Insect-Mediated Dispersal of the Rhizobacterium *Pseudomonas chlororaphis*

William E. Snyder, David W. Tonkyn, and Daniel A. Kluepfel

First and second authors: Department of Biological Sciences; and third author: Department of Plant Pathology and Physiology, Clemson University, Clemson, SC 29634.

Current address of W. E. Snyder: Center for Ecology, Evolution and Behavior, and Department of Entomology, College of Agriculture, University of Kentucky, Lexington 40546-0091.

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ABSTRACT

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The southern corn rootworm, *Diabrotica undecimpunctata* subsp. *howardi*, a common and mobile insect pest, was shown to transmit the rhizobacterium *Pseudomonas chlororaphis* strain L11 between corn plants. Strain L11 has been genetically modified to contain the *lacZY* genes from *Escherichia coli*. It can reach high densities on roots and invade the roots and move into the foliage. *D. undecimpunctata* subsp. *howardi* became infested with L11 as larvae while feeding on roots of seed-inoculated corn and retained the bacteria through pupation, molting to the adult

stage, and emergence from the soil. Bacterial densities on or in the insects increased 100-fold after they fed again as adults on L11-infested foliage. Adults retained the bacteria for at least 2 weeks after last exposure and could transmit L11 to new plants. The likelihood of transmission decreased with time since last exposure to L11, but increased with time spent on the new plants. This research demonstrates that rhizobacteria can escape the rhizosphere by moving in or onto foliage, where they can then be acquired and transmitted by insects. This transmission route may be common among naturally occurring rhizobacteria and facilitate the dispersal of both beneficial and harmful soilborne microorganisms.

Additional keywords: insect vector, microbial ecology, microcosm.

The use of microorganisms to perform such utilitarian functions as biological control, phytoremediation, and the reclamation of hazardous waste sites holds tremendous potential (2,9,10,32). However, as we begin to expand our use of native and genetically modified microorganisms, it will be useful to gain a greater understanding of the basic biology of these organisms including their dispersal ecology. Here we describe experiments designed to examine insect-mediated dispersal of the rhizobacterium *Pseudomonas chlororaphis* (formerly *P. aureofaciens*) strain L11, an effective rhizosphere colonizer isolated from the roots of corn.

L11 possesses no known biological control functions, but rather serves as a model organism for studying the dispersal ecology of soilborne Gram-negative bacteria in the rhizosphere. Rhizobacteria have been shown to travel less than 1 m laterally through the soil via colonization of intertwined roots or passive groundwater transport (18). Percolation vertically through the soil also is well documented. For example, under field conditions, Natsch et al. (26) reported that *P. fluorescens* moved 150 cm down into the soil profile 24 h after application to the soil surface. These and other reports demonstrate that microbial transport through soil will occur frequently with many soilborne Gram-negative bacteria under field conditions. However, this sort of passive transport will usually be over limited distances (17,39,40).

Transmission of phytopathogenic bacteria by insects has been well documented (14). For example, transmission of *Erwinia amylovora*, the causative agent of fire blight, has been reported for over 50 species of insects belonging to eight orders (14). However, beneficial bacteria can be transported as well. For example, Thomson et al. (37) and Johnson et al. (16) found that honey bees (*Apis mellifera*) could transport bacterial biocontrol agents of *Erwinia*

amylovora. Transmission of beneficial microorganisms may be as common as transmission of pathogens, although the former has been less studied.

Since rhizobacteria reside below the ground, it seems less likely they would be acquired and transported by winged insects, which are important vectors for many foliar bacterial pathogens (14). However, several recent studies have shown that rhizobacteria can be recovered from the foliage of plants (7,19,20,22,24), apparently entering through root wounds or by contamination of the cotyledons during germination. Even though seed-transmitted foliar bacterial pathogens also move from the soil into the foliage, it is perhaps more surprising that soilborne nonpathogens can also move into the foliage and survive (1). Once in or on the foliage, rhizobacteria could be acquired by foliar-feeding insects that often have wide host ranges and can travel long distances (23,28,33,36).

