Establishment and Vertical Passage of Enterobacter (Pantoea) agglomerans and Klebsiella pneumoniae through All Life Stages of the Mediterranean Fruit Fly (Diptera: Tephritidae)

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ABSTRACT We investigated the fate of ingested Enterobacter (Pantoea) agglomerans and Klebsiella pneumoniae within adult Mediterranean fruit fly, Ceratitis capitata (Wiedemann) (Diptera: Tephritidae), in a mass rearing facility. This examination revealed the establishment of both bacterial strains as biofilms within the adult intestines, on the apical end of developing and developed eggs, and throughout all subsequent life stages. The bacteria were detected in adults through two generations. Irradiation treatment for the sterile insect technique did not disrupt the vertical transmission of E. (P.) agglomerans or K. pneumoniae. This is the first demonstration of maternal spread of Enterobacter/Pantoea spp. and Klebsiella spp. through populations of C. capitata. A mixed pattern of vertical and horizontal transmission of symbionts associated with tephritids may be one explanation for the difficulty in defining the symbiotic associations of tephritids.

KEY WORDS symbiosis, reproduction, sterile insect technique

The importance of bacteria in the life history of certain pest Tephritidae has been in question since the 1930s when Allen et al. (1934) described the association between Phytomonas (Pseudomonas) melophora and Rhagoletis pomonella Walsh, the apple maggot. Since that time, the main focus of work into the nature of tephritid–bacteria interactions has been the attraction of certain pest Tephritidae to bacteria (MacCollom et al. 1992, 1994; Lauzon et al. 1998, 2000) or to odors produced by bacteria in culture (Bateman and Morton 1981, Drew and Faye 1988, Martinez et al. 1994, Robacker and Flath 1995, Robacker and Bartelt 1997, Robacker et al. 1998, Epsky et al. 1998, Robacker and Lauzon 2002). This work sought to establish lures for detection of these important agricultural pests rather than to determine the intimacy and meaning of any symbiotic relationship that exists in nature.

The mechanisms by which tephritids acquire and maintain their symbionts have not been fully defined. Although ingestion of bacteria is one way that tephritids acquire their normal gut bacteria, it has not been established that this is the sole mechanism. Moreover, the type of symbiotic relationships tephritids possess with bacteria, i.e., facultative or obligatory, has never been conclusively determined.

Tephritids consume a variety of microorganisms in their natural food (Drew et al. 1983, Drew and Lloyd 1987, Prokopy et al. 1993). Of these, species in two bacterial genera Enterobacter/Pantoea and Klebsiella consistently inhabit the tephritid gut (Lauzon et al. 1998). Lauzon et al. (2000) suggested that these two bacteria participate in nitrogen cycling within the tephritid gut and serve as important contributors to tephritid survival in nature. Artificial rearing of fruit flies, such as the Mediterranean fruit fly, Ceratitis capitata (Wiedemann) (Diptera: Tephritidae), for control programs precludes the exposure to, and establishment of, these bacterial genera from natural sources. C. capitata are cultured in large-scale production facilities around the world and typically in these situations normal gut microbiota are absent (C.R.L., unpublished data). If specific bacteria in the gut of C. capitata confer a fitness advantage, then their absence in mass production may result in decreased fitness of sterile males released in control programs. Indeed, reports exist that describe poor performance of sterile male flies in the field (Shelly and Whittier 1996). Understanding the relationship between bacteria and C. capitata in nature may allow for improvements to mass production protocols (e.g., probiotic diets) that would increase the effectiveness of sterile males.

