by a PBS rinse. To protect the fluorescent samples from bleaching, a drop of Vectashield (Vector Labs, Burlingame, CA) was added. Samples were viewed under a Zeiss 510 Meta confocal microscope. Esophageal bulbs and cultured cells were both hybridized with a 6-carboxyfluorescein isothiocyanate-diluted labeled EUB338 (2) probe (data not shown), EdF1-Cy5, and EnF2-Cy3.

Identification of olive fly symbiont species using phylogenetic analysis. Nucleotide sequences from related bacteria, including free-living and insect-associated taxa, were deposited in GenBank and phylogenetic trees have been constructed in TreeBASE. Sequences were aligned separately for each locus and as a concatenated matrix using ClustalW (54) with default parameters. Codons of rca and ompA were translated to amino acids and converted back to nucleotides after amino acid alignment. All alignments were manually edited in Mesquite, version 3.1 (25). (i.e., realigned by hand to ensure correct orientation when dozens or hundreds of samples were excluded from phylogenetic analysis (see Table S1 in the supplemental material). Only coding regions of rca and ompA were included for these loci. ModelTest (48) was used to compare models of evolution for individual loci (see Table S1 in the supplemental material), and the best model was chosen on the basis of the Akaike information criterion. A Bayesian analysis using MrBayes, version 3.1 (25), was performed to infer topologies, branch lengths, and support values for each locus and for the concatenated matrix. Table S1 in the supplemental material lists the partitioning and nucleotide evolution models as implemented by MrBayes. Two Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses were run with four chains each for 5 million generations, with sampling every 1,000 generations. Burn-in periods (see Table S1 in the supplemental material) were estimated by graphing likelihood values and were defined by the generation at which the likelihood had increased (in each MCMC run) to a level that corresponded with the average likelihoods of the trees in the burn-in MCMC samples. The taxa in clades I and II were selected as outgroups and were used to root the tree (Fig. 1).

Identification of bacteria by FISH. Fluorescent in-situ hybridization (FISH) was used to confirm the identity of the bacteria present in the esophageal bulbs of adults and the cultured bacterial isolates. Cultured Enterobacter sp. cells isolated from olives were grown overnight at 28°C, pelleted to remove medium, and washed twice in phosphate-buffered saline (PBS) at pH 7.0. Cells were fixed in 4% paraformaldehyde for 30 min at 22°C and were washed twice with PBS. Cells were dried on microscope slides (Fisherbrand Super Frost Plus; Fisher Scientific, Pittsburgh, PA) for FISH. Insect tissues fixed in parafomaldehyde had high levels of autofluorescence. Therefore, a different fixation protocol (19) was used. Flies were surface sterilized, and the esophageal bulb was dissected as described above. Esophageal bulbs were fixed in Carnoy’s fixative for 4 days, rinsed twice for 5 min with 100% ethanol, and transferred to 6% H2O2 in ethanol for 4 days at 22°C. Bulbs were rinsed in 100% ethanol, followed by three washes, each 5 min long, in PBS plus 0.01% Tween. For hybridization, bulbs were rinsed in hybridization solution three times (for 5 min each time) and incubated overnight at 40°C in a mixture of the FISH probe, 20% formamide, and hybridization buffer. Samples were rinsed in 3 M sodium chloride and 0.3 M sodium citrate, followed by a PBS rinse. To protect the fluorescent samples from bleaching, a drop of Vectashield (Vector Labs, Burlingame, CA) was added. Samples were viewed under a Zeiss 510 Meta confocal microscope. Esophageal bulbs and cultured cells were both hybridized with a 6-carboxyfluorescein isothiocyanate-diluted labeled EUB338 (2) probe (data not shown), EdF1-Cy5, and EnF2-Cy3.
FIG. 1. Phylogenetic identification of *B. oleae* symbionts. The results of Bayesian analysis of the concatenated sequences of the *recA* (~870 bp), *ompA* (~900 bp), and 16S rRNA (~1,400 bp) genes are shown. Six groups (I to VI) that also occur in analyses of individual loci (see Table S2 in the supplemental material) are identified. Group V is an unresolved grade in all analyses. Posterior probabilities above 0.85 appear above and to the left of the corresponding nodes. The six clades have different grayscale values, and taxa incertae sedis are on black branches, as they change position from locus to locus. Designations beginning with “i” represent cultured isolates; those beginning with “b” represent bacteria amplified from the insect host named; and those beginning with “C” represent bacteria of *Candidatus* status. Samples of bacteria from *Bactrocera oleae* are in boldface, with the life stage (l, larva; p, pupa) or adult gender (m, male; f, female) or organ (o, ovipositor [female]) indicated.
The G+C content of each gene was determined by dividing the number of G+C nucleotides present by the total number of nucleotides in a given sequence. The G+C contents for the three loci were compared to the genome characteristics of the 32 sequenced genomes (free-living bacteria, plant pathogens, and obligate mutualists) used in our phylogenetic analyses. Regression analysis was used in each comparison to determine if the G+C contents of these loci were representative of the GC content of the whole genome (StatView, version 5.0; SAS Institute, Cary, NC). The mean and range of the G+C percentage for each locus were determined for the groups identified in the three-gene concatenated phylogeny (Table 1).

