ELISA Versus Immunolocalization to Determine the Association of *Erwinia tracheiphila* in *Acalymma vittatum* (Coleoptera: Chrysomelidae)

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ABSTRACT DAS-ELISA, immunohistochemistry and electron microscopy were used to investigate the association of the causal agent of bacterial wilt, *Erwinia tracheiphila* (Smith), within the beetle *Acalymma vittatum* (F.). After a 24-h acquisition period, a high percentage of individuals tested positive for *E. tracheiphila* antigen using both immunohistochemistry (100%) and DAS-ELISA (70–60%). Both assays showed that the antigen remained in beetles long after the initial acquisition, with the percentage declining during incubation. Using ELISA, the percentage decreased to 4.7% within 3 d after acquistion, then increased to 10% within 10 d and remained at 10% for 30 d. Immunoperoxidase assays of paraffin embedded gut sections were more sensitive, and showed that 95% of the beetles harbored the pathogen after 10 d and 20% after 30 d. *E. tracheiphila* antigen was present throughout the digestive tract soon after acquisition, but only small clusters of *E. tracheiphila* were observed along the alimentary canal 3 d after transfer onto clean plants. After 10 and 30 d on clean plants, *E. tracheiphila* antigen reaction was stronger and clusters of bacteria were more numerous, primarily in the posterior midgut and anterior portion of the hindgut. Scanning electron microscopy and TEM photomicrographs confirmed the presence of bacterial cells resembling *E. tracheiphila* associated with the intima of the hindgut 1 and 30 d after acquisition. This demonstrated the sensitivity of immunohistochemisty for detecting *E. tracheiphila* within its vector, and suggests a long-term extracellular endosymbiotic association of *E. tracheiphila* with the alimentary canal of *A. vittatum.*

KEY WORDS Coleoptera, Chrysomelidae, *Acalymma vittatum*, vector-pathogen association, *Erwinia tracheiphila*

THE BACTERIUM *Erwinia tracheiphila* (Smith), the causal agent of bacterial wilt in cucurbits, has long been associated with the striped cucumber beetle, *Acalymma vittatum* (F.) (Rand 1915). Rand and Enlows (1916) showed that *A. vittatum* remained infective up to 6 wk after *E. tracheiphila* acquisition. Rand and Cash (1920) isolated *E. tracheiphila* from the guts of two out of 551 field-collected beetles in spring, and inoculation of indicator plants with these isolates resulted in bacterial wilt symptoms. No symptomatic plants were present at the time of collection, therefore Rand and Cash (1920) suggested that *E. tracheiphila* survived in the gut of overwintering beetles. More recently, Fleischer et al. (1999) found serological (ELISA) evidence of *E. tracheiphila* overwintering in 7–10% of the *A. vittatum* collected from overwintering cages placed on bare soil where cucurbits were grown the previous fall. Yao et al. (1996) demonstrated a positive correlation between the incidence of bacterial wilt of cucumbers and population densities of *A. vittatum* under field conditions. Brust (1997) and Lukezic et al. (1997) showed that the severity and time to development of bacteria wilt symptoms in cucurbits was positively correlated with inoculum concentration, the size of the wounds, and timing of application of inoculum to wounds.

This association between *E. tracheiphila* and *A. vittatum*, however, has not always been consistent in published reports. Leach (1964), using paraffin embedded guts and gram-negative staining, found no evidence of bacterial multiplication or long-term retention in *A. vittatum* fed on *E. tracheiphila* infected cucumber plants. Even in beetles fed for 48 h on infected tissues, the amount of bacteria present in the alimentary canal was low. Bassi (1983) was unable to detect *E. tracheiphila* in overwintering beetles using immunofluorescence, but he did find immunofluorescent evidence of the pathogen in weeds, and Blua et al. (1994) found that weeds injected with *E. tracheiphila* produced serological evidence of the pathogen 3 wk after injection. These discrepancies may be the result of serological detection of nonviable bacteria, and varying sensitivity of assays. De Mackiewicz et al. (1998) showed that the serological response in weeds to injected pathogen was most easily explained by the presence of nonviable antigen. Fleischer et al.

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(1999) showed that ELISA assays were three to five times more likely to suggest the presence of the pathogen in beetles than caged transmission bioassays of individual beetles, and that more beetles harbor the pathogen than are able to transmit sufficient doses to cause disease in single-beetle bioassays.

Plant pathogens in the genus *Erwinia* are known for establishing long-term associations within the alimentary canal of insect vectors. *E. carotovora* (Jones) Winslow et al., the causal agent of potato blackleg, multiply and survive the winter in the alimentary canal of the seedcorn maggot, *Hylemyia cilicrura* (Rond.) (Leach 1940, Carter 1973). *E. stewartii* (Erw. Smith), responsible for bacterial wilt of corn, has been reported inhabiting and overwintering in the digestive tract of *Chaetocnema pulicaria* Melsh. (Elliott and Poos 1934). Viable *E. amylovora* (Burr.) (Winslow et al.) has been recovered from the digestive tract of *Apis mellifica* L., 6 and 9 d after feeding on an *E. amylovora* contaminated diet (Beyme et al. 1975). Schalk et al. (1987) isolated 22 species of bacteria from the abdominal flora of *Diabrotica belteata* LeConte, a species closely related to *A. vittatum.* Those isolates included *E. herbicola* (Lohnis) in 9 and 25% of all field-collected and laboratory-reared beetles, respectively.

