Differential Colonization Dynamics of Cucurbit Hosts by *Erwinia tracheiphila*

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ABSTRACT

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Bacterial wilt is one of the most destructive diseases of cucurbits in the Midwestern and Northeastern United States. Although the disease has been studied since 1900, host colonization dynamics remain unclear. *Cucumis*- and *Cucurbita*-derived strains exhibit host preference for the cucurbit genus from which they were isolated. We constructed a bioluminescent strain of *Erwinia tracheiphila* (TedCu10-BL#9) and colonization of different cucurbit hosts was monitored. At the second-true-leaf stage, *Cucumis melo* plants were inoculated with TedCu10-BL#9 via wounded

leaves, stems, and roots. Daily monitoring of colonization showed bioluminescent bacteria in the inoculated leaf and petiole beginning 1 day postinoculation (DPI). The bacteria spread to roots via the stem by 2 DPI, reached the plant extremities 4 DPI, and the plant wilted 6 DPI. However, *Cucurbita* plants inoculated with TedCu10-BL#9 did not wilt, even at 35 DPI. Bioluminescent bacteria were detected 6 DPI in the main stem of squash and pumpkin plants, which harbored approximately 10^4 and 10^1 CFU/g, respectively, of TedCu10-BL#9 without symptoms. Although significantly less systemic plant colonization was observed in nonpreferred host *Cucurbita* plants compared with preferred hosts, the mechanism of tolerance of *Cucurbita* plants to *E. tracheiphila* strains from *Cucumis* remains unknown.

Bacterial wilt of cucurbits, caused by *Erwinia tracheiphila* (E. F. Smith) Holland, occurs in the United States, Europe, South Africa, and Japan (Agrios 2005). It is considered one of the most destructive diseases of cucurbits in the Midwestern and Northeastern United States, causing losses of up to 80% (Rojas et al. 2015). Cucurbits are important crops in the United States agricultural economy, with more than 110,000 ha of cucumber, melon, pumpkin, and squash cultivated annually for fresh and processing markets (NASS 2015).

E. tracheiphila can infect a host at any time during the growing season; however, young plants represent a higher epidemic risk because they are more susceptible to the disease than older plants (Liu et al. 2013). The pathogen is most commonly introduced into host tissues via feeding wounds caused by its insect vectors. Cucumber beetle density per plant is positively correlated with bacterial wilt incidence (Yao et al. 1996). *E. tracheiphila* survives in the alimentary canal of striped (*Acalymma vittatum* (F)) and spotted (*Diabrotica undecimpunctata howardi* Barber) cucumber beetles but survival rate inside the vectors is strain dependent (Mitchell and Hanks 2009). In previous studies, only 7 to 11% of the adult striped cucumber beetles that emerged in the spring were positive for *E. tracheiphila* and the proportion of beetles that tested positive for *E. tracheiphila* was five times greater than the proportion of beetles that actually transmitted bacterial wilt (Fleischer et al. 1999; Garcia-Salazar et al. 2000).

Cucumber beetles feed on all cucurbit plant parts, from seedling emergence in the spring through the end of the growing season. The pathogen is transmitted from an infested vector to cucurbit plants through the vector's feces (frass) when deposited in or near beetle

feeding wounds (Brust 1997a,b; Yao et al. 1996). The bacterium can be present in the frass of *A. vittatum* at concentrations as high as 10^5 colony forming units (CFU) per beetle 28 days after exposure to an infected plant (Shapiro et al. 2014). Sasu et al. (2010) also found that 95% of Texas gourd flowers contained cucumber beetle frass positive for *E. tracheiphila*. Using a strain transformed with green fluorescent protein inoculated onto the nectaries, they showed that 42% of the plants contracted the disease before the formation of an abscission layer. The presence of water on the leaf surface is a key factor in the survival of *E. tracheiphila*. Dry periods in the first 24 h after inoculation can drastically decrease the epiphytic survival of the pathogen (Rojas and Gleason 2012). *E. tracheiphila* can survive in dried plant tissue for a limited time and does not survive in the soil for more than 2 or 3 months (Babadoost et al. 2004; Koike et al. 2007).

Systemic infection of cucurbits by *E. tracheiphila* occurs once the bacteria reach the xylem. Wilting occurs due to the multiplication of the bacteria inside xylem tissue and secretion of an exopolysaccharide resin by the bacteria that blocks water flow in the vascular tissue (Koike et al. 2007; Sasu et al. 2010). Rojas et al. (2013) reported a preference among *E. tracheiphila* strains for colonizing hosts in the genus from which they were isolated. Genetic variability was reported among 69 strains isolated from five different Cucurbitaceae genera, with distinct repetitive-element polymerase chain reaction (rep-PCR) fingerprint profiles of isolates originating from *Cucumis* versus *Cucurbita*. There have been no studies reported to date to describe the differences in preferred versus nonpreferred host colonization dynamics in this pathosystem.

The use of bacteria transformed with a bioluminescent marker to monitor the interaction between plant and pathogen is an innovative approach to documenting colonization dynamics nondestructively in real time (Kassem et al. 2014). Actively metabolizing transformed cells express *lux* genes and emit photons that can be captured by a sensitive charge-coupled device camera, which allows the visualization of bacterial cells within plants. The camera is fitted into a chamber large enough to accommodate entire plants up to approximately 24 cm in height. This technique provides a sensitive

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*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary video and one supplementary figure are published online.

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means of visualizing bacterial invasion and colonization of hosts, and allowing real-time representation of plant–pathogen interactions (Xu et al. 2010).