**Nucleotide sequence accession numbers.** The sequences generated during this study have been deposited in GenBank under accession numbers GQ478372 to GQ478388 and GQ487328. Phylogenetic trees have been deposited in TreeBASE under study number S2481.

**RESULTS**

Specific bacteria were associated with olive fly populations. Bacteria from surface-sterilized wild olive flies were identified by using sequences generated by PCR amplification of 16S rRNA, *ompA*, and *recA*. Phylogenetic analyses identified five clades of bacteria and one unresolved grade that appeared in allcomp consensus trees of the *ompA* (see Fig. S1B in the supplemental material), *recA* (see Fig. S1C in the supplemental material), and concatenated (Fig. 1; see also Table S1 in the supplemental material) analyses. Phylogenetic analyses of 16S rRNA alone (see Fig. S1A in the supplemental material) resulted in poorly resolved trees. Four bacterial species were recovered from olive flies (Fig. 1).

The most abundant bacterial species was identified from sequences obtained from whole animals (larvae, adults) and ovipositors (Fig. 1, samples b1 to b8). This abundant species had high sequence similarity to the entire 16S rRNA (99% BLAST identity) of “Ca. Erwinia dacicola” from Italian olive flies (9). Phylogenetic analyses of bacterial sequences amplified from adult and larvae olive flies (Fig. 1, samples b1 to b8) and

<table>
<thead>
<tr>
<th>Species</th>
<th>16S rRNA Mean (range) % G+C content</th>
<th>ompA</th>
<th>recA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade I</td>
<td>54.5 (53.8–55.9) (n = 6)</td>
<td>un</td>
<td>60 (58.2–63.9) (n = 4)</td>
</tr>
<tr>
<td>Clade II</td>
<td>54.5 (53.8–54.96) (n = 4)</td>
<td>65.5 (63–68) (n = 2)</td>
<td>59.2 (52.1–63.3) (n = 3)</td>
</tr>
<tr>
<td>Clade III</td>
<td>55.0 (54.2–55.6) (n = 12)</td>
<td>52.7 (51.9–54.3) (n = 5)</td>
<td>52.6 (49.8–55.4) (n = 11)</td>
</tr>
<tr>
<td>“Ca. Erwinia dacicola,” Italy</td>
<td>54.7 (n = 1)</td>
<td>un</td>
<td>un</td>
</tr>
<tr>
<td>“Ca. Erwinia dacicola,” southwestern United States</td>
<td>54.2 (n = 1)</td>
<td>51.9 (51.8–52.2) (n = 3)</td>
<td>53.6 (53.4–53.7) (n = 2)</td>
</tr>
<tr>
<td><em>Enterobacter</em> sp.</td>
<td>54.5 (48.9–55.7) (n = 21)</td>
<td>54.6 (52.1–56.6) (n = 17)</td>
<td>56.1 (51.9–61.2) (n = 16)</td>
</tr>
<tr>
<td><em>Enterobacter</em> sp.</td>
<td>56.1 (cultured), 54.7 (uncultured) (54.6–54.9) (n = 3)</td>
<td>55.19 (n = 1)</td>
<td>56.1 (n = 1)</td>
</tr>
<tr>
<td>Clade V</td>
<td>52 (36–55) (n = 26)</td>
<td>54.0 (14–57.2) (n = 16)</td>
<td>47.9 (19–57.8) (n = 15)</td>
</tr>
<tr>
<td>Clade VI</td>
<td>54.8 (53.8–55.8) (n = 7)</td>
<td>52.1 (50.3–53.8) (n = 7)</td>
<td>50.6 (48.4–54.0) (n = 7)</td>
</tr>
</tbody>
</table>