Although published studies show that *E. tracheiphila* is associated with and overwinters in *A. vittatum,* biological details about the distribution of *E. tracheiphila* within the vector over time have not been clearly defined. Rand and Cash (1920) isolated *E. tracheiphila* from dissected whole guts and mouth parts and Leach (1964) visualized gram-negative bacteria in the midgut of beetles, but did not define where bacterial retention or replication occurs within the beetle. Garcia-Salazar et al. (2000) used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to describe the alimentary canal morphology in *A. vittatum* and observed cells resembling *E. tracheiphila* floating in the gut lumen and associated with the intima of the hindgut 1 and 30 d after feeding on *E. tracheiphila* sandwiches. The objective of this study was to compare the fate of *E. tracheiphila* within its vector measured with ELISA and an immunoperoxidase localization technique. We used DAS-ELISA to determine percentages of *A. vittatum* that acquired and retained *E. tracheiphila* over time after acquisition, and adapted the avidin-biotin-peroxidase technique to localize the bacterial antigen along the alimentary canal. Electron microscopy was used to confirm our immunological findings, and to help verify the location of *E. tracheiphila* in the gut.

Materials and Methods

Acquisition of *E. tracheiphila*. We used beetles from a laboratory colony initiated with *A. vittatum* collected from cucurbits in Center County, PA (Ellers-Kirk 1996). Voucher specimens were deposited in the Frost Entomology Museum, Pennsylvania State University. Also, a laboratory culture of *E. tracheiphila* (ATCC 33245) was maintained according to De Mackiewicz et al. (1998) for inoculation of beetle vectors and plant material. Cohorts of 3-d-old adults were inoculated with *E. tracheiphila* by feeding them sandwiches prepared with 7-d-old cultured *E. tracheiphila* smeared onto disks (0.5 cm diameter) of healthy cucumber cotyledons. Beetles were fed individually in small (3.5 by 1.0 cm) vials for 24 h, which we refer to as the access acquisition period. Beetles were then transferred to 14- to 21-d-old healthy cucumber or squash plants for an incubation period ranging from 0 to 35 d after acquisition. Simultaneously, individuals from the same cohort of 3-d-old beetles were given a 24-h acquisition period on healthy cucumber cotyledon disks and used as negative controls. Individuals from the same cohort removed from the experiment right after the acquisition period on *E. tracheiphila* sandwiches were used as positive controls. Insects were caged on the cucumber plants in screened Plexiglas cylinders (11 cm diameter by 10 cm high) and maintained at 27 ± 1°C, 65% RH, and a photoperiod of 16:8 (L:D) h. Plants used as feeding substrate were replaced twice weekly in the shorter incubation treatment and weekly in the longer incubation treatment to prevent beetles from reacquiring bacteria by feeding on newly infected plants. Upon completion of each incubation period, beetles were allocated to DAS-ELISA, immunohistochemistry, or electron microscopy for testing.

ELISA Detection of *E. tracheiphila* in *A. vittatum* Over Time After Acquisition. The presence of *E. tracheiphila* antigen in single inoculated and noninoculated beetles was assessed using an antiserum against *E. tracheiphila* (ATCC 33245) described in De Mackiewicz at al. 1998. Animal use was approved by the university animal care committee, and a copy of the guidelines is retained on file. Beetles were assayed in a DAS-ELISA following methods described in De Mackiewicz at al. 1998 and Fleischer et al. 1999 as follows. Briefly, microtitre plates were coated with the primary antibody and incubated at 4°C overnight, rinsed with the corresponding buffer, the antigen applied, and incubated for another 24 h. Plates were rinsed to remove unbound antigen and an alkaline phosphatase antibody conjugate applied to the antigen. Incubation continued for another 24 h at low temperature before rinsing and color development. To determine the detection limit of the antiserum, a standard curve was developed with a 10-fold serial dilution of *E. tracheiphila* (ATCC 33245) cultured in broth and streaked on NAP plates (De Mackiewicz 1998). After a 3-d incubation at 28 ± 1°C the number of colony forming units per dilution was counted and their corresponding absorbance values at 405 nm obtained. The detection threshold was established using nonlinear regression of the number of colony forming units ($\log_{10}$) with their corresponding absorbance values. To discriminate between ELISA positive and false-positive individuals, a threshold was established as the mean absorbance of noninoculated colony beetles plus three standard deviations (Gillett et al. 1986). *E. tracheiphila* inoculated beetles showing absorbance values below or equal to this threshold were considered noninfested.

To elucidate the short-term association of *E. tracheiphila* within *A. vittatum*, 60 adult *A. vittatum* (3 d old) were inoculated with the plant pathogen, and then divided into six groups of 10 beetles each. Each group was randomly assigned to one of six incubation treatments (0, 1, 3, 4, 5, and 10 d after acquisition) on healthy cucumber plants. Another 10 noninoculated beetles were used as negative controls. Beetles allocated to the 0-d incubation treatment were used as positive controls. Thus, each cage held 10 beetles per plant. This whole experiment was replicated five times.