Bioluminescent *E. amylovora* was used to study colonization (Bogs et al. 1998) and invasion of mature and immature apple fruit through pedicels (Azegami et al. 2004), while bioluminescent *E. ananas* was used to document its colonization of rice plants (Hasegawa et al. 2003). Xu et al. (2012) visualized for the first time the colonization, in real time, of tomato plants by *Clavibacter michiganensis* subsp. *michiganensis*, reporting acropetal and basipetal movement of the pathogen. Roots were entirely colonized by 10 days postinoculation (DPI). Using a bioluminescent strain of *Ralstonia solanacearum*, latent infections of asymptomatic potato plants were detected before development of the disease (Cruz et al. 2014).

Understanding the colonization dynamics of *E. tracheiphila* inside its hosts is essential to elucidate the interaction between pathogen and host that leads to productive colonization and symptom expression and the development of approaches to manage the disease. The objectives of this study were to (i) develop a *Cucumis*-derived bioluminescent strain of *E. tracheiphila* marked with the *lux* operon, (ii) provide detailed observations of melon plant colonization by bioluminescent *E. tracheiphila* via different routes of entry using real time imaging, and (iii) describe *E. tracheiphila* colonization dynamics in preferred and nonpreferred cucurbit hosts.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. tracheiphila* strains were obtained from a collection at Iowa State University (Rojas et al. 2013) (Table 1).

Broth cultures of *E. tracheiphila* strains were grown aerobically overnight at 200 rpm and 28°C in either nutrient broth yeast extract (NBY) medium with glucose (5 mg/ml) or nutrient peptone broth (NPB). The growth medium was supplemented with chloramphenicol (5 µg/ml) or kanamycin (5 µg/ml), depending upon the plasmid used. *Escherichia coli* strains (DH5α and ER2925) were grown overnight in Luria-Bertani (LB) medium at 37°C.

Plasmids. Transposon plasmids pXX2 and pXX3, constructed with the *lux* operon (*luxCDABE*) and associated chloramphenicol resistance gene, were used (Xu et al. 2010). The plasmid pUWGR4 (Rajashekara et al. 2005) was used for transposome engineering using the EZ::Tn5 system (Epicentre, Madison, WI). The plasmid pUWGR4 was digested with *Pvu*II, generating a linear DNA fragment of approximately 7 kb containing the *lux* operon and kanamycin resistance gene. The fragment was purified (Qiagen, Valencia, CA) and mixed with the transposase enzyme. Briefly, 1 µl of the purified DNA was mixed with 2 µl of the enzyme and 1 µl of 100% sterile glycerol. The reaction was incubated for 3 days at room temperature, then stored at –20°C. Broad-host-range plasmids pBBR1MCS1-*lux* and pBBR1MCS1 without *lux* (the control) were also evaluated in this study. Plasmids or a transposome complex were introduced into the cells via electroporation.

Bacterial transformation. Bioluminescent strains of *Erwinia tracheiphila* TedCu10 were generated by electroporation of the transposon plasmids pXX2 or pXX3, pBBR1MCS1-*lux*, pBBR1MCS1, or the EZ::Tn5 transposome into electrocompetent cells prepared by inoculating a flask containing NPB medium with a 1:100 dilution of TedCu10 bacterial suspension at an optical density of 600 nm (OD₆₀₀) of 1.0. The culture was grown until OD₆₀₀ of 0.5 to 0.7 was achieved. The culture was kept at 4°C for 30 min to slow the growth. Bacterial cells were harvested by centrifugation at 8,000 × g for 15 min at 4°C. The pelleted cells were first washed with cold sterile MilliQ water using the same initial volume. A second wash with sterile water was done using 50% of the initial volume. A third wash was done with 10% sterile glycerol, using 10% of the original volume. The final cell pellet was resuspended in 10% sterile glycerol to 0.2% of the original volume. Electrocompetent cells (100 µl) were mixed separately with 1 µl of plasmids pXX2, pXX3,

pBBR1MCS1-*lux*, or pBBR1MCS1, or with 2 µl of the EZ::Tn5 transposome complex (1 µg total). Electroporation was performed in a 0.2-cm cuvette using a Gene Pulser Xcell Electroporator (Bio-Rad, Hercules, CA) with the following settings: 2.5 kV, 25 µF, and 200 Ω. Immediately after electroporation, 500 µl of room-temperature Super Optimal broth with Catabolite repression (S.O.C.) medium (Corning, Corning, NY) was mixed with the cells in the cuvette, transferred to a 10-ml sterile disposable tube, and incubated with horizontal shaking for 2 h at room temperature for cell recovery. After incubation, 100 µl of the suspension was spread on a plate containing either chloramphenicol or kanamycin. Plates were incubated at 28°C for 3 to 5 days. Colonies were screened for bioluminescence using the In Vivo Imaging System (IVIS) Lumina Series III Imaging System (PerkinElmer, Shelton, CT). Bioluminescent colonies were purified and stored at –80°C in cryogenic vials containing 500 µl of NBY and 500 µl of 30% glycerol. The negative control was electroporation with sterile water.

PCR analysis of transformed colonies. PCR with specific primers targeting the *carbamoyl phosphate synthetase* gene, ETC-1 (5'-GCACCAATTCCGCAGATCAAG-3') and ETC-2 (5'-CGCAGGATGTTACGCTTAACG-3'), was performed to confirm the identity of transformed colonies as *E. tracheiphila* (Mitchell and Hanks 2009). To confirm the introduction of the *lux* operon into the cells, direct-colony PCR was performed using LuxCF (5'-GTGTGATGAATATCCACCTCT-3') and LuxCR (5'-GGACATACATTCGTGACTTA-3') primers (Xu et al. 2010). PCR was performed using a GoTaq Green Master Mix kit (Promega Corp., Madison, WI).