We studied insect-mediated dispersal of the rhizobacterium *P. chlororaphis* strain 3732RNL11 (hereafter called L11) in the work reported here. In a previous field release, L11 never spread more than 20 cm through the soil from the point of release (18). However, in subsequent work, Lamb et al. (22) found that L11 did move from the rhizosphere into the foliage of all 15 plant species tested. Once in the foliage, L11 might be acquired by foliar-feeding insects and transported to new sites and plants. Here we present data on the likelihood of acquisition and transmission of L11 by insects as a function of bacterial density in the plant hosts, time since last exposure to infested plant hosts, and feeding time on new, noninoculated plants.

Diabrotica undecimpunctata subsp. *howardi* (Coleoptera: Chrysomelidae), the southern corn rootworm, was used as the model dispersal agent. Beetles in this genus are typical of many crop pests in having a wide host range and long-range dispersal ability (4,8,13,25,30). *Diabrotica* spp. feed on roots as larvae, while the adults feed on leaves, stems, and flowers. *D. undecimpunctata* subsp. *howardi* larvae and adults are highly polyphagous, feeding upon plant species in the families Graminae, Cucurbitaceae, Leguminosae, So-

Corresponding author: D. A. Kluepfel; E-mail address: dklpfl@clemson.edu

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lanaceae, Compositae, Cyperaceae, and Convolvulaceae and are pests of economic importance on corn, cucurbit crops, and legumes (6,21).

In all experiments, *D. undecimpunctata* subsp. *howardi* were reared on *P. chlororaphis* L11-inoculated corn plants (*Zea mays*), both as larvae and as young adults, and then transferred to plants that were free of L11, mimicking typical exposure patterns in a field crop. By sampling *D. undecimpunctata* subsp. *howardi* regularly for L11, we were able to monitor the appearance and persistence of L11 in the insects. By housing L11-infested *D. undecimpunctata* subsp. *howardi* on noninoculated corn plants and later assaying the roots of those plants for L11, we were able to record transmission of L11 by the insects. Experiments were conducted to answer the following three questions. (i) Can *D. undecimpunctata* subsp. *howardi* acquire and transmit L11? (ii) If reinfestation from their food plants is blocked, does the population of L11 in the insects, and the likelihood of transmission, decline through time? (iii) How long must infested *D. undecimpunctata* subsp. *howardi* feed on nonexposed plants to transmit L11?

MATERIALS AND METHODS

Bacterial strain. The parent strain of L11, *P. chlororaphis* (formerly *P. aureofaciens*) PS3732, was originally isolated near St. Charles, MO, from the roots of corn (12), after which spontaneous mutants resistant to two antibiotics, rifampicin and nalidixic acid, were selected to create strain PS3732RN. The *lacZ* and *lacY* genes of *Escherichia coli* then were inserted into the chromosome using a Tn7-delivery cassette, creating L11 (5,11). This modification gives L11 the ability to utilize lactose as a sole carbon and energy source, an ability not known in other fluorescent pseudomonads (15). When L11 was plated onto media containing the chromogenic dye 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), a lactose analog, distinctive blue colonies were formed. This color, in conjunction with antibiotic selection in the media, facilitated identification of L11. Neither the genetically modified strain, L11, nor its nonmodified parental strain possess any known biological control capabilities.

L11 from freezer stocks (-80°C in 15% glycerol) was grown in broth containing 10 g of Bacto tryptone per liter, 20 g of proteose peptone no. 3 (Difco Laboratories, Detroit) per liter, 1.5 g of K_2HPO_4 per liter, and 1.5 g of MgSO_4 per liter amended with nalidixic acid (100 $\mu\text{g}/\text{ml}$), rifampicin (100 $\mu\text{g}/\text{ml}$), and cycloheximide (100 $\mu\text{g}/\text{ml}$). Bacteria were centrifuged ($4,000 \times g$ for 15 min), and pellets were then resuspended in sterile distilled water (sdH_2O). Bacterial cell concentration was measured spectrophotometrically (optical density at 600 nm).