To determine the long-term association of *E. tracheiphila* within *A. vittatum*, a cohort of 120 beetles inoculated with the 24-h acquisition period were placed in two separate screened cages with healthy cucumber plants for incubation. Immediately after acquisition, 20 individuals were removed for use as positive controls. From the remaining 100 individuals, subsamples of 13, 10, and 9 beetles were taken 8, 15, and 35 d after acquisition. The negative control consisted of 13 adult *A. vittatum* fed for 24 h on healthy cucumber cotyledon disks. Upon completion of the incubation treatment, individuals were freeze-killed and stored at −4°C until assayed with DAS-ELISA.

Percentages of ELISA positive individual were transformed to angular values [arcsine (square root)] and regressed against incubation periods using linear regression. Regressions were compared between the short and long-term retention studies (Neter and Wasserman 1974).

Immunoperoxidase Localization of *E. tracheiphila* in the Alimentary Canal of *A. vittatum*. Digestive tracts of healthy and *E. tracheiphila* fed beetles were dissected and fixed overnight in saline buffer (0.15 M sodium chloride, 0.15 M calcium chloride, 0.02 M potassium chloride, 0.025 M sodium bicarbonate) containing 4% formaldehyde and 0.2% glutaraldehyde. Fixed samples were dehydrated for 10 min in 30, 70, 90, and 100% ethanol (three changes per dilution) and then twice in xylene. Dehydrated samples were infiltrated overnight in 50/50 (vol:vol) paraffin-xylene in a vacuum oven at 56°C, embedded in Paraplast-X-TRA (Oxford Labware, St. Louis, MO), and refrigerated at 4°C until sectioned. Embedded tissues were cut into 2- to 4-μm sections in a AO 820 rotary microtome (American Optical, Buffalo, NY) and fixed to slides previously dipped in a solution containing 5% gelatin and 0.5% chromium potassium sulfate.

Detection of *E. tracheiphila* in paraffin sections of *A. vittatum* by immunostaining was accomplished by coupling the *E. tracheiphila* ATCC 33245 rabbit antiserum, at a concentration of 1:400, to a biotinylated goat anti-rabbit antibody and to an avidin/biotinylated peroxidase complex reacted with a 3, 3′ diaminobenzidine tetrahydrochloride (DAB) substrate (ABC method, Hsu et al. 1981). Paraffin sections were immunostained according to the ABC protocol (Vector Laboratories, Burlingame, CA) with modification as follows: the rinsing buffer was PBS/Tw 20/Gelatin buffer, pH 7.5 (10 mM sodium phosphate, 0.9% sodium chloride, 0.1% Tween 20, 1.0% gelatin from fish), and antibodies as well as ABC regents were diluted in PBS, pH 7.5. After blocking and rinsing, slides were incubated in the primary antibody, then in rabbit-anti-*E. tracheiphila* and in the avidin/biotinylated peroxidase complex. The presence of *E. tracheiphila* was revealed by incubating the slides in 200-250 μl of DAB substrate in 0.1 M Tris-HCl, pH 8.2 buffer. The reaction stained the cells brown, and colonies of *E. tracheiphila* immunobound to the detecting substrate. Slides were counterstained blue with Harris modified hematoxylin stain according to the manufacturer instructions (Fisher, Pittsburgh, PA). Slides were then dehydrated in ethanol and xylene, drained, and a coverslip adhered with a droplet of Permount (Stephene Scientific, Riverdale, NJ).

Nonspecific binding of the biotinylated secondary antibody, the avidin/biotinylated peroxidase complex, and DAB with the digestive tract tissue was evaluated. Seven slides with sections from one healthy beetle and nine slides from one *E. tracheiphila* fed beetle were selected at random. These 16 slides and an additional four slides with smears of *E. tracheiphila* were treated as follows: (1) cultured *E. tracheiphila* complete ABC treatment, (2) cultured *E. tracheiphila;* rabbit anti-*Erwinia* omitted, (3) cultured *E. tracheiphila;* goat anti-rabbit omitted, (4) healthy beetle; complete ABC treatment, (5) healthy beetle; goat anti-rabbit omitted, (6) *E. tracheiphila* fed beetle; complete ABC treatment, (7) *E. tracheiphila* fed beetle; goat anti-rabbit omitted, and (8) *E. tracheiphila* fed beetle; rabbit anti-*Erwinia* omitted. After treatment, the slides were counterstained, dehydrated and mounted as above. Treatments 1 and 6, where *E. tracheiphila* was present, were the positive controls and were predicted to stain brown after bacterial cells immunobounded to the DAB detection substrate. All other treatments were negative controls.

To study the short-term association of *E. tracheiphila* within the alimentary canal of *A. vittatum*, 45 adult *A. vittatum* (3 d old) inoculated with a 24-h acquisition period were randomly allocated to three incubation treatments (0, 3, and 10 d after acquisition, n = 15, 10, and 20, respectively). Another 15 noninoculated individuals were used as negative controls. Beetles allocated to the 0-d incubation treatment after the 24-h acquisition period were used as positive controls. All individuals were frozen at the completion of the incubation period and held at −4°C until paraffin-embedded, sectioned and immunostained.