Bioluminescence analysis. Aliquots (10 µl) of suspensions of bioluminescent colonies adjusted to OD₆₀₀ = 1.0 were added to individual wells of a 96-well deep-well microtiter plate (Thermo Scientific, Waltham, MA) containing 990 µl of NBY plus glucose amended with chloramphenicol. The plate was sealed and incubated at 200 rpm and 28°C. Measurements of light intensity (average radiance, maximum radiance, and total radiance per well) using IVIS with open filter OD₆₀₀ were taken over time from a 100-µl sample. Bioluminescence intensity was compared among transformed strains at an OD₆₀₀ of 0.2. The three categories of light intensity mentioned above were used together for selection. Four strains were selected for pathogenicity testing.

Growth characterization. The growth characteristics of the selected transformed strains were compared with those of the nontransformed wild type using cultures grown in flasks containing NBY + glucose, on a horizontal shaker at 200 rpm and 28°C in the dark. Light intensity (average radiance, maximum radiance, and total radiance per well) and OD₆₀₀ were measured at 0, 8, 10, 12, 14,

TABLE 1. Bacterial strains and plasmids used in this study

Strain	Description	Reference or source
<i>Erwinia tracheiphila</i>		
TedCu10	Wild type isolated from <i>Cucumis sativus</i> L.	Rojas et al. 2013
BHKY	Wild type isolated from <i>Cucurbita moschata</i>	Rojas et al. 2013
<i>Escherichia coli</i>		
DH5α	Used for plasmid propagation	Invitrogen
ER2925	Used for plasmid propagation	New England Biotechnology
Plasmids		
pUWGR4	Source of EZ::Tn/lux transposon	Rajashekara et al. 2005
pXX2	Source of <i>TN1409::lux</i> ; gram positive	Xu et al. 2010
pXX3	Source of <i>TN1409::lux</i> ; gram negative	Xu et al. 2010
pBBR1MCS1- <i>lux</i>	pBBR plasmid carrying the <i>lux</i> operon	Laboratory collection
pBBR1MCS1-nolux	Control for pBBR- <i>lux</i>	Laboratory collection

16, 18, 20, 22, and 24 h after inoculation. The experiment was conducted twice, with three biological replications.

Minimum detection limit. Aliquots of bioluminescent *E. tracheiphila* adjusted to $OD_{600} = 0.3$ (approximately 10^8 CFU/ml) were added to four (experiment 1) or eight (experiments 2 and 3) individual wells of a 96-well microtiter plate (black, with clear, flat bottom; Corning). Eleven dilutions (1:3) were performed with a final volume of 100 μ l in each well. The plate was photographed using IVIS with open filter, and the minimum detection limit was determined based on the presence of light in the well at 22°C. The experiment was performed three times.

Pathogenicity assay. Seed of hybrid melon (*Cucumis melo* 'Athena F1') and cucumber (*C. sativus* 'Intimidator') were planted in 50-cell flats containing Baccto professional grower's potting mix (Baccto, Houston, TX) comprising 75 to 85% peat moss and perlite limed to pH 5.5 to 6.5. Plants were maintained in a greenhouse at 28°C under a 14-h photoperiod, and fertilized with N-P-K (20-20-20 at 4 g/liter; Plant Marvel, Chicago Heights, IL) weekly. When the second true leaf was completely unfolded, cells containing individual seedlings were cut from the tray and moved to new trays for inoculation and randomization.

Wild-type TedCu10 (positive control) and transformed strains TedCu10-BL#6, -9, -19, and -32 were recovered from -80°C storage, plated onto NBY with glucose, and incubated for 2 days at 28°C. Bacterial suspensions were prepared in sterile 10 mM potassium phosphate buffer (pH 7.4) and adjusted to approximately 10^8 CFU/ml. Negative control plants were inoculated with the buffer only. A 100- μ l aliquot of inoculum was distributed in 10 approximately equal volumes on the surface of the second true leaf of each melon and cucumber plant ($n = 4$). Leaves were wounded slightly through each drop of inoculum with a sterile razor blade. Plants were monitored for symptoms at 2, 4, 6, and 8 days DPI. The number of wilted leaves and total number of leaves were recorded, and these data were used to calculate percent wilt per plant. Area under the wilting progress curves (AUWPC) were calculated based on the formula $\sum[(x_i + x_{i+1})/2](t_{i+1} - t_i)$, where x_i is the percentage of wilted leaves per plant at each evaluation time and $(t_{i+1} - t_i)$ is the number of days between evaluations. Plants were photographed using IVIS with a 570 filter at each evaluation time.

Plants were randomized in a complete block design in a greenhouse room, where each block was a replication composed of a tray containing all treatments. Four replications, treated as random effects, were used for each treatment. The experiment was performed twice for each crop.

In vitro and in vivo stability of the insertion. The in vitro stability of the plasmid insertion was verified by growing each of the four bioluminescent strains for 24 h in 5 ml of NBY without antibiotics in a 14-ml sterile tube, with four replicates. Growth conditions were as described above. A 5- μ l aliquot of the culture was transferred to a new tube containing 5 ml of the same medium every 24 h, for a total of 10 passages. At the end of each passage, OD_{600} and total radiance were measured from an aliquot of 100 μ l of the bacterial suspension.