L11 was recovered from plant and insect samples on *Pseudomonas* agar F (PAF) (Difco Laboratories) amended with nalidixic acid (100 $\mu\text{g}/\text{ml}$), rifampicin (100 $\mu\text{g}/\text{ml}$), and cycloheximide (100 $\mu\text{g}/\text{ml}$). Agar plates containing these antibiotics and X-Gal (20 $\mu\text{g}/\text{ml}$) were used to identify L11. Bacterial colonies were tallied after incubation for 48 h at 28°C .

Insect sampling. Insects to be assayed were collected with flame-sterilized forceps and placed individually in 1.5-ml Eppendorf tubes. The insects were cold-anaesthetized (5°C for 1 h) and then individually ground into a uniform homogenate using an ice-cold mortar and pestle containing 1 ml of phosphate buffer (0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 , pH 7.2). The homogenate was serially diluted (replicate 10-fold dilutions in sdH_2O) and plated onto selective media as described above.

Root sampling. Sections of root 1 cm long taken just below the seed were collected using sterile forceps and scissors and placed individually into ice-cold 1.5-ml Eppendorf tubes. Tubes were weighed, and samples were then transferred into ice-cold mortars and ground into a uniform homogenate in 2 ml of phosphate buffer. The tubes were then reweighed, yielding root weights by subtraction. Well-mixed root homogenate (1 ml) was serially diluted and plated onto selective media as described above.

Microcosm and insect handling. Larval-feeding microcosms each contained 50 corn seeds (Pioneer cultivar 3165; Pioneer Hi-Bred International Inc., Johnston, IA) that had been surface-sterilized by 30 s of immersion in 70% EtOH, followed by 6 min of immersion in 10% Clorox solution (The Clorox Co., Oakland, CA), and then five rinses with sdH_2O . The seeds were arranged in five rows of 10 seeds each in plastic boxes (30 by 15 by 10 cm) containing a 3-cm layer of twice-autoclaved potting soil (autoclaved 1 h at 121°C and 15 lb/in^2 , cooled for 24 h, and then autoclaved again for 1 h). Each seed was inoculated with a 1-ml suspension of L11 (containing 10^8 CFU/ml of sdH_2O) and then immediately covered with soil. Plants were grown in a controlled environment chamber at 27°C with a 14-h light/10-h dark cycle, and watered with sdH_2O amended with fertilizer (3 g of Peters Solution per liter, 20-20-20 N-P-K; Grace-Sierra Horticultural Products Co., Milpitas, CA). Plants were allowed to grow for 10 days before the addition of *D. undecimpunctata* subsp. *howardi* larvae.

D. undecimpunctata subsp. *howardi* were purchased as eggs (French Agricultural Research Service, Lamberton, MN) and incubated on moist filter paper in closed petri dishes (hatch occurred on day 7 at 27°C). Approximately 35 newly hatched larvae (>6 h from hatch) were moved to each tray of L11-inoculated corn seedlings, where the larvae developed and pupated. Three days before emergence, trays were placed into 42.4-liter (10-gallon) aquaria covered with tight-fitting screen lids. Emerging adults were collected using a respirator.

Acquisition and maintenance, with reinfestation possible. In the first experiment, *D. undecimpunctata* subsp. *howardi* that had been exposed to L11 as larvae, as described above, were collected as adults upon emergence from the soil; transferred into new aquaria containing new trays of 10-day-old, L11-inoculated corn plants; and allowed to feed for 52 h. The adults were then moved into new aquaria with trays of noninoculated corn. Every 4 days, the insects were collected with an aspirator and moved to new trays of noninoculated corn. Samples of insects were removed from these aquaria and tested for L11 infestation at 5 days prior to molting to the adult stage (eclosion; -5 days), at eclosion (0 days), and at 0.5, 1.5, and 2.17 days (52 h) after eclosion, while on L11-inoculated corn, and then at 1, 4, 8, 15, and 22 days after the insects were moved onto noninoculated corn. In this trial, the noninoculated corn was planted in soil, allowing *D. undecimpunctata* subsp. *howardi* to transmit L11 to their noninoculated food plants, providing an additional means to sustain infestations in the insects.