This work is part of an investigation to determine whether artificial introduction of symbionts (i.e., probiotic diets) will appreciably improve the fitness of mass-reared sterile males. Three experiments are described in this article and begin to address the question of symbiont establishment and spread. The first experiment was conducted to monitor the fate of two
endosymbionts that were ingested by *C. capitata* for the purposes of describing endosymbiont establishment and retention in the adult *C. capitata* gut. Our findings also indicated that the endosymbionts migrated to the ovaries and established on the apical end of eggs. These findings led to the second experiment where we monitored the fate of the endosymbionts through successive life stages of *C. capitata* starting with females that ingested the endosymbionts, and two successive generations of *C. capitata* thereafter. The third experiment was designed to determine whether radiation exposure at 145 Gy used in the sterile insect technique would disrupt the vertical transmission of the endosymbionts.

### Materials and Methods

#### Insect Rearing

All experiments were conducted at the USDA APHIS Hawaii Fruit Fly Production Facility (HFFPF) in Waimanalo, HI. The standard Maui-93 strain of *C. capitata* (McInnis et al. 1996) was used in experiments 1 and 2. Maui-93 had been in production at HFFPF for ≈9 yr. The Vienna VII temperature-sensitive lethal genetic sexing strain (Franz et al. 1996) used in experiment 3 was obtained from the California Department of Food and Agriculture Medfly Rearing Facility in Waimanalo, HI.

#### Transformation of Endosymbionts

Strains of *P. (E.) agglomerans* herein referred to as *E. agglomerans*, and *K. pneumoniae* of tephritid origin were previously transformed to express the fluorescent protein enhanced green fluorescent protein (EGFP) (Peloquin et al. 2000), or they were transformed (Sambrook et al. 2000) to express the fluorescent protein DsRed as follows: bacterial cells were grown to late-stationary phase in Luria Bertani (LB) broth (Difco, Detroit, MI) and centrifuged at 10,000 *g* for 15 min at 18°C. The bacterial pellet was resuspended in 250 µl of ice-cold calcium chloride (aq) in microfuge tubes and kept on ice. Ten microliters of DsRed plasmid DNA (Invitrogen, Carlsbad, CA.) was added to the bacteria:calcium chloride solution, the solution was mixed well, and returned to ice for 15 min. Bacterial cells were then heat-shocked in a 42°C water bath for 90 s under gentle agitation. Cells were placed in ice for 1 min and received 250 µl of LB broth. After 10 min, cells were plated onto LB agar and incubated overnight at 24°C. Transformed bacterial cells displayed a pink-red colonial phenotype and were confirmed by fluorescence using a fluorescent microscope (Leica, Heerbrugg, Switzerland). Transformation was determined to be stable through numerous subcultures.

#### Experiment 1

Six hundred (100 flies × 3 replicates × 2 groups) newly eclosed adults were provisioned with the following diets for 2 d: sucrose/yeast hydrolysate enzymatic (3:1, vol:vol), or the same sucrose/yeast hydrolysate enzymatic diet containing fluorescently marked *E. agglomerans* and *K. pneumoniae* (e.g., probiotic mixture). The flies had a continuous water supply. The probiotic mixture was added to the dry diet and included 750 µl of individual preparations of bacteria grown to mid-log phase, cleansed of medium, and resuspended in sterile water to an optical density of 0.40 at 550 nm. Quality control was conducted on aliquots of the bacterial diets sampled before and after use for the presence of the probiotics following standard methods (Brennan 1992) and using API 20E biochemical identification strips (bioMerieux Vitek, Inc., Hazelwood, MO).

After 2 d of feeding ad libitum, 30 individual male and female Mediterranean fruit fly from each group were dissected, and their alimentary canal and reproductive organs were removed (Lauzon et al. 1998) and prepared for examination using confocal scanning laser microscopy (CLSM).

#### CLSM

Alimentary canal and reproductive organs were placed onto clean, glass slides. Intestines were often teased apart using sterile needles (Yale hypodermic needles, BD Biosciences, Franklin Lakes, NJ). Approximately 20 µl of anti-fade reagent (Citißuor, Ltd., City University, London, United Kingdom) were added to each sample. A coverslip was gently applied, and the edges were sealed to the slide using fingernail polish. The samples were optically sectioned using a 510 confocal laser scanning microscope (Zeiss Optical Systems, Inc., Thornwood, NY) located at The Biological Imaging Facility at The University of California in Berkeley, CA.