In vivo stability was verified in Athena F1 melon plants, grown as described above. *E. tracheiphila* TedCu10-BL#9 was inoculated on the second true leaf of plants, as described above. Plants were processed 2, 4, and 6 DPI for bacterial quantification. Whole plants were photographed using IVIS, then separated into leaves, stem, and roots, each of which were photographed. Roots were processed separately for bacterial quantification. In the first experiment, the three replicate whole-stem samples were processed for bacterial quantification. In the second experiment, four replicate stem sections (approximately 0.2 g each) were processed. Replications were treated as random effects in a randomized complete block design. Plant samples were ground in a mesh sample bag (Agdia Inc., Elkhart, IN) with 3 ml of sterile 100 mM potassium phosphate buffer (pH 7.4). Extracts were serially diluted and selected 10-fold dilutions were plated on NBY plus glucose, with and without chloramphenicol. The number of bioluminescent and nonbioluminescent

colonies growing on each plate was compared to assess the percentage of cells that retained the lux insertion in each part of the plant. The experiment was performed twice. Bacterial populations were $\log_{10}[(CFU/g) + 1]$ transformed prior to analysis.

Colonization of melon via stem and root inoculation. Seed of Athena F1 melon were sown as described above. When the second true leaf was fully expanded, v-shaped wounds approximately 1 mm in length were cut into stems approximately 1 cm below the cotyledon with a sterile razor blade to mimic cucumber beetle feeding damage. An approximately 10^8 CFU/ml suspension of *E. tracheiphila* TedCu10-BL#9 was prepared as described above and an aliquot of 10 μ l was placed on the wound. Four plants were inoculated, and four negative control plants, mock-inoculated with buffer, were used for each experiment. The experiment was performed three times. In the third experiment, plant sap that oozed out of the stem when wounded was removed with a sterile paper towel before inoculation.

Roots were cut with a sterile scalpel approximately 3 cm deep, 1 cm from the stem. A suspension of *E. tracheiphila* TedCu10-BL#9 was prepared as described above and 1 ml was used to drench the soil where the roots had been wounded. Four plants were inoculated in experiments 1 and 2 and eight plants were inoculated in experiment 3. Four negative control plants were mock inoculated with buffer. For the third experiment, four additional plants were inoculated by drenching the soil with inoculum or buffer (control) near the crown of the plant without wounding.

Cucurbit host preference by *E. tracheiphila*. Seed of Athena F1 melon, Intimidator cucumber, pumpkin (*Cucurbita pepo* 'Charisma F1'), and zucchini squash (*C. pepo* 'Dunja F1') were sown as described above. Plants were transplanted to 10.2-cm pots before inoculation at the second-true-leaf stage.

Strains of *E. tracheiphila* (TedCu10-BL#9 and BHKY) were recovered from -80°C storage, plated onto NBY plus glucose, and incubated for 2 days at 28°C. Plants were inoculated on the second true leaf as described above.

For the first experiment, 10 *Cucurbita* and 5 *Cucumis* plants of each species were inoculated with TedCu10-BL#9 as described above, and 4 plants of each were mock inoculated with sterile buffer as negative controls. Plants were imaged in IVIS 1, 2, 3, 4, 5, 6, 10, 12, 14, and 21 DPI. The experiment was monitored for 35 days.

For the second experiment, five melon and five cucumber plants were inoculated with TedCu10-BL#9, and five of each were inoculated with buffer. In all, 10 pumpkin and 10 squash plants were inoculated with TedCu10-BL#9, 5 of each were mock inoculated with buffer, and 5 of each with the *Cucurbita*-derived strain BHKY. For the third experiment, six melon and six cucumber plants were inoculated with TedCu10-BL#9, and six of each were mock inoculated with buffer. In total, 12 pumpkin and 12 squash plants were inoculated with TedCu10-BL#9, and 6 of each were mock inoculated with buffer and 6 of each with the *Cucurbita*-derived strain BHKY. Plants were photographed using the IVIS 2, 4, 6, 8, 10, 14, 18, 21, 25, 28, 31, and 35 DPI. Bacterial populations were quantified from three plants from each treatment at 6 DPI. Plants were processed as described above; stem sections were selected at the origin of the second true leaf. Bioluminescent signals were detected 8 DPI from the stem of one squash plant in experiment 2, and the bioluminescent portion of the stem was processed. Populations of TedCu10-BL#9 were determined in three additional squash and pumpkin plants 21 and 35 DPI (experiment 2) or nine plants of each species at 35 DPI (experiment 3). Roots of squash and pumpkin were photographed in IVIS at each processing time point and bacterial populations were quantified 35 DPI (experiment 3). Bacterial populations were $\log_{10}[(CFU/g) + 1]$ transformed prior to analysis. All experiments were randomized in a complete block design. Bacterial quantification data for experiments 2 and 3 were analyzed together, with blocks and experiment treated as random factors. Treatments were defined as each host inoculated with TedCu10-BL#9 or BHKY.

Data analysis. Data were analyzed using PROC GLIMMIX in SAS (version 9.4; SAS Institute, Cary, NC), and means were separated by a least significance difference test at the 5% level of significance.

Real-time imaging of colonization. To gain insights into *E. tracheiphila* colonization in noninterrupted real-time, in a separate experiment, seed of Athena F1 melon were sown and leaf inoculated with bioluminescent *E. tracheiphila*, as described above. Four replicate plants were photographed every 4 h for 9 days. Photographs were converted to a video (Supplementary Video S1).