To study the long-term association of *E. tracheiphila* within the vector's digestive tract, we used individuals from the same cohort of *E. tracheiphila* inoculated beetles prepared for the long-term study using DAS-ELISA. This study comprised three incubation periods after the 24-h acquisition period on *E. tracheiphila* sandwiches and a positive and negative control. A sample of 10, 13, 10, and 9 individuals was removed from the cohort at 0, 8, 15, and 35 d after acquisition. Negative controls were 13 *A. vittatum* fed for 24 h on healthy cucumber cotyledon disks. Treatment beetles assigned to the 0-d incubation treatment were used as positive control. Both negative and positive control

Table 1. ELISA positive *A. vittatum* after a 24-h acquisition period on *E. tracheiphila* and incubated for different periods on healthy cucumber or squash plants (mean ± SD)

Incubation time, d	Short-term retention			Long-term retention		
	n	% ELISA positive	Abs 405 nm	*n*	% ELISA positive	Abs 405 nm
0 (Positive control)	40	60.0	0.61 ± 0.42	10	70.0	0.22 ± 0.02
1	57	14.1	0.55 ± 0.32			
3	42	4.8	0.60 ± 0.30			
4	49	10.2	0.28 ± 0.09			
5	34	11.8	0.16 ± 0.02			
8				13	46.2	0.24 ± 0.05
10	44	9.1	0.18 ± 0.04			
15				10	50.0	0.53 ± 0.31
35				9	11.1	0.43 ± 0.00
Healthy beetles[a]	49	2.0	0.03 ± 0.03	13	7.7	0.08 ± 0.03

[a] Healthy beetles fed for 24 h on healthy cucumber cotyledon disks and freeze-killed after the acquisition period were negative controls. Their absorbence values correspond to the entire sample, which includes any false positive individuals.

beetles were frozen at the end of the acquisition period and held at −4°C until processed. Beetles incubated for longer periods were also frozen but immediately dissected, embedded, and immunostained. Immunostained sections were examined by light microscopy and photographed.

Electron Microscopic Detection of *E. tracheiphila* in the Alimentary Canal of *A. vittatum*. For SEM, digestive tracts were removed and placed in fixative (0.1 M sodium cacodylate, 2.5% formaldehyde, and 1.5% glutaraldehyde). After overnight fixation at 4°C, digestive tracts were postfixed for 2–3 h in 1% osmium tetroxide in 0.1 M sodium cacodylate. After postfixation, specimens were incubated overnight at 5°C in 2% tannic acid in 0.1 M sodium cacodylate buffer. After a second postfixation in 1% osmium tetroxide, digestive tracts were washed in 0.1 M sodium cacodylate and dehydrated in a ethanol gradient (25–100%). After two additional changes in 100% ethanol, fixed tissues were critical-point dried in a Polaron E300 Critical-Point Dryer (Bio-Rad/EBS, Agawam, MA). Specimens were mounted on SEM stubs, dissected further to open the digestive tract, sputter-coated with 10 nm (100 A) of gold-palladium, and viewed in a JEOL JSM 5400 (JEOL, Peabody, MA) SEM. Four *E. tracheiphila* inoculated beetles incubated for 1 d, three incubated for 30 d after acquisition, and two noninoculated beetles were examined.

For TEM observations, two noninoculated beetles, two beetles inoculated and incubated for 1 d, and three individuals inoculated and incubated for 30 d after the acquisition period were processed in similar manner as for SEM with modifications. After fixation and postfixation, the samples were "en bloc" stained for 1 h in 1% uranyl acetate and dehydrated in a ethanol gradient (50–100%) followed by ethanol (EM grade) and acetone (3 times, 5 min each), and embedded in Spur resin and polymerized at 60°C. Spur blocks were sectioned on a KLB III-8800 (LKB Instruments, Rockville, MD) ultramicrotome. Thin sections displaying silver to light-gold interference color were collected on 200-mesh copper grids, stained with lead citrate and uranyl acetate, and photomicrographs of the cellular structure taken with a JEOL 1200EXII (JEOL, Peabody, MA) TEM.

Results

Serological Detection of *E. tracheiphila* in *A. vittatum* Over Time After Acquisition. The sensitivity of our DAS-ELISA was modeled with the exponential regression model $y = 0.000068 \pm 0.00001 (e^{1.4388 \pm 0.0294*CFU})$, where y = ELISA absorbance values and CFU is the $\log_{10}$ of the colony forming units per milliliter (Fleischer et al. 1999). We were able to detect down to $10^{3.14}$ CFU per ml. However, because of cross-reactivity with other bacteria harbored by *A. vittatum*, we established a higher threshold based on the mean + 3*SD from negative control beetles, which were $10^{5.32}$ and $10^{5.50}$ CFU/ml of beetle homogenate for the study of short and long-term association, respectively. These thresholds corresponded to 0.144 and 0.188 absorbance at 405 nm. Assayed beetles were declared infested with *E. tracheiphila* when their absorbance was higher then these thresholds.