#### Scanning Electron Microscopy (SEM)

Intestines from 15 males and 15 females from each group were removed and placed individually on Thermaxo plastic sterile coverslips (Nunc Brand Products, Nalge Nunc International, Naperville, IL) coated with poly-L-lysine (Ted Pella, Inc., Redding, CA). Once the samples were affixed to coverslips, they were immersed into 2.5% glutaraldehyde (Ted Pella, Inc.) in 0.05 M cacodylate buffer, pH 7.2, for 1 h at 4°C. Eggs were removed and placed into microfuge tubes containing the same fixative in buffer and held for 1 h at 4°C. All samples were rinsed twice for 10 min each using 0.05 M cacodylate buffer, pH 7.2, for 1 h at room temperature, followed by two 10-min rinses using the cacodylate buffer, and two additional 10-min rinses using distilled water, pH 7.0. Samples were postfixed using osmium tetroxide and dehydrated in a graded ethanol series: 50, 70, 95 (two times), 100% (three times) for 10 min each. Samples were infused with CO₂ and processed accordingly in a critical point dryer (Polaron Instruments, Inc., Hatfield, PA). The samples were then individually mounted on aluminum stubs (Ted Pella, Inc.) with carbon conductive adhesive (Ted Pella, Inc.). The edges of the coverslips were tacked down to the stub with either colloidal silver liquid or Electrodeg 520 (Ted Pella, Inc.). Samples were sputter-coated (Hummer VII Sputter Coating System, Anatech, Ltd., Springfield, VA) with 30 nm of gold-palladium. A Philips XL-40 scanning electron microscope (FEI/Philips, Eindhoven, The Netherlands) at an accelerating voltage of 12 kV was used to view these samples.

#### Experiment 2

Sixty adult females fed ad libitum on either probiotic-amended standard diet or standard diet, and water for 2 d. Females were transferred to clean cages containing males, standard diet and water. Fertil-
ized eggs were collected, seeded onto larval diet, and pupae were collected. Pupae were held until eclosion. Some adults were killed and examined for the presence of the fluorescent probiotics. Others were kept, and the process was repeated for two subsequent generations. Adult flies were transferred to previously unused cages and given fresh standard adult diet and water routinely.

**Experiment 3: Irradiation of Pupae Containing** *E. agglomerans* **and** *K. pneumoniae*. One hundred Mediterranean fruit fly eggs were artificially seeded with *E. agglomerans* and *K. pneumoniae* and reared to the pupal stage. Fifty microliters of a mixture of the bacteria was applied to eggs on filter paper. Specimens from all life stages were sampled to confirm the presence of the fluorescently marked bacteria by using CLSM. Pupae were placed into petri dishes and sealed within Zip-Loc bags (S.C. Johnson & Son, Inc., Racine, WI) for 30 min. Pupae were irradiated at 145 Gy following the standard protocol for Mediterranean fruit fly pupae used in sterile insect release programs. Equal numbers of irradiated and nonirradiated pupae were placed within separate insect cages that contained standard adult diet and water until they emerged as adult flies. Adults were allowed to feed for 2 d on standard diet and water and then were sacrificed by freezing. The flies were dissected and examined using CLSM described above. CLSM will confirm the presence, location, and arrangement of transformed endosymbionts.

**Results**

**Fate of** *E. agglomerans* **and** *K. pneumoniae* **in** *C. capitata*. *E. agglomerans* and *K. pneumoniae* established as biofilms, or aggregates adhered to tissue surfaces, within the alimentary canal and on the egg surface of adult *C. capitata* after ingestion (Figs. 1–3). The biofilm filled the insect lumen (Fig. 2). The biofilms within esophageal bulbs, and crops were not as dense and seemed architecturally less complex than biofilms within the intestines of the flies. The distribution of *E. agglomerans* and *K. pneumoniae* seemed random throughout most areas of the biofilms. However, using software for the CLSM that determines overlap of emission signals, we found that *E. agglomerans* and *K. pneumoniae* colocalized at openings within biofilms (Fig. 4).