Acquisition, maintenance, and transmission, with reinfestation blocked. The above experiment was repeated, with the only changes being that adult *D. undecimpunctata* subsp. *howardi* were allowed to feed on inoculated corn for only 1 day (versus 52 h in the first experiment) and were then maintained on leaf clippings from noninoculated corn plants (versus whole plants, which might themselves become infested with L11 and reinfest the insects). The leaf clippings were changed daily. A total of 75 insects were removed in each sample: at eclosion (0 days); 1 day after feeding on inoculated corn; and 1, 2, and 6 days after the last exposure to L11. In each sample, 30 insects were assayed immediately for L11, and the remaining 45 were placed in groups of three into 15 transmission microcosms for 24 h. In a final collection, 13 days after the insects' last exposure to L11, only 45 insects remained, and all were placed into transmission microcosms.

Transmission microcosms contained surface-sterilized corn seeds placed singly in 236-ml (8-oz.) plastic drinking cups containing twice-sterilized potting soil (10 cm deep). Each cup had a hole in the bottom for drainage and sat in a separate petri dish lid to catch water run-off and avoid cross-contamination. Plants were grown for 10 days and then enclosed singly in 20-cm long, 7-cm diameter, open-ended plastic tubes. Each tube had four 5-cm-diameter ventilation holes drilled in the side and covered with fine plastic mesh. The bottom of the tube was inserted several centimeters into the soil to hold the tube upright and prevent beetles from burrow-

Fig. 1. Acquisition and maintenance. L11 densities per adult *Diabrotica undecimpunctata* subsp. *howardi* through time, scaled so that eclosion is day 0. Insects at -1 day were pupae; all later samples were of adult insects. **A**, Trial 1. *D. undecimpunctata* subsp. *howardi* adults fed on L11-inoculated plants from day 0.5 (*) through day 2 (**), after which they were maintained on noninoculated, potted corn. L11 densities in or on the insects increased significantly when they first started to feed as adults, leveling off at 12 h (data analyzed by linear regression). The increase in L11 densities in the third week (***) represents secondary reinfestation. **B**, In the minimum transmission time trials, *D. undecimpunctata* subsp. *howardi* fed on inoculated corn clippings from eclosion (*) through day 1 (**), fed for 18 h on L11-free leaf-clippings, and then were maintained in groups of three on singly potted plants. Increases in L11 densities in or on *D. undecimpunctata* subsp. *howardi* at later sampling dates may represent secondary reinfestation. Squares indicate first replicate and triangles indicate second replicate. **C**, Loss of L11 from *D. undecimpunctata* subsp. *howardi* from time of last exposure for all trials combined. Data points that were known or thought to represent secondary reinfestation were not included. Bacterial densities decreased exponentially through time ($R^2 = 0.75$, $P = 0.05$, regression line shown). Squares denote experiment 1 with L11 reinfestation possible, triangles denote experiment 2 with L11 reinfestation blocked, and hexagons denote minimum transmission time experiments.

ing under the base of the tube. A petri dish lid on top of the tube prevented insects from climbing or flying out. Insects could move freely over the plant and could walk on or burrow into the soil at the base.

At the end of the 1-day exposure to new, noninoculated corn in the transmission microcosms, the insects were collected and assayed for L11 as described above. The plants were then incubated for 2 days at 27°C, to allow time for any L11 on the roots to multiply to detectable levels (≈ 50 CFU/g of root), after which their roots were collected and assayed, also as described above. Three control plants that had been grown alongside the experimental plants and treated identically, except that no insects were placed on them to feed, were also sampled for L11 contamination each time. L11 was never found on the roots of the control plants. This experiment yielded data on the density of L11 in *D. undecimpunctata* subsp. *howardi* 5 days before eclosion and emergence from the soil; at eclosion; following 1 day of feeding on inoculated corn; and at 1, 2, 3, 6, 7, and 14 days beyond the last exposure to L11, as well as on the roots of plants exposed to these insects during the intervals -1 to 0, 0 to 1, 2 to 3, 6 to 7, and 13 to 14 days after the insects' last exposure.