RESULTS

Plasmid insertion into *E. tracheiphila*. In total, 259 bioluminescent colonies were obtained when plasmids pXX2 (159 colonies) and pXX3 (100 colonies) were used for transformation. Electroporation of *E. tracheiphila* cells with the EZ::TN/lux transposome complex prepared from the pUWGR4 plasmid did not yield transformed colonies. The average total flux of light normalized for the OD₆₀₀ at early log phase (approximately OD₆₀₀ = 0.2) of 100 colonies transformed with pXX3 was 8.3×10^8 photons/s. The average light flux of 26 colonies transformed with pXX2 was 5.6×10^7 photons/s, and for 4 colonies transformed with pBBR1MCS1-lux was 4.8×10^6 photons/s (Fig. 1). Colonies transformed with pBBR1MCS1-lux were unstable in the presence of antibiotics and did not grow during subculturing.

Effect of plasmid pXX3 insertion on virulence of bioluminescent *E. tracheiphila* strains. Transformed *E. tracheiphila* strains TedCu10-BL#6, TedCu10-BL#9, TedCu10-BL#19, and TedCu10-BL#32 were selected, based on high bioluminescent signal intensity,

to test for pathogenicity. These strains were confirmed as *E. tracheiphila* based on PCR with the ETC primer set. There were no significant differences among the wild-type strain and transformed strains in wilting progress (AUWPC; Fig. 2). Bioluminescent signals were detected in plants inoculated with each of the four transformed strains, which were confirmed to harbor the *lux* operon by PCR with the LuxC primer set.

Growth, minimum detection limit, and stability in vitro and in vivo of bioluminescent *E. tracheiphila* strain TedCu10-BL#9. The in vitro growth curve for bioluminescent *E. tracheiphila* TedCu10BL#9 was similar to that of wild-type TedCu10 based on the OD₆₀₀ when grown in NBY + glucose medium. Bioluminescence increased as TedCu10-BL#9 grew in log phase. Similar growth of TedCu10-BL#9 was observed in rich medium (NBY plus glucose; Fig. 3). The minimum detection limit of TedCu10-BL#9 in vitro was $6.7 \times 10^3 \pm 2.2 \times 10^3$ CFU at 22°C.

Bioluminescence was relatively unstable in vitro in rich medium (NBY plus glucose) at 28°C; a decrease in bioluminescence after 10 passages was observed. A reduction of approximately 1.7 log photons/s was observed for TedCu10-BL#9 after 10 passages (data not shown).

The percentage of bioluminescent *E. tracheiphila* colonies recovered from stems of TedCu10-BL#9 leaf-inoculated plants did not differ statistically 2, 4, and 6 DPI in the first experiment ($P = 0.1791$), whereas, in the second experiment, the number of bioluminescent cells recovered 6 DPI was lower than 2 and 4 DPI ($P = 0.0117$). The plasmid was stable in roots in the second experiment, with no statistical differences in bioluminescent colony recovery over time ($P = 0.3868$) (Table 2).

Colonization of melon plants by bioluminescent *E. tracheiphila* after leaf inoculation. Bioluminescent *E. tracheiphila* was detected in the petiole of inoculated leaves 1 DPI and in the main stem 2 DPI. Inoculated leaves began wilting 2 DPI. Plants were completely colonized 4 DPI (Fig. 4).

Fig. 1. *Erwinia tracheiphila* colonies transformed with pXX3 and pXX2 plasmids containing the *lux* operon. Microtiter plate on the top: columns 1 to 4, *E. tracheiphila* colonies transformed with pXX3; columns 6 to 9, transformed with pXX2; and column 11, wild type. Plate on the bottom: columns 1 to 10, transformed with pXX3; column 11, transformed with pBBR1 plasmids; and column 12, wild type.

Fig. 2. Area under the wilting progress curve (AUWPC) for cucumber and melon plants inoculated on the second true leaf with 10^7 CFU of bioluminescent *Erwinia tracheiphila* strains TedCu10-BL#6, -9, -19, or -32 or the nontransformed wild-type TedCu10. Negative control plants were mock inoculated with sterile buffer. Columns with the same letter and case represent AUWPC values that are not significantly different. Lowercase: Intimidator cucumber and uppercase: Athena F1 melon.

Bacterial populations were higher at 4 than 6 DPI in the first experiment, with reduction of approximately two log CFU/g from 4 to 6 DPI (Table 2). Bacterial populations inside stem sections were not different in the second experiment. Low TedCu10-BL#9 populations were detected in the roots 2 DPI and populations increased at 4 DPI (Fig. 5; Table 2).

Colonization of melon plants inoculated with bioluminescent *E. tracheiphila* via stems and roots. Results of three experiments in which melon plants were stem and root inoculated with TedCu10-BL#9 were inconsistent. In the first experiment, none of the plants inoculated via the stem with the bioluminescent strain were colonized, and only one plant inoculated with wild-type TedCu10 wilted. In the second experiment, three of four inoculated melon plants were colonized by bioluminescent *E. tracheiphila*. One plant was fully colonized 8 DPI (Fig. 6). Two additional plants were fully colonized 12 DPI. In the third experiment, only one plant was fully colonized at 8 DPI. In total, 33.3% of melon plants inoculated via wounded stems were colonized by *E. tracheiphila*.

Only one root-inoculated plant was colonized by bioluminescent *E. tracheiphila* in the first experiment, with upward movement in the plant detected 5 DPI (Fig. 7). None of the plants inoculated with wild-type TedCu10 wilted. In the second and third experiments, no colonization was detected after monitoring plants for 14 days.