*E. agglomerans* and *K. pneumoniae* were found in ovaries and assembled as biofilms on the surface of developing eggs, developed eggs, or both (see below; Fig. 10a and b). The two bacterial species colocalized, and the degree of colocalization was greater than that observed with digestive biofilms (data not shown). Biofilms on egg surfaces were less dense than those observed in intestines, but they were thicker than those observed in esophageal bulbs and crops. *E. agglomerans* and *K. pneumoniae* were not found in male reproductive organs. Neither fluorescently marked bacteria were found in adults that fed on standard diet.

**Fate of** *E. agglomerans* **and** *K. pneumoniae* **in Consecutive Generations of** *C. capitata*. *E. agglomerans* and *K. pneumoniae* were detected in all life stages of Mediterranean fruit fly through two generations. Upon visual inspection, adult *C. capitata* that acquired *E. agglomerans* and *K. pneumoniae* through feeding had the greatest amount of each bacterial species within their alimentary canal organs. Two consecutive generations of *C. capitata* both had fluorescent *E. agglomerans* and *K. pneumoniae* in each life stage.
We detected only a few bacterial cells in each stage, with the exception of adults which had moderate numbers of each bacteria established within their gut.

**Scanning Electron Microscopic Examination of Eggs and Intestines.** Intestinal biofilms were massive and seemed to occlude the lumen (Fig. 8). The ridged surface of the apical end of dissected *C. capitata* eggs supported the attachment of bacteria (Fig. 9). Bacteria were observed within the confines of the micropyle (Fig. 10a). A similar pattern of establishment is shown using CLSM (Fig. 10b).

**Radiation Effects on the Spread of* E. agglomerans* and* K. pneumoniae.* E. agglomerans and *K. pneumoniae* were detected in adult *C. capitata* intestines that were irradiated previously as pupae (Fig. 11). No appreciable variation between nonirradiated and irradiated flies was seen.

**Discussion**

We found that *E. agglomerans* and *K. pneumoniae* establish rapidly after ingestion and form a biofilm in the gut of female and male *C. capitata*. The biofilm was extensive and contained many open pores, channels, and arteries that ranged in length and width. *E. agglomerans* and *K. pneumoniae* colocalized near the entry sites of the biofilm. The rapid establishment and coordinated assembly of microorganisms as biofilms (Costerton et al. 1987, 1995; Davies et al. 1998; O’Toole and Kolter 1998) come only after prerequisite criteria have been satisfied, such as responses of planktonic bacteria to each other, the environment, and a surface (Loo et al. 2000). Thus, the establishment of *E. agglomerans* and *K. pneumoniae* as a biofilm in the *C. capitata* gut suggests that these bacteria naturally and routinely assemble there. *Enterobacter* and *Klebsiella* spp. are bacterial species most consistently isolated from several tephritid species, and their assembly as a biofilm may explain why.

Biofilms are metabolically active structures (Costerton et al. 1995) that often operate as a coordinated unit, i.e., a digestive organ, and they are complex and diverse in their metabolic abilities. Lauzon et al. (2000) suggested that *E. agglomerans* and *K. pneumoniae* jointly participate in the catabolism of nitrogen in the gut of *R. pomonella*. Behar et al. (2005) demonstrated nitrogen fixation in the gut of *C. capitata* in which *Enterobacteriaceae* were the dominant microbiota. The colocalization of *E. agglomerans* and *K. pneumoniae* at areas where food from the gut lumen would enter the biofilm supports this hypothesis. Biofilms within *C. capitata* may serve functions beyond that of nitrogen cycling (Lauzon et al. 2000), although we can only speculate about these functions now. Lauzon et al. (2003) found that *E. agglomerans* degrades and detoxifies phloridzin, a toxic and potent antifeeding compound typical of *Malus* spp. found in plant leachate. The intestinal biofilm likely catabolizes a variety of compounds that enter the insect gut, such as plant antifeeding compounds, before they reach epithelial tissue. The thickness of the intestinal biofilm...
may physically protect epithelial tissue from toxins, toxicants, or irritants that C. capitata might ingest.