Minimum transmission time. *D. undecimpunctata* subsp. *howardi* were reared on L11-inoculated corn as described above. After 1 day of feeding on inoculated corn, all adult insects were moved to new aquaria and fed for 18 h on leaf clippings of L11-free corn plants and then transferred in groups of three to transmission microcosms as described above. The experiment was conducted twice. Similar feeding periods were used in both replications. In the first replicate, insects were allowed to feed on the new plants in the transmission microcosms for 3, 6, 9, 12, 24, or 48 h. In a second replicate, the insects were allowed to feed for 3, 24, or 48 h. After feeding, insects were collected and sampled for the presence of L11. The plants were incubated for 3 days from the time the insects were removed, and then assayed for L11 in the roots as described above.

Route of transmission. In all experiments, we measured L11 densities on root and stem samples from inoculated corn immediately before it was offered to *D. undecimpunctata* subsp. *howardi*. In the acquisition, maintenance, and transmission experiment in which reinfestation was blocked, five corn clippings that had only been exposed to L11 through feeding by infested *D. undecimpunctata* subsp. *howardi* were collected separately with flame-sterilized forceps, weighed, and sampled for L11. This allowed us to determine if *D. undecimpunctata* subsp. *howardi* were transmitting L11 to the noninoculated corn clippings, which might reinfest the insects. Concurrently, frass from the bowl that had held those clippings were poured into a single, sterile 1.5-ml Eppendorf tube and weighed. The frass were ground in 1 ml of buffered solution with an ice-cold mortar and pestle as a group (one sample per container per 1-day period) and dilution-plated to quantify L11.

Statistics. Because L11 counts are highly variable and positively skewed, the data were always transformed to $\log_{10}(\text{CFU} + 1)$ per insect or per gram of plant tissue for analysis and reporting (22). Using the data from the acquisition and maintenance experiment in which reinfestation was possible, we examined the change in L11 densities through time within *D. undecimpunctata* subsp. *howardi*, while still on inoculated plants, with linear regression. We compared the loss of bacteria through time across the trials using analysis of covariance (ANCOVA), with time from last exposure and trial as independent variables. For this analysis, all data were standardized so that the time of last exposure was the zero point. Also, we excluded from the analysis later data points for the acquisition and maintenance experiment in which reinfestation was possible and the minimum transmission time replicates in which *D. undecimpunctata* subsp. *howardi* were known to have become reinfested with L11, or in which this possibility could not be excluded. We used ANCOVA to identify factors that would predict transmission in the acquisi-

tion, maintenance, and transmission experiments in which reinfestation was blocked. The mean density of L11 in the three insects on each plant (insect bacterial density assayed after the insects had finished feeding) and the time from the insects' last exposure to L11 were the independent variables. Data from the minimum transmission time replicates were analyzed similarly; however, our statistical model included the mean density of L11 in the insects feeding on a plant as a covariate, with trial and feeding time as the independent variables. All statistical tests were performed using SAS (version 6; SAS Institute, Cary, NC).