* The G+C composition was determined for each species in the clades identified in the three-gene concatenated phylogeny. Shown here are the mean, range, and number of species (n) for each clade. Also included are the statistics for the *Enterobacter* sp. and “Ca. Erwinia dacicola” sequenced from U.S. flies (this study) and for “Ca. Erwinia dacicola” from Italian flies (9).

* un, unknown.

![Image](http://aem.asm.org/)

FIG. 2. FISH of an adult esophageal bulb. (A) Whole mount of an adult esophageal bulb labeled with EdF1-Cy5, specific to “Ca. Erwinia dacicola.” Bar, 20 μm. (B) Higher magnification of “Ca. Erwinia dacicola” cells found within the esophageal bulb and labeled with EdF1-Cy5. Bar, 2 μm.

TABLE 1. Percent G+C contents of *recA*, *ompA*, and 16S rRNA for species used in the phylogenetic analysis

<table>
<thead>
<tr>
<th>Species</th>
<th>16S rRNA</th>
<th><em>ompA</em> (n)</th>
<th><em>recA</em> (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade I</td>
<td>54.5 (%)</td>
<td>un</td>
<td>60 (%)</td>
</tr>
<tr>
<td>Clade II</td>
<td>54.5 (%)</td>
<td>65.5 (%)</td>
<td>59.2 (%)</td>
</tr>
<tr>
<td>Clade III</td>
<td>55.0 (%)</td>
<td>52.7 (%)</td>
<td>52.6 (%)</td>
</tr>
<tr>
<td>“Ca. Erwinia dacicola,”</td>
<td>54.7 (%)</td>
<td>un</td>
<td>un</td>
</tr>
<tr>
<td>“Ca. Erwinia dacicola,”</td>
<td>54.2 (%)</td>
<td>51.9 (%)</td>
<td>53.6 (%)</td>
</tr>
<tr>
<td>southwestern United States</td>
<td>54.5 (%)</td>
<td>54.6 (%)</td>
<td>56.1 (%)</td>
</tr>
<tr>
<td><em>Enterobacter</em> sp.</td>
<td>56.1 (%)</td>
<td>55.19 (%)</td>
<td>56.1 (%)</td>
</tr>
<tr>
<td><em>Enterobacter</em> sp.</td>
<td>52 (%)</td>
<td>54.0 (%)</td>
<td>47.9 (%)</td>
</tr>
<tr>
<td>Clade V</td>
<td>54.8 (%)</td>
<td>52.1 (%)</td>
<td>50.6 (%)</td>
</tr>
</tbody>
</table>
previously characterized “Ca. Erwinia dacicola” placed this bacterium within a clade III (Erwinia plus Pantoea) (posterior probability [PP] in concatenated analyses, 1.0). Phylogenetic analyses using ompA (see Fig. S1B in the supplemental material) and recA (see Fig. S1C in the supplemental material) revealed the monophyly of these samples and showed them to be allied to other Erwinia species. In concatenated analyses, the “Ca. Erwinia dacicola” clade was sister to a clade with plant pathogens *Brenneria (Erwinia) rhapontici* and *Erwinia persicina* (PP = 1.0) (Fig. 1). “Ca. Erwinia dacicola” is thus far unculturable (9; also data not shown).