The proportion of ELISA positive individuals decreased over time after the 24-h acquisition period. The rate of decrease in the short- ($Y = 0.553–0.037x$, $r^2 = 0.30$, $n = 6$, $P = 0.26$) and long-term study ($Y = 0.972–0.018x$, $r^2 = 0.98$, $n = 4$, $P = 0.03$) differed significantly ($F = 13.57$; df = 2, 6; $P < 0.05$). In the short-term (0–10 d) study, a sharp reduction from 60 to 4.7% occurred within 3 d after the 24-h acquisition period. However, between 4 and 10 d after acquisition the percentage increased from 4.7 to 9.0–10% (Table 1). In the long-term study, ELISA positive individuals decreased from 70.0% immediately after the acquisition period to 11.1% within 35 d after acquisition and remained at 46–60% 8–15 d after acquisition (Table 1). This difference may be the result of the sample size ($n = 10–13$ at the shorter intervals in the 35-d study, versus 44–49 in the 10-d study). Other factors could have been differential consumption of the *E. tracheiphila* sandwich and virulence of the bacterial cul-

Table 2. Treatments to evaluate the unspecific reaction of the VECTASIN *Elite* ABC System, rabbit IgG Kit (Vector Laboratories, Burlingame, CA)

Treatment	Slides processed	Antibody treatment[a]		DAB	Color developed
		Rabbit anti-*E.t.*[b]	Goat anti-rabbit		
Cultured *E. tracheiphila*	2	+++	+++	+++	Brown
	1	−−−	+++	+++	No color
	1	+++	−−−	+++	No color
Healthy beetle	4	+++	+++	+++	No color
	3	+++	−−−	+++	No color
E. tracheiphila fed bettle	4	+++	+++	+++	Brown
	3	+++	−−−	+++	No color
	2	−−−	+++	+++	No color

[a] Antibody applied (+++); no antibody applied (−−−).
[b] Rabbit anti-*E. tracheiphila* antiserum.

ture used to inoculate individuals assigned to the long-term study.

Immunoperoxidase localization of *E. tracheiphila* in the alimentary canal of *A. vittatum*. The ABC method and the anti-*E. tracheiphila* IgG resulted in a specific strong staining of lumen tissues containing *E. tracheiphila* (treatments 1 and 6 of the test of specificity of the immunoperoxidase method, Table 2). The intense, irregular or spotted brown DAB reaction in the lumen of the alimentary canal in beetles fed *E. tracheiphila* was the same as that observed in smears of cultured *E. tracheiphila.* In treatments 4 and 5, tissues of healthy beetles did not react with the antibody or any detection component. In this experiment, biotinylated goat anti-rabbit IgG as well as the avidin/biotinylated peroxidase complex were not reactive to tissues other than those treated with and binding the primary antibody. In negative control treatments (2, 3, 7, and 8), the elimination of rabbit anti-*E. tracheiphila* IgG or the biotinylated goat anti-rabbit IgG resulted in a failure to stain any tissues in beetles having fed upon *E. tracheiphila* or the bacteria themselves (Table 2).

However, we did see nonspecific reaction outside of the alimentary canal, primarily with fat bodies. That reaction could be caused by the endogenous peroxidase present in some animal tissues (Hsu 1993). However, the brown coloration was distinctively different from the DAB reaction with the substrate that immunobound to *E. tracheiphila* cells. Thus, several criteria were established to differentiate false-positive or background reactions from positive immunostaining. First, only the reaction occurring in the lumen of the alimentary canal was considered. Second, because most slides contained 10–20 serial tissue sections, a tissue was considered positive only if the DAB staining observed in one section occurred precisely in the same place in two previous and two posterior sections. This helped rule out false positives produced as an artifact during processing.

The bacterial antigen was detected in the whole alimentary canal after a 24 h acquisition period on *E. tracheiphila* sandwiches (Table 3). Beetles killed immediately after the acquisition period revealed *E. tracheiphila* antigen attached to the pharynx, esophagus, crop, midgut, and hindgut. The whole lumen of the digestive tract was brown after immunostaining, indicating that the *E. tracheiphila* antigen was present throughout the alimentary canal, as well as in the fecal pellets.

Incubation for 3 and 10 d on healthy plants eliminated most of the ingested *E. tracheiphila*, allowing for a more precise identification of retention sites in the gut. Beetles fed for 24 h on *E. tracheiphila* and transferred to healthy plants for a 3-d incubation period showed a light staining along the alimentary canal, with small pockets of *E. tracheiphila* antigen in the foregut, midgut, and hindgut in all beetles examined. In the foregut, the antigen was localized in the rows of spines of the pharynx and in folds and wrinkles of the crop. The antigen also associated with the microvilli at the distal end of the midgut and in folds and wrinkles of the hindgut. In contrast, a 24-h acquisition period followed by 10-d incubation on healthy plants yielded an increased amount of bacterial antigen, es-

Table 3. Immunoperoxidase localization (ABC method) over time of *E. tracheiphila* in the alimentary canal of *A. vittatum* after a 24-h acquisition period on *E. tracheiphila* and subjected to various incubated periods

Incubation time, d	*n*	% ABC positive	Intensity of DAB stain[a]		
			Foregut[b]	Midgut	Hindgut
0 (Positive control)	15	100.0	+++	+++	+++
3	8	100.0	+	+	+
10	20	95.0	+	++	++
30	10	20.0	−−−	+	++
Healthy beetles[c]	15	0.0	−−−	−−−	−−−

[a] Strong (+++); moderated (++); light (+); negative (−−−).
[b] Foregut included the pharynx oesophagus, and crop.
[c] Healthy beetles fed for 24 h on healthy cucumber cotyledon disks and freeze-killed after the acquisition period were negative controls.