RESULTS

Acquisition and retention of L11. In the first experiment, *D. undecimpunctata* subsp. *howardi* pupae collected 0.5 days before eclosion contained moderate populations of L11 (mean $\pm$ standard error = $1.34 \pm 0.173 \log_{10}[\text{CFU} + 1]/\text{insect}$). Immediately after eclosion, but before adults were moved to inoculated corn, the level of L11 infestation was slightly higher (2.09 ± 0.26). After 0.5 days of feeding on inoculated plants, the average level of infestation increased by 2.5 $\log_{10}$ units (to 4.56 ± 0.13). Linear regression showed that bacterial densities increased significantly through time from the end of pupation through the end of the acquisition period ($R^2 = 0.76$, $P = 0.05$; $t = -1$ through 2.17 days) (Fig. 1A). A high level of infestation was maintained until the end of the 52-h feeding period on the inoculated corn; there was no significant increase in bacterial densities through time after insects fed for 0.5 days ($R^2 = 0.12$, $P = 0.78$) and remained on infested corn. L11 densities in *D. undecimpunctata* subsp. *howardi* fell quickly once they were removed from noninoculated plants, returning to levels similar to those seen in pupae and newly emerged adults (0.98 ± 0.21) (Fig. 1A). This pattern of rapid acquisition and loss was repeated in the second experiment and in both replicates of the minimum transmission time experiment. When newly emerged adults fed on inoculated corn, L11 reached high densities (between 3 and 5 log units) in those insects. Bacterial densities in *D. undecimpunctata* subsp. *howardi* then declined rapidly after they were removed from a source of inoculum and leveled off at values similar to those seen in pupae (around 1 $\log_{10}[\text{CFU} + 1]/\text{insect}$) (Fig. 1A to C).

In the first experiment, *D. undecimpunctata* subsp. *howardi* adults were maintained on noninoculated, potted corn after their last exposure to L11. Up to 15 days after their last exposure to L11-inoculated corn, the insects contained low levels of L11 (1.28 ± 0.13), but these levels increased by 1.5 $\log_{10}$ units (to 2.72 ± 0.10) at 22 days (Fig. 1A). In this case, the insects transmitted L11 to their food plants and then may have become reinfested. In fact, L11 had colonized the roots of these plants to densities of $6.70 \pm 0.30 \log_{10}(\text{CFU} + 1)/\text{g}$ (mean of four root samples collected from the tray), at which density L11 typically invades the foliage (22). In the second experiment, when the *D. undecimpunctata* subsp. *howardi* were prevented from reinfesting their food plants, the patterns of L11 acquisition and decline were similar to the first experiment, except that L11 continued to decline, reaching below-detection limits on day 14 (Fig. 2A). In both replicates of the minimum transmission time experiment, a slight increase in L11 densities was detected in the final sample (Fig. 1B). This increase may have been due to insects becoming reinfested, as we saw in trial 1, since the insects were housed on potted corn while we were testing for minimum transmission time.

Bacterial loss data from all experiments fit a model of exponential decay through time ($R^2 = 0.89$, $P = 0.0001$) (Fig. 1C); trial and interaction effects were not significant (ANCOVA, $F = 1.87$, $P = 0.185$ for experiment; $F = 0.72$, $P = 0.556$ for interaction). That is, the rate of decay was adequately described by the exponential decay model with no significant differences in the rate constants between experiments.

Transmission of L11. In the second experiment (acquisition, maintenance, and transmission with reinfestation blocked), when-

ever L11 was detected in *D. undecimpunctata* subsp. *howardi*, the insects were able to transmit the bacterium to noninoculated plants (Fig. 2A). Immediately following eclosion but before exposure to L11 as adults, *D. undecimpunctata* subsp. *howardi* were able to transmit L11 to 5 of 15 noninoculated plants (33%). Immediately after a 1-day exposure to L11 as adults, the beetles were highly infested and transmitted L11 to 12 of 15 plants (80%). One day after the last exposure, the beetles transmitted L11 to 13 of 15 plants (86.7%), but this number dropped to 1 of 15 plants (6.7%) 6 days later, when bacterial densities in the insects themselves had dropped to low levels. Insects 14 days from last exposure to L11 did not contain detectable levels of the bacterium and did not transmit it. Time from insects' last exposure to L11 was a statistically significant predictor of transmission success (ANCOVA, $F = 25.97$, $P = 0.0002$). The mean den-