The biofilm filled the gut lumen forming both narrow and large passages for food and water to meander or flow through. The thickness and architecture of the biofilm may regulate the transit time of food and water passing through the gut (Gjersing et al. 2005). This would allow for maximized digestion and absorption of nutrients, efficient removal of materials, or both. It is possible that the biofilm acts both as a nonspecific digestive organ and a protective barrier for C. capitata and other tephritids.

We found that E. agglomerans and K. pneumoniae migrate per os to the ovaries and assemble in a biofilm at the apical end of developing and developed eggs. Thus, E. agglomerans and K. pneumoniae are vertically transmitted in C. capitata. To our knowledge, this is the first report of maternal transmission of symbionts in tephritids. The passage of these two bacteria from female to egg strongly suggests that these bacteria are important in the life history of C. capitata.

It is generally accepted if symbionts are spread maternally through a host population, then two host-symbiont relationships likely occur, mutualism or parasitism (Dedeine et al. 2003). Mutualism includes a reciprocal increase in fitness for the host and the symbiont and is the likely association that exists between E. agglomerans, K. pneumoniae, and C. capitata. Several observations support the hypothesis that the presence of E. agglomerans and K. pneumoniae increase the fitness of C. capitata. The addition of E. agglomerans and K. pneumoniae to the diet of mass-reared male C. capitata increases their mating efficiency (Niyazi et al. 2004). Lauzon and Potter (2008) also found that the gut of C. capitata damaged by irradiation at the standard 145 Gy for sterilization of males released in control programs improved when these irradiated adults consumed a diet that contained E. agglomerans and K. pneumoniae.

The question remains, however, of how the fitness of E. agglomerans and K. pneumoniae increases. Both bacterial species can, and do, exist in a free-living state in nature (Brennan 1992). It is possible that these bacteria survive and reproduce more readily in the gut of C. capitata than in water, soil, or plant environments.

Tephritids consume a variety of microorganisms while feeding on natural food substrates (Lauzon et al. 1998) Adult C. capitata and other tephritids are known to acquire E. agglomerans and K. pneumoniae through natural food sources that have a high nitrogen content, i.e., bird feces and insect frass on leaf surfaces (unpublished data). We found that E. agglomerans and K. pneumoniae ingested by adult C. capitata are passed vertically through all life stages and along successive generations. The numbers of fluorescently marked bacteria varied among life stages and tissues. The adult intestine, the egg surface in the female reproductive tract, and the egg surface postoviposition seem to support the greatest numbers of bacteria, respectively. Very few bacteria were detected in larval and pupal samples and were not detected at all in the testes.

Fig. 3. CLSM image of fluorescent bacteria, EGFP K. pneumoniae and DsRed E. agglomerans, assembled as a biofilm on an egg surface. Magnification, 63×. (Online figure in color.)
Mixed patterns of symbiont transmission behavior in insects are not uncommon (Agnew et al. 2003). A mixed pattern of vertical and horizontal transmission of symbionts associated with tephritids may be one explanation for the difficulty in defining the symbiotic associations of tephritids. This mixed pattern of sym-

Fig. 4. CLSM image of colocalization of EGFP *K. pneumoniae* and DsRed *E. agglomerans*, shown as blue regions. Notice localization dominates near openings within the biofilm. Magnification, 63X. (Online figure in color.)