Table 4. Immuno and electron microscopic detection of *E. tracheiphila* in *A. vittatum* incubated for 30 d on healthy cucumbers after a 24-h acquisition period

Incubation time, d	ABC staining (+/*n*)	SEM (+/*n*)	TEM (+/*n*)	Total (*n*)	% *Erwinia* positive
0 (Positive control)	10/10			10	100.0
1	10/10	4/4	2/2	16	100.0
30	2/10	2/3	2/3	16	37.5
Healthy beetles[a]	0/10	0/2	0/2	14	0.0

[a] Healthy beetles fed for 24 h on healthy cucumber cotyledon disks and positive control individuals fed on *E. tracheiphila* sandwiches were freeze-killed after the acquisition period and fixed immediately.

pecially in the posterior portion of the midgut and in the hindgut. In 19 out of 20 beetles immunostained 10 d after the acquisition period, the *E. tracheiphila* antigen was observed clumped in the crop and posterior portion of the midgut (above the base of the Malpighian tubules), but mainly in the hindgut. In the hindgut, the antigen was observed in the colon attached to the folds of the intima, and in the peritrophic membrane wrapping the fecal pellets. In the long-term association study, two out of 10 beetles incubated for 30 d on healthy plants after a 24-h acquisition showed the *E. tracheiphila* antigen in the posterior portion of the midgut and in the hindgut (Table 3 and 4). Surprisingly, no bacterial antigen was observed inside the ventricular crypts, even in cases of a heavy *E. tracheiphila* infestation after the 24-h acquisition period and before transfer to clean plants.

Electron Microscopic Detection of *E. tracheiphila* in the Alimentary Canal of *A. vittatum*. The electron microscopic analysis of inoculated and noninoculated *A. vittatum* incubated for 1 and 30 d on healthy plants confirmed the presence of bacteria resembling *E. tracheiphila* in the posterior part of the midgut, the hindgut and fecal pellets. One day after a 24-h acquisition on *E. tracheiphila*, rod-shaped bacteria (*Et*) were found around the pyloric valve (Fig. 1A). Thirty days later, two out of three beetles examined with SEM showed rod-shaped bacteria (*Et*) attached to the microvilli in the midgut near to the pyloric valve (Fig. 1B) and in fecal pellets of the same individuals. Inoculated beetles examined at the cellular level with TEM from the same group (incubated for 1 and 30 d on healthy plants) revealed that most of the bacteria resembling *E. tracheiphila* was attached to the intima in the pyloric valve and colon of inoculated individuals (Fig. 1C and 1D). In both SEM and TEM, control beetles fed for 24 h on healthy cucumber cotyledon disks and incubated on healthy plants showed no bacterial accumulation in the same regions (Table 4).

Discussion

Because of their sensitivity, immunoperoxidase localization techniques are widely used in human and animal research (DeLellis et al. 1979, Imam and Tokes 1981, Robinson 1982). The avidin-biotin-peroxidase complex (ABC) method (Hsu et al. 1981) proved sensitive enough to detect small amounts of *E. tracheiphila* antigen in specific tissues of single beetles, allowing detection in beetles at levels that escaped detection using DAS-ELISA. After the initial 24-h acquisition period, immunoperoxidase localization assays yielded a higher percentage of individuals infested with *E. tracheiphila* (100%) than did DAS-ELISA (70–60%). Both assays show that the *E. tracheiphila* antigen remained in beetles long after the initial acquisition, with the percentage declining during incubation, but the pattern of decline differed. With DAS-ELISA, the percentage of beetles that tested positive for the *E. tracheiphila* antigen declined steadily, but reached an asymptote consistent in both short- (9.1% at 10 d) and long-term association studies (11.1% at 35 d). This lower asymptote is similar to the 7–10% obtained by Fleischer et al. (1999) in overwintering field-collected *A. vittatum* using the same technique. With the immunoperoxidase assays, the percentage of *A. vittatum* positive for the *E. tracheiphila* antigen remained almost constant within the first 10 d after the 24 h acquisition period (≈95%), although the intensity of the DAB reaction decreased sharply 3 d after inoculation. That coincides with the sharp decline in the number of ELISA-positive beetles observed 3 d after acquisition. After 30 d of feeding on healthy plants, ≈20% of the inoculated population tested positive for the *E. tracheiphila* antigen. The difference in the percentages of acquisition and retention reported by ELISA and the ABC method are the result of their difference in sensitivity. Gillett et al. (1986) indicated that, in establishing an ELISA detection threshold using a population of positive and negative individuals, we are choosing a threshold that yields the fewest false negative or false positive results. Accordingly, our detection threshold based on the mean plus three standard deviations of negative samples underestimated the percentage of individuals carrying the *E. tracheiphila* antigen. Microscopic analysis of immunostained sections of the alimentary canal can detect small packets of bacteria that may go undetected by ELISA.