Cucurbit host preference by *E. tracheiphila*. Although both melon and cucumber plants inoculated with TedCu10-BL#9 wilted within 6 DPI, none of the 10 TedCu10-BL#9-inoculated squash

or pumpkin plants wilted up to 35 DPI (experiment 1). The stems of all melon and cucumber plants were colonized and bioluminescent within 4 DPI but bioluminescence was only detected in the main stem of one squash and two pumpkin plants 4 and 6 DPI. However, the signal disappeared by 8 DPI (Supplementary Fig. S1).

Similar results were observed for *Cucumis* plants in the second experiment. However, bioluminescence was not detected 4 and 6 DPI in the stems of squash and pumpkin inoculated with TedCu10-BL#9. Petioles of the inoculated leaf of five squash and three pumpkin plants were bioluminescent 2 DPI. In the third experiment, bioluminescence was detected in the stem of two squash plants 4 and 6 DPI. None of the plants wilted within 35 DPI (experiment 2 and 3). The results for bacterial quantification are shown in Table 3. The effect of treatment (defined as each host inoculated with TedCu10-BL#9 or BHKY) was highly significant ($P < 0.001$). Bacterial populations were $>10^8$ CFU/g of tissue in *Cucumis* or *Cucurbita* plants inoculated with TedCu10-BL#9 or BHKY, respectively, within 6 DPI. Bacterial populations were 1.1×10^4 and 1.1×10^1 CFU/g in squash and pumpkin plants, respectively, inoculated with TedCu10-BL#9 within 6 DPI (Table 3). Additionally, one squash plant (experiment 2) bioluminescent in the stem 8 DPI harbored *E. tracheiphila* TedCu10-BL#9 at 4.5×10^7 CFU/g; however, the plant did not wilt. No bacteria were detected 21 and 35 DPI in the second experiment or in the mock-inoculated controls in either experiment.

Fig. 3. Growth curves (OD) and bioluminescence (BL) of *Erwinia tracheiphila* wild type TedCu10 and TedCu10-BL#9 strains. Both strains were inoculated into 100 ml of nutrient broth yeast extract broth with glucose. Optical density at 600 nm (OD₆₀₀) and photon emissions were measured for 26 h. Error bars represent standard deviations.

TABLE 2. In vivo stability of plasmid pXX3 insertion conferring bioluminescence in *Erwinia tracheiphila*^a

DPI	Stem				Roots	
	Experiment 1		Experiment 2		Log ₁₀ CFU/g	Nonbioluminescent (%)
	Log ₁₀ CFU/g	Nonbioluminescent (%)	Log ₁₀ CFU/g	Nonbioluminescent (%)		
2	7.8 ± 0.36 b	1.3 a	4.3 ± 4.98 a ^y	1.1 b	1.8 ± 3.63 b ^y	0.0 a
4	9.5 ± 0.17 a	3.7 a	8.3 ± 0.16 a	6.8 b	8.4 ± 0.11 a	6.3 a
6 ^z	7.6 ± 1.13 b	17.2 a	8.0 ± 0.37 a	22.7 a	8.6 ± 0.03 a	6.9 a

^a Populations were recovered 2, 4, and 6 days postinoculation (DPI) from stems and roots of Athena F1 melon plants leaf inoculated with *E. tracheiphila* TedCu10-BL#9. Columns with the same letter represent log₁₀ CFU/g or percent nonbioluminescent values that are not significantly different.

^y No colonies were recovered from two stem and three root replicate samples 2 DPI.

^z Average log₁₀ CFU/g and standard deviation calculated from two replications at 6 DPI in experiment 1.

For the third experiment, TedCu10-BL#9 at $1.6 \times 10^5 \pm 2.3 \times 10^5$ CFU/g (average $\pm$ standard deviation of colonized plants only) was detected 35 DPI in five of nine squash plants. Three of nine pumpkin plants were colonized with $4.7 \times 10^3 \pm 4.2 \times 10^3$ CFU/g. Bioluminescence was detected in three of nine squash roots 35 DPI (Fig. 8). The bacteria were recovered from only one root sample with a population of 1.2×10^6 CFU/g. Bioluminescence was not detected and bacterial colonies were not isolated from pumpkin roots or any mock-inoculated control plants.

DISCUSSION

Bacterial wilt is a major disease of cucurbits in the Midwestern and Northeastern United States and can cause significant economic impact, especially when infection occurs early in the season, when

plants are highly susceptible. Little is known about the biology of this pathogen and how it colonizes its hosts from different ports of entry. The use of the *lux* operon as a bioluminescent reporter facilitates the characterization of the colonization of plants by bacterial pathogens and has been used previously for other pathosystems (Azegami et al. 2004; Bogs et al. 1998; Cruz et al. 2014; Hasegawa et al. 2003; Xu et al. 2010, 2012). This technique enabled visualization of colonization dynamics of *E. tracheiphila* in planta in real time from different points of entry and the interaction between a *Cucumis*-derived strain with different hosts within the genera *Cucumis* and *Cucurbita*.

The construction of bioluminescent *E. tracheiphila* strains required testing different approaches. The goal was to generate a strain that was stably bioluminescent and for which growth or virulence was not affected. Initially, the EZ::Tn5 system, using the

Fig. 4. Colonization of Athena F1 melon with bioluminescent *Erwinia tracheiphila* TedCu10-BL#9 after inoculation of the second true leaf with 10^7 CFU evenly distributed in 10 locations on the leaf surface. Leaves were wounded with a sterile razor blade through the inoculum droplet. Plants were photographed using IVIS Lumina System III and all images were corrected to the same light scale. The rainbow scale represents light intensity in photons per second (represented on the right). DPI = days postinoculation.