Fig. 5. CLSM image of a second generation larval gut containing EGFP *K. pneumoniae* and DsRed *E. agglomerans* cells indicated by arrows. Magnification, 63X. (Online figure in color.)
biont transmission behavior may indicate a compromise between vertical transmission efficiency and cost to the host. The variation in symbiont abundance among life stages may be related to stage-specific differences in nutritional requirements. Adults require high levels of protein (i.e., nitrogenous compounds),

Fig. 6. CLSM image of internal structures within a second generation Mediterranean fruit fly pupa showing the presence of fluorescent bacteria, EGFP K. pneumoniae and DsRed E. agglomerans. (Online figure in color.)

Fig. 7. CLSM image of a second generation adult Mediterranean fruit fly intestine containing fluorescent bacteria EGFP K. pneumoniae and DsRed E. agglomerans. (Online figure in color.)
for completion of sexual maturation and egg production. Adults not only enhance symbiont horizontal transmission efficiency by attraction to, and feeding on, these bacteria in nature (MacCollom et al. 1992, 1994; Prokopy et al. 1993, Lauzon et al. 1998) but also ingest the carrier substrates with a high nitrogen content. That is, the flies are simultaneously ingesting high levels of nitrogenous compounds and the symbionts that facilitate nitrogen catabolism. The observed role of *E. agglomerans* in detoxification (Lauzon et al. 2003) suggests that larger numbers of this species would be beneficial to the host during the adult stage when the insect is exposed to a diverse environment. Similarly, the low abundance of these bacteria in the larval stages may reflect a compromise between vertical transmission efficiency and cost to the fruit fly host. The insect does not maintain a high level of symbionts during the larval stage that requires relatively low levels of nitrogenous compounds and when it is exposed to a more homogeneous environment within the fruit. In addition, the highly active larval gut from prolific feeding may physically diminish the ability of the symbionts to establish. Turbulence and flushing are known factors that can interfere with biofilm formation (Sutherland 2001). This may be another factor associated with the larval stage that diminishes symbiont attachment.
transmission efficiency. Although few bacteria are present in the pupal stage, bacteria are likely carried into the adult gut in the meconium. Symbionts transmitted in this way have an opportunity to attach and proliferate in the relatively calm gut of newly eclosed adults. Nutrients, such as precursor materials of the peritrophic matrix, would support bacterial growth. A more steady flow of nutrients would come later in the gut with feeding behavior changes that meet nutritional demands of flies as they age and mature reproductively.

The presence of *E. agglomerans* and *K. pneumoniae* on the egg surface pre- and postoviposition suggests another role for the symbionts in larval development. Bacteria were observed within the micropyle, suggesting that this may be an entry point into the egg for maternal transmission. Bacteria on the surface of the egg may also inoculate the larval host at oviposi-

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**Fig. 10.** (a) SEM image of bacteria near and within micropyle of a Mediterranean fruit fly egg. Magnification, 12,000×. (b) CLSM image of bacteria, EGFP *K. pneumoniae* and DsRed *E. agglomerans* on the surface of a Mediterranean fruit fly egg showing a similar pattern of placement as seen in Fig. 12a. Magnification, 63×. (Online figure in color.)

**Fig. 11.** CLSM image of fluorescent bacteria EGFP *K. pneumoniae* and DsRed *E. agglomerans* within the gut of an adult sterile male Mediterranean fruit fly. Magnification, 25×. (Online figure in color.)
tion, providing a second means of transmission to the immature insect through feeding. Furthermore, these bacteria may play an important role in the breakdown of the host fruit and subsequent development of the larvae.

Mixed patterns of symbiont transmission behavior in insects are not uncommon (Agnew et al. 2003) and likely reflect a host’s adaptation to various ecological niches. Multiple acquisitions and replacements of symbionts in *C. capitata* suggest that these bacteria serve important roles in overcoming biological constraints encountered by these insects in nature. Knowledge and understanding of symbiont inheritance should lead to a more complete understanding of *C. capitata* dispersal and biology.

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