The second most abundant bacterial species was identified from sequences obtained from larvae, pupae (Fig. 1, samples b10 to b12), and one cultured isolate (Fig. 1, sample i1) from the abdomen of a wild male olive fly. Several isolates from both larvae and male flies had a round, smooth colony morphology on Luria-Bertani (LB) and *Erwinia*-selective media. The cultured isolate and the 16S rRNA sequences amplified from olive fly larvae and pupae form clade IV (*Enterobacteriaceae*) (Fig. 1). The cultured isolate is a member of an unsupported sister clade that contains *Enterobacter cancerogenus*, *Enterobacter agglomerans*, *Enterobacter cloacae* (from the midgut of *Leptinotarsa decemlineata*), and a bacterium isolated from the cobblomblan *Folsomia candida* (PP in concatenated analyses, 1.0). Initial sequence similarity results (99% BLAST match) and phylogenetic analysis using 16S rRNA alone (see Fig. S1A in the supplemental material) suggested that the *Enterobacter* sp. isolate was closely related to *Enterobacter cloacae* or *Enterobacter agglomerans*. The recA analysis (see Fig. S1C in the supplemental material) groups the cultured bacterium with *Enterobacter cancerogenus* and *Enterobacter cloacae* (PP = 0.95). Further resolution was not obtained using ompA alone (see Fig. S1B in the supplemental material).

Two other bacterial species (Fig. 1, samples b9 and b13) were amplified only once in adult flies. Sample b9, from a wild female fly, was closely related to the plant pathogen *Citrobacter freundii*, although relationships were weak using ompA alone (see Fig. S1B in the supplemental material). Sample b13, from a wild California male fly, could not be identified using the taxon sampling of this phylogeny. BLAST results showed 99% similarity to *Rauouella ornithinolytica*.

“Ca. Erwinia dacicola” and the *Enterobacter* sp. were associated with all olive fly life stages. PCR analysis revealed that “Ca. Erwinia dacicola” and the *Enterobacter* sp. were present in all life stages of wild olive flies surveyed, suggesting that they persist through metamorphosis (data not shown). Sequence analysis revealed that “Ca. Erwinia dacicola” was present in 94% of the females and 86% of the males. The sex of the flies did not influence the presence or absence of “Ca. Erwinia dacicola” (P = 0.2932; n = 113). In 9.8% of the adults, neither “Ca. Erwinia dacicola” nor the *Enterobacter* sp. was found. There were no flies with both bacteria. One male had only the *Enterobacter* sp. There was no difference in the frequency of “Ca. Erwinia dacicola” between individuals from California and those from Arizona (P = 0.7213; n = 113). “Ca. Erwinia dacicola” and the *Enterobacter* sp. were not present in either ripe or unripe olives without fly larvae. The rot tunnels that the larvae made while feeding on the olive always contained “Ca. Erwinia dacicola” in ripe (n = 20) and unripe (n = 20) olives (data not shown).

“Ca. Erwinia dacicola” and the *Enterobacter* sp. are located within larvae and adults. “Ca. Erwinia dacicola” was consistently amplified from several structures of the *B. oleae* digestive tract. The endosymbiont was always present in the larval midgut (mycetome) (n = 10) and the evagination of the adult foregut (esophageal bulb) (n = 10). More than 70% of the 8 rectal sacs and 10 crops sampled had “Ca. Erwinia dacicola” (data not shown). None of the sampled larval salivary glands (n = 10) or adult testes (n = 8) or ovaries (n = 5) had either bacterium. Ovipositors of four of the five females sampled had “Ca. Erwinia dacicola.” The fifth ovipositor had only the *Enterobacter* sp.

**Endosymbionts are both intracellular and extracellular.** Bacterial species present in adult esophageal bulbs were identified using FISH probes specific to “Ca. Erwinia dacicola” and *Enterobacter* 16S rRNA. In adults, all bacterial cells in the esophageal bulb hybridized with the general enteric bacterial probe EUB338 and the “Ca. Erwinia dacicola”-specific probe EdF1-Cy5 (Fig. 2A and B) but not with the *Enterobacter* specific EnF2-Cy3 probe, demonstrating that these cells were “Ca. Erwinia dacicola.” The EnF2-Cy3 and EUB338 probes labeled the cultured *Enterobacter* sp. cells. Both of these results were confirmed using PCR. No labeling was seen in negative controls, and autofluorescence was very low (data not shown).