Fig. 5. Colonization of Athena F1 melon roots 2 and 4 days postinoculation (DPI) with 10^7 CFU of bioluminescent *Erwinia tracheiphila* strain TedCu10-BL#9 distributed in 10 locations on the second true leaf. The rainbow scale represents light intensity in photons per second.

lux operon from pUWGR4, was used but bioluminescent colonies were not generated. Plasmids pXX2 and pXX3 were previously developed by Xu et al. (2010) and were used in direct electroporation. Plasmids pBBR1MCS1-*lux* and pBBR1MCS1-*nolux* were used as controls because pBBR1MCS1 cloning vector has been previously reported with *E. amylovora* (Edmunds et al. 2013). Electroporation of pXX2 and pXX3 yielded 159 and 100 transformed colonies, respectively, that carried the *lux* operon. The low efficiency of transformation could be attributed to the large size of the plasmids and the *lux* operon. The total light emission in photons per second for colonies transformed was highest for pXX3 and lowest for pXX2 and pBBR1MCS1-*lux* ($P < 0.001$). Xu et al. (2010) reported that pXX3 was optimized for use with gram-negative bacteria, which explains why we observed a 93.3% reduction in luminescence in *E. tracheiphila* strains transformed with pXX2 compared with strains transformed with pXX3.

E. tracheiphila strain TedCu10-BL#9 exhibited growth similar to that of the wild-type strain TedCu10, and no differences in virulence compared with each other or the wild type in melon and cucumber. Colonization of melon plants was initiated within 1 DPI, with bacteria moving from the inoculated leaf to the leaf petiole; root colonization was detected as early as 2 DPI. There is a paucity of information about *E. tracheiphila* colonization of cucurbit plant organs. A 1970 study indicated that the pathogen was never observed in hypocotyl or root tissue of inoculated plants (Main 1971), although very early work demonstrated root colonization when roots were directly inoculated (Rand and Enlows 1916). To our knowledge, this is the first report of root colonization resulting from an aboveground inoculation.

Although bioluminescence intensity in TedCu10-BL#9 decreased in vitro over time, we found that, on average, about 95% of colonies reisolated from infected melon stems 4 DPI were bioluminescent. Maximum colonization of plant stems was observed 4 DPI (experiment 1). In our in vitro assessment of *E. tracheiphila* growth dynamics, we found that bioluminescence intensity peaked at the end of an exponential growth phase. The decrease in bioluminescent intensity in planta after the *E. tracheiphila* colonization peak might have been the result of reduction in bacterial cell metabolism due to lower availability of resources concomitant with the collapse of the plant. Expression of bioluminescence is ATP dependent, and energy from cell metabolism is required for luciferin conversion into light (Hakkila et al. 2002).

Cucumber beetles tend to prefer leaves as feeding sites (Smith 1911). The majority of melon plants inoculated with TedCu10-BL#9 via a wounded stem or roots were not colonized productively and, therefore, did not wilt. Bioluminescence was not detected in these plants through 14 DPI. When the same amount of inoculum applied to the stem was used on the second true leaf, a productive colonization was observed and all tested plants wilted 6 DPI (data not shown). The depth of the stem wound or other factors not tested in this study may be important in colonization success. Rand and Enlows (1916) demonstrated 62.5% *E. tracheiphila* colonization after inoculation of root-pruned cucumber, and 12.5% when roots were not pruned. In a second experiment with cucumber inoculated with strains isolated from squash, cucumber, and cantaloupe, the authors detected 25 and 22% of wilting when inoculated roots were pruned or not pruned, respectively. It is not known whether *E. tracheiphila* is transmitted from cucumber beetle adults to eggs

Fig. 6. Athena F1 melon plant stem inoculated with 10^6 CFU of bioluminescent *Erwinia tracheiphila* TedCu10-BL#9 and photographed using the IVIS Lumina System III 4, 8, and 12 days postinoculation (DPI). The stem was wounded about 1 mm deep with a sterile razor blade prior to inoculation. The majority of inoculated plants were not colonized and did not wilt after stem inoculation. The rainbow scale represents light intensity in photons per second.

Fig. 7. Athena F1 melon plant root inoculated with bioluminescent *Erwinia tracheiphila* strain TedCu10-BL#9 and photographed 2, 6, and 10 days postinoculation (DPI) with IVIS Lumina System III. Roots were wounded with a sterile scalpel approximately 3 cm deep, 1 cm from the stem. Only 1 plant among 16 across three experiments was colonized and wilted after root inoculation. The rainbow scale represents light intensity in photons per second.

and to larvae, which feed on roots, or if the pathogen can be transmitted to plants via root feeding. These are potentially important aspects of the epidemiology of this pathosystem yet to be studied.

The striped cucumber beetle is the more common of the two cucumber beetle species found in the Northeastern United States (Shapiro 2012) and Wisconsin (Delahaut 2005). Striped cucumber beetles have a preference for feeding on cucurbits, whereas spotted cucumber beetles can feed on more than 200 crops (Snyder 2015). Adult beetles diapause in the soil until spring, when they emerge and threaten cucurbit fields (Shapiro 2012). It is known that *E. tracheiphila* overwinters only in the digestive tracks of adult cucumber beetles in diapause in the soil (de Mackiewicz et al. 1998). *E. tracheiphila* can be found in the foregut and hindgut of striped cucumber beetles. Previous reports showed that beetles can harbor the pathogen for 30 days after feeding on infected leaves (Garcia-Salazar et al. 2000; Shapiro et al. 2014). According to de Mackiewicz et al. (1998), the pathogen cannot survive in weeds. Researchers were not able to isolate the pathogen from inoculated weeds, even though serological assays were positive for *E. tracheiphila*. There is no report of the pathogen overwintering in the root system of cucurbit crops left on the ground after harvest.