We were aware that a low percentage of false positives observed with ELISA (2.0–7.6%) could be the result of a crossreaction of *E. tracheiphila* antiserum with other bacteria in the beetle's gut. Because we used the same antibody in both ELISA and immunolocalization studies, we expected similar results with the ABC method. However, we consistently found the *E. tracheiphila* antigen only in the gut of beetles fed bacteria and not in noninoculated individuals. The

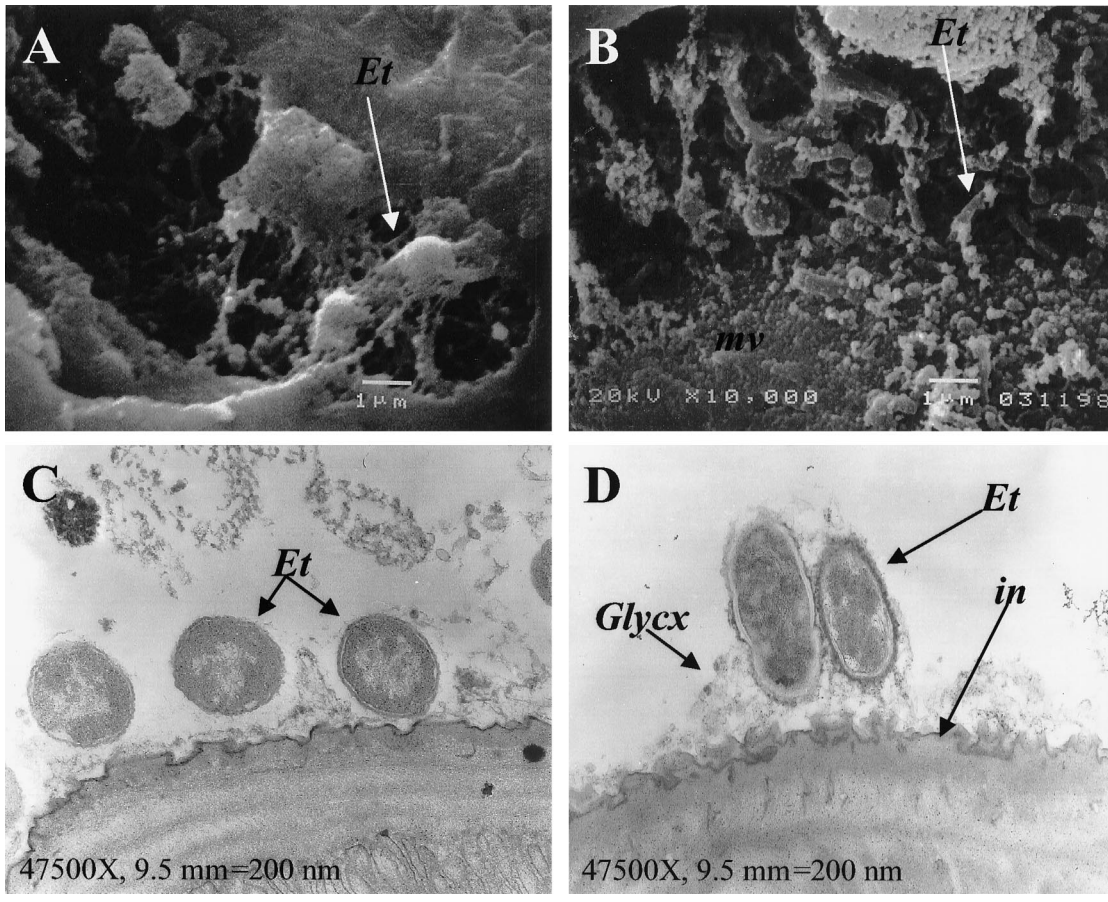

Fig. 1. SEM and TEM composite image showing (A) SEM of the pyloric valve region of *A. vittatum* with bacteria resembling *E. tracheiphila* (*Et*) 1 d after a 24-h acquisition period on *E. tracheiphila* sandwiches. (B) SEM of the posterior portion of the midgut of *A. vittatum* showing rod-shaped bacteria resembling *E. tracheiphila* (*Et*) after a 24-h acquisition period and incubated for 30 d on healthy cucumber plants. (C) TEM photomicrograph of the intima of *A. vittatum* with bacteria resembling *E. tracheiphila* (*Et*) 1 d after a 24-h acquisition period on *E. tracheiphila* sandwiches. (D) TEM photomicrograph of the intima (*in*) in the hindgut of *A. vittatum* showing bacteria resembling *E. tracheiphila* (*Et*) and the glycocalyx (*Glycx*) after a 24-h acquisition period on *E. tracheiphila* and incubated for 30 d on healthy cucumber plants.

stringency of the fixation, embedding and deparaffination process to which inoculated tissues were subjected before immunostaining may reduce nonspecific binding of the antiserum. Furthermore, SEM and TEM observations in beetles fed bacteria and incubated for 1 and 30 d agree with the antigenic data found with ELISA and the ABC method. We found bacterial cells resembling *E. tracheiphila* attached to the midgut and hindgut of *E. tracheiphila* inoculated beetles but not in noninoculated individuals (Fig. 1).