TEM revealed bacteria that were intracellular residents of larval cells (see Fig. 4A to D) (e.g., residing within the cellular membrane of epithelial cells of the digestive tissue) and extracellular residents of adult tissues (e.g., residing in the digestive system lumen but outside the cellular membrane of host cells) (Fig. 2A and B and 3A to D). In the adult, bacteria within the esophageal bulb (Fig. 3A to D) and ovipositor diverticula (Fig. 3E) had the classic rod-shaped morphology of an enteric bacterium (M1 type cells) and were surrounded by a matrix, suggesting biofilm development. These bacteria were longitudinal nearest the host epithelium, creating a very densely packed array of cells in cross section (Fig. 3B and E). In the esophageal bulb center, bacteria were more randomly oriented and may have included cells dispersing from the biofilm, since bacteria were observed in the stem connecting the esophageal bulb to the rest of the digestive system (data not shown). Other studies have noted bacteria dispersing from the esophageal bulb (9).

Although microscopy of the olive fly pupal stage was unsuccessful, recently eclosed (<24 h posteclosion) adults were examined to determine the number of bacteria present immediately after pupation. The esophageal bulbs of recently eclosed adults contained fewer than 10 bacterial cells closely associated with the bulb epithelium (Fig. 3C), and a few bacteria were loosely associated with membranes. In contrast, bulbs of older, fed adults were filled with bacteria (Fig. 3A and B).

In larvae, bacterial cells were present within host cells (intracellular) (Fig. 4A to D), instead of inside the digestive system lumen (extracellular), as in the adult esophageal bulb. Within host cells, the endosymbiont exhibited two bacterial morphologies. The majority of bacteria in host cells had an amorphous morphology (M2-type cells) (Fig. 4A, B, and D) similar to that of other insect intracellular endosymbionts, such as *Buchnera* (41), “* Candidatus Canidatus Carso nella*” (53), and “* Candidatus Ishikawaella*” (24). The cytoplasm of the amorphous morphology was diffus and granular, with electron-dense inclusion bodies clustered at one
region of the cell (Fig. 4D). A second bacterial morphology, circular in cross section with a denser cytoplasm, typical of enteric bacteria and similar to what is seen in the adult stage (M1-type cells) (Fig. 4C), was rarely present. Although two distinct bacterial morphologies were present, PCR confirmed that “Ca. Erwinia dacicola” was the only bacterium found in the mycetome.

The G+C compositions of recA, ompA, and 16S rRNA from olive fly symbionts are similar to those of closely related, free-living species. The G+C nucleotide contents of recA, ompA, and 16S rRNA were correlated with the overall genome G+C content \((n = 32; R^2 = 0.94, 0.97, \text{ and } 0.70, \text{ respectively}; \ P < 0.0001 \text{ in all cases}), and the whole-genome G+C content...
is positively correlated with the genome size \( (n = 32; R^2 = 0.90; P < 0.0001) \) in the sequenced bacterial genomes used in our phylogenetic analysis. The G+C contents of recA, ompA, and 16S rRNA were correlated with the genome size \( (n = 32; R^2 = 0.76, 0.9, \) and 0.63, respectively; \( P < 0.0001 \) in all cases). The G+C compositions of recA, ompA, and 16S rRNA of both “Ca. Erwinia dacicola” and the Enterobacter sp. associated with olive flies are similar to those of closely related, free-living enteric bacteria (Table 1; see also Table S3 in the supplemental material). The genome sizes of the bacteria, estimated from the equation of the regression line of all three loci, were similar to the genome sizes of their free-living enteric bacterial relatives (Table 1; see also Table S3 in the supplemental material).

**DISCUSSION**

*Ca. Erwinia dacicola* is a tightly associated symbiont of the olive fly. Culturable bacterial endosymbionts of the olive fly were first described by Petri in 1909 (47). A century later, Capuzzo et al. used molecular techniques to identify a specific uncultivable bacterium, “Candidatus Erwinia dacicola,” in Italian adult olive flies (9). Other studies have identified distinct gammaproteobacterial species acquired during feeding (5, 52), as well as the alphaproteobacteria *Acetobacter tropicallis* (29) and *Asaia* spp. (52), within the olive fly.