E. tracheiphila was thought to be a single species, regardless of *Cucumis* or *Cucurbita* host. It was reported in 1911 that strains isolated from cucumber led to no symptoms or slower development of symptoms when inoculated in pumpkin and squash (Smith 1911). Rojas et al. (2013) recently observed that *E. tracheiphila* strains isolated from *Cucumis* plants are genetically different from strains isolated from *Cucurbita* plants based on genomic fingerprint analysis. They also showed that some *Cucurbita* plants inoculated with a *Cucumis*-derived strain wilted 35 DPI but the majority of the plants did not wilt. Our study is the first to show that nonproductive colonization occurs inside *Cucurbita* plants inoculated with a *Cucumis*-derived bioluminescent *E. tracheiphila* strain. We observed that, when plants were colonized by the strain isolated from the same genus (*Cucumis* plants inoculated with TedCu10-BL#9 and *Cucurbita* plants inoculated with BHKY), the bacterial population was $>10^8$ CFU/g of tissue within 6 DPI. When *Cucurbita* plants were inoculated with TedCu10-BL#9, the bacterial population was lower 6 DPI, 1.1×10^4 CFU/g in squash and 1.1×10^1 CFU/g in pumpkin. The population of *E. tracheiphila* in the stem of a squash plant where bioluminescence was observed (experiment 2, 8 DPI) was 4.5×10^7 CFU/g, approximately 28% of the population observed in a BHKY-inoculated *Cucurbita* stem 6 DPI. These results suggest that some interaction between *Cucurbita* host and *Cucumis*-derived pathogen is taking place that does not allow productive colonization and subsequent disease development. This phenomenon is also observed in other pathosystems involving aggressive vascular

TABLE 3. Quantification of *Erwinia tracheiphila* in stem tissues of melon, cucumber, squash, and pumpkin plants 6 days postinoculation (DPI) with bioluminescent strain TedCu10-BL#9^w

Crop ^x	Strain inoculated	Log ₁₀ CFU/g 6 DPI	Plants yielding <i>E. tracheiphila</i> colonies/total plants
Melon	TedCu10-BL#9 ^y	8.2 ± 0.32 a	6/6
Cucumber	TedCu10-BL#9	8.6 ± 0.19 a	6/6
Squash	TedCu10-BL#9	4.0 ± 2.03 b	5/6
Squash	BHKY ^z	8.2 ± 0.38 a	6/6
Pumpkin	TedCu10-BL#9	1.0 ± 1.62 c	2/6
Pumpkin	BHKY	8.2 ± 0.75 a	6/6

^w Squash and pumpkin were also inoculated with BHKY, a nonbioluminescent squash-derived strain (positive control). All negative controls were mock inoculated with sterile buffer. Columns with the same letter represent log₁₀ CFU/g values that are not significantly different.

^x No bacteria were isolated from mock-inoculated controls.

^y Bioluminescent *E. tracheiphila* strain transformed from strain originally isolated from cucumber.

^z *Erwinia tracheiphila* strain originally isolated from *Cucurbita pepo*.

bacterial pathogens such as *R. solanacearum* in solanaceous plants. Strains of *R. solanacearum* vary in their ability to colonize pepper, tomato, and eggplant genotypes, and latent infections in which the bacteria colonize the vascular system but do not cause the plant to wilt are common (Lebeau et al. 2011). Colonization of nonpreferred host (squash) roots by *E. tracheiphila* TedCu10-BL#9 may indicate a strong source-sink relationship in the plant but the significance of nonpreferred host root populations in the survival and dissemination of the pathogen is yet to be determined. There have been no reports of *E. tracheiphila* in the Andes and Mesoamerica, the geographical center of origin of *Cucurbita* spp. The pathogen was reported recently to infect pumpkin and watermelon (*Citrullus lanatus* var. *lanatus*) in New Mexico (Sanogo et al. 2011). This finding is particularly noteworthy because *E. tracheiphila* is generally thought not to cause bacterial wilt disease in watermelon in areas in which the disease has traditionally been a problem (Latin 2000). Shapiro (2012) has suggested that the pathogen may be undergoing a rapid evolution, leading to adaptation to different hosts. The *E. tracheiphila*–Cucurbitaceae pathosystems are not highly explored and advanced knowledge of these systems could improve management approaches, including disease resistance, which are not currently available in commercial cultivars. Bio-luminescent *E. tracheiphila* strains for both *Cucumis* and *Cucurbita* hosts can be efficient tools to screen plant introductions, lines, and cultivars for colonization; elucidate the dynamics of colonization inside weeds; and assess the efficacy of antibiotics and bactericides in planta in real time.

Fig. 8. Roots of Dunja F1 squash plants 35 days postinoculation (DPI) with bioluminescent *Erwinia tracheiphila* strain TedCu10-BL#9, with 10^7 CFU evenly distributed in 10 locations on the second true leaf surface. Leaves were wounded with a sterile razor blade through the inoculum droplet. Plants were photographed using IVIS Lumina System III. Roots of three of nine plants inoculated with TedCu10-BL#9 were colonized by the bacterium. The rainbow scale represents light intensity in photons per second.

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