The immunoperoxidase localization studies revealed the distribution of the antigen in the alimentary canal of *A vittatum* over time. Immediately after acquisition, the *E. tracheiphila* antigen was distributed throughout the alimentary canal except in the ventricular crypts of the midgut. In contrast, beetles transferred to healthy plants for 3 d after acquisition showed only small clusters of antigen, mainly in the foregut, the hindgut, and distal portions of the midgut around the pyloric valve. Assuming that these areas lack natural barriers to prevent bacterial attachment, then these are appropriate sites for initial retention of *E. tracheiphila.*

An important feature of the alimentary canal of *A. vittatum* is the peritrophic membrane (Garcia-Salazar et al. 2000). This membrane may protect midgut cells from damage by the gut contents during transit and digestion, and provide a barrier to pathogens (Walters et al. 1993, Barbehenn and Martin 1995, Walters et al. 1995). The small amount of antigen detected only in the distal region of the midgut 3 d after acquisition suggests that the peritrophic membranes kept most of the initial inoculum from attaching to the midgut epithelium or entering the ventricular crypts.

Erwinia tracheiphila attachment to the distal portion of the midgut 3, 10, and 30 d after acquisition (Fig. 1B) suggests that the plant pathogen was gaining entrance into the ectoperitrophic spaces of the alimentary canal of *A. vittatum.* Richards and Richards (1977) indicated that parasites might enter ectoperitrophic

space through the fluid anterior end of the peritrophic membrane or by forward migration from the open end of the membrane or from the hindgut.

Costerton et al. (1978, 1981) indicated that plant and animal tissue colonization by bacteria depends on its adhesion and persistence provided by the production of a bacterial glycocalyx. Accordingly, a bacterial cell adheres to a plant cell, an animal cell, or another bacterial cell by juxtaposing its own glycocalyx to the surface of the cell it adheres to. Our SEM and TEM observations provide very clear images of bacterial cells attached to the posterior end of the midgut and to the chitin of the hindgut intima of *E. tracheiphila* inoculated individuals. In TEM microphotographs taken 30 d after inoculation, the bacterial cell attachment via the glycocalyx is clearly visible (*Glycx* in Fig. 1D). The presence of bacteria resembling *E. tracheiphila* in the posterior portion of the midgut 30 d after acquisition (Fig. 1C) can be best explained as migration and colonization of live *E. tracheiphila* that avoided being expelled with the passage of food by the production of a glycocalyx. The detection of the *E. tracheiphila* antigen and bacterial cells resembling *E. tracheiphila* 30 d after the acquisition period lends credibility to early claims that the bacteria could live and survive in the alimentary tract of *A. vittatum* (Rand and Enlows 1916). Thus, our results indicate a long-term *E. tracheiphila–A. vittatum* extracellular symbiotic association, the nature of which (e.g., mutualism, commensalism) is still unknown (Steinhaus 1946, Carter 1973).

Although the immunoperoxidase technique is not suitable for epidemiological studies, it is interesting to compare the higher rate of positives using immunolocalization (90% after the 24-h acquisition period) with the much lower rate of beetles resulting in plant disease when caged in greenhouse bioassays on susceptible cucurbits. For example, in a preliminary study using single-beetle caged bioassays (unpublished data) only 10% of the beetles were able to cause disease after a 24-h acquisition period on *E. tracheiphila* inoculated plants, and only 30% after a 24-h acquisition period on *E. tracheiphila* sandwiches. The immunolocalization data suggest a higher rate of *E. tracheiphila* acquisition and retention within the beetle than rates of transmission under these caged bioassays, which agrees with a similar relationship between ELISA-positive beetles and caged bioassays (Fleischer et al. 1999). If we assume that *E. tracheiphila* plant infection is a dose dependent process (Lukezic et al. 1997), then even low levels of inoculum, such as those seen in beetles 3 and 10 d after aquisition, may contribute to transmission by incremental addition achieved by increasing beetle density. This may be the process underlying the positive relationship between beetle density and disease transmission reported by Yao et al. (1996) under field conditions and Brust and Rane (1995) under caged bioassay conditions. However, the relationship of either assay and the ability to transmit sufficient pathogen titers to cause disease was not determined in this study.

Our research has demonstrated the greater sensitivity of the ABC method over ELISA for detecting *E. tracheiphila* within its vector. Both methods have utility for studying the *E. tracheiphila–A. vittatum* association. Although less sensitive, DAS-ELISA is more suitable for population level studies, whereas the ABC technique is more appropriate for localization of the pathogen to specific tissues. This research also demonstrated the suitability of the alimentary canal of *A. vittatum* for survival or multiplication of *E. tracheiphila* and we defined the posterior midgut and hindgut as sites within the canal that harbor most of the bacterial cells over time. The detection of the antigen 30-35 d after acquisition suggests a long-term endosymbiotic association of *E. tracheiphila* with its beetle vector. This is the type of association described for other *Erwinia* species and insects (Elliott and Poos 1934, Leach 1940, Steinhaus 1946, Carter 1973, Harrison et al. 1980, Schalk et al. 1987). This type of association allows for the conclusion that, in nature, *A. vittatum* is both a vector and a reservoir for *E. tracheiphila* and makes possible the overwintering of the pathogen in its invertebrate vector.

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