Our study found two bacteria, a culturable *Enterobacter* sp. and the unculturable “Ca. Erwinia dacicola,” present in all life stages of wild olive flies, suggesting that these bacteria can be maintained through changes in diet and host metamorphosis. Although other low-abundance species that our universal 16S rRNA primers do not amplify may be present, we focused on symbionts consistently present at high density, not on all species within the olive fly digestive system.

“Candidatus Erwinia dacicola” is found both in the TLP and in 90% of wild flies surveyed. It is possible that the 10% of wild individuals testing negative for “Ca. Erwinia dacicola” were recently eclosed individuals with endosymbiont tilters below the threshold detectable by PCR. qPCR of other recently eclosed flies showed extremely low densities of “Ca. Erwinia dacicola” (data not shown), and TEM revealed fewer than a dozen bacteria in the esophageal bulbs of recently eclosed flies (Fig. 3B), so PCR screens may underestimate endosymbiont presence. “Ca. Erwinia dacicola” has also been found within adult olive flies in Italy (9, 52) and Greece (29). The high frequency of association of “Ca. Erwinia dacicola” with the fly, its presence in different populations and in all life stages, its inability to be cultured on standard microbiological media, its vertical transmission to offspring, and its ability to reside within larval host cells suggest that this bacterium is a tightly associated endosymbiont of olive flies (46). To our knowledge, intracellular endosymbionts have not been reported in other tephritids (30), and other olive fly-associated bacteria, such as *Acetobacter tropicalis*, are exclusively extracellular (29, 52). An intracellular stage of “Ca. Erwinia dacicola” implies a more specific and longer-term interaction with the host (26).

The *Enterobacter* sp. is only occasionally found in the olive flies screened and is likely to be a nonspecific microbe. *Enterobacter* spp. are common in insects, including the other tephritids *Rhagoletis completa* (49), *Rhagoletis pomonella* (30), and *Bactrocera tau* (C. S. Prabhakar, P. Sood, B. A. Padder, S. S. Kanwar, V. Kapoor, P. K. Metha, and P. N. Sharma, unpublished data); cockroaches (12; also C. M. Gibson, unpublished data); fire ants (31); and cucumber beetles (D. R. Herndon and K. D. Spence, unpublished data). The *Citrobacter* sp. and sample b13 were thought to be phylloplane bacteria acquired during feeding (5, 14), since they were PCR amplified only once in wild-caught adults.

Our phylogenetic analyses demonstrate that “Ca. Erwinia dacicola” is closely related to several insect-vecotred, plant-pathogenic *Erwinia* species, such as *Erwinia amylovora*, *Erwinia pyrifoliae*, *Erwinia tracheiphila*, and *Pantoea* (*Erwinia*) *stewartii*. These pathogens persist primarily in association with their plant hosts and insect vectors, with limited survival in the soil (50). These closely related plant pathogens differ from each other and from “Ca. Erwinia dacicola” in the duration and the intimacy of their association with their insect versus plant hosts. *Erwinia amylovora* and *Erwinia pyrifoliae* overwinter at canker margins and in plant host buds, causing wilt (34, 51). Insect vectors are attracted to these bacterium-filled exudates and transmit the pathogens to new infection sites (16, 23, 51). In contrast, *Erwinia tracheiphila* and *Pantoea stewartii* overwinter in beetle digestive systems, and disease incidence on host plants is correlated with insect survival (11, 20, 40). “Ca. Erwinia dacicola” is found (via PCR) only in the rot tunnels where larvae are present, presumably deposited in the olive through regurgitation or defecation. Whether the bacterium is metabolically active in rot or whether the host or symbiont is responsible for the rot is unknown.

“Ca. Erwinia dacicola” transitions between an intracellular and an extracellular existence. During olive fly development, “Ca. Erwinia dacicola” resides intracellularly within larval midgut cells and extracellularly in the adult foregut. Other insect endosymbionts survive both intracellularly and extracellularly (10, 45); however, the ramifications of this transition for symbiosis and for endosymbiont evolution have not been discussed. The transition from an intracellular to an extracellular existence within the digestive system during insect development could be essential to endosymbiont survival in a polyphagous, holometabolous host. Prior to pupation, *Diptera* void their digestive tracts, reducing the number of extracellular microbes (22). During metamorphosis, larval digestive system tissues degrade, and adult tissues are assembled from larval midgut regenerative cells (27). Vertically transmitted endosymbions must have mechanisms for surviving metamorphosis and reestablishing themselves in the adult. Although the mechanisms of endosymbiont survival during complete metamorphosis are unknown, we hypothesize that bacterial cells present in the regenerative cells recolonize the adult gut.

In adults, “Ca. Erwinia dacicola” resides in a bacterial biofilm in the digestive system lumen. Nutrient acquisition for opportunistic foragers, such as the adult olive fly, is often spatially and temporally patchy. The formation of bacterial biofilms may provide resistance to periods of low nutrients and other stresses (13). Olive fly endosymbionts could not be removed from adult flies fed bleach and antibiotics (rifampin [rifampicin], streptomycin, tetracycline, and ampicillin) (data not shown) once biofilms were established. The extracellular lifestyle could also be important for transmission to offspring. The presence of “Ca. Erwinia dacicola” in the ovipositor, but not in the ovaries or testes, suggests that olive fly endosymbi-
nts (47) are vertically transmitted via smearing as the egg passes by the symbiont-rich ovipositor diverticulum (Fig. 3E) (52). Extracellular bacteria have more rigid and well-formed cell walls that may facilitate egg-smearing transmission.

The endosymbiont environment hypothesis and bacterial genome characteristics. The olive fly, “Ca. Erwinia dacicola,” provides, to our knowledge, the first example of a tightly associated symbiosis in a polyphagous, holometabolous insect. This study expands our knowledge of the types of environments in which endosymbionts survive and leads us to propose that symbioses be discussed in terms of the endosymbiont environment the host provides. In insects, the endosymbiont environment ranges from constant to variable based on at least three axes: the host diet (monophagous versus polyphagous), the host life cycle (hemimetabolous versus holometabolous), and the location of the endosymbiont within host tissues (intracellular versus extracellular). The most constant environment for endosymbionts would be provided by monophagous, hemimetabolous insects, such as aphids and sharpshooters, where endosymbionts reside exclusively within bacteriomes and hosts feed exclusively on one food type. The most variable environment would be found in polyphagous, holometabolous insects, such as the olive fly. Endosymbionts of polyphagous, holometabolous insects must survive changes in insect diet and changes to tissues during complete metamorphosis. We suggest that the environment an insect endosymbiont experiences may greatly influence its genome composition, genome size, and evolution (A. M. Estes, unpublished data).

The nucleotide compositions and genome sizes of bacteria are directly related to the degree of environmental variability, since bacteria quickly accumulate mutations and lose genes that are not under selection (6, 43). Free-living bacteria experiencing a diversity of environmental conditions have more G+C-rich genes and larger genomes than obligately intracellular endosymbionts (3, 6, 21). Thus, we predicted that endosymbionts of polyphagous, holometabolous insects, such as the olive fly, would have genomes that are less A+T biased and larger than those of obligate endosymbionts of monophagous, holometabolous insects, such as aphids. Indeed, the nucleotide compositions of the three loci analyzed from “Ca. Erwinia dacicola” and the Enterobacter sp. were more similar to those for plant-pathogenic and free-living relatives than to those for obligately intracellular endosymbionts (Table 1; see also Table S3 in the supplemental material). The G+C nucleotide compositions of the recA, ompA, and 16S rRNA genes were shown to be a good proxy for genome size, and the genome sizes of both bacteria were estimated to be similar to those of free-living and plant-pathogenic relatives.

The data presented here suggest that polyphagous, holometabolous insects associate with specific microbes. This study of the olive fly endosymbiont, “Ca. Erwinia dacicola,” provides detailed microscopy to show that the bacterium associates with different host tissues and transitions between intracellular and extracellular stages during host development. This is one of the few examples of an insect endosymbiont that makes such a transition. Examination of digestive-system endosymbionts of other polyphagous, holometabolous insects will be essential to understanding the mechanisms that influence bacterial genome structure and evolution.

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