Fig. 2. Transmission experiments. **A**, Experiment 2, acquisition, maintenance, and transmission with reinfestation blocked. Adult beetles fed on inoculated corn from emergence (*) through day 1 (**), and then were maintained on noninoculated corn clippings, preventing secondary reinfestation. Density of L11 (reported as mean $\pm$ standard error $\log_{10}[\text{CFU} + 1]/\text{insect}$) in the *Diabrotica undecimpunctata* subsp. *howardi* placed on singly potted, noninoculated corn plants (left axis) and the proportion of those plants whose roots become infested with L11 as a result (right axis), both as a function of days since eclosion. In all cases, the insects only had access to the plants for 1 day. Transmission likelihood was correlated with bacterial densities in the insects; likelihood of transmission decreased significantly through time (ANCOVA). Triangles represent proportion of plants with L11 and circles represent L11 densities in insects. **B**, Proportion of corn plants whose roots become infested in two replicates of the minimum transmission time experiment, plotted against the duration of the feeding period by adult *D. undecimpunctata* subsp. *howardi*. The likelihood of transmission increased with increased feeding time; the two trials did not differ significantly from one another (ANCOVA). Circles indicate replicate 1 and squares indicate replicate 2.

sity of bacteria in insects after feeding did not predict transmission success (ANCOVA, $F = 1.41$, $P = 0.2543$ for mean $\log_{10}[\text{CFU} + 1]$ per three insects per plant), and the interaction term was non-significant ($F = 2.30$, $P = 0.1518$).

We conducted two trials measuring minimum transmission time (Fig. 2B). Since these trials encompassed similar feeding times, we will discuss the two trials together. In both trials, some transmission occurred in the shortest time period tested (3 h). In both trials, feeding times of 3 to 12 h resulted in less than 50% of the plants becoming infested. Feeding times of greater than 30 h resulted in a greater than 50% chance of transmission, with nearly 80% of the plants becoming infested after 45 h of feeding in trial 1 (Fig. 2B). The bacterial densities in the insects, measured after feeding in transmission microcosms, were not correlated with the likelihood of bacterial transmission to noninfected plants ($F = 0.00$, $P = 0.966$). Likelihood of transmission increased significantly with increased feeding time ($F = 15.36$, $P = 0.0001$); trial and interaction effects were both nonsignificant ($F = 1.96$, $P = 0.1628$; and $F = 0.80$, $P = 0.3704$, respectively).

Transmission route. Bacterial densities were high in the roots and stems of corn provided to infest the beetles (Table 1). In the second acquisition trial, L11 was never detected in the corn clippings provided as food after the period of inoculation, indicating that the clippings were not sustaining L11 in the microcosms and could not reinfest the beetles. L11 was detected in *D. undecimpunctata* subsp. *howardi* frass collected concurrently with the corn clippings on most sample dates. L11 densities in the frass were 0 and 3.76 $\log_{10}(\text{CFU} + 1)/\text{g}$ wet weight at 24 and 48 h from last exposure, respectively, and were 4.42 and 3.74 $\log_{10}(\text{CFU} + 1)/\text{g}$ wet weight in two samples taken at 168 h from last exposure.

DISCUSSION

We can delineate four stages in the L11 infestation of *D. undecimpunctata* subsp. *howardi* adults. First, L11 densities increase rapidly in adults that have recently emerged and begun feeding on L11-infested plants. Second, within a day of feeding, L11 densities in the insects reach a plateau. Third, immediately after *D. undecimpunctata* subsp. *howardi* leave a source of inoculum, bacterial densities decline exponentially, although L11 can be detected in the insects for up to 2 weeks. A fourth stage will occur if insects remain on the same plants, either in the laboratory or, presumably, in the field. If *D. undecimpunctata* subsp. *howardi* transmit L11 to those plants before losing their own infestations, the beetles may become reinfested from feeding on those same plants.

Bacterial densities on roots of L11-infested plants were as much as 10,000 times those in the foliage (Table 1), yet levels of L11 infestation of *D. undecimpunctata* subsp. *howardi* were lower in pupae than in recently eclosed adults. Although, as larvae and pupae, the beetles inhabit a bacteria-rich environment, they do not feed during the 2-week pupation period and may lose bacteria acquired as larvae. However, as adults, the beetles feed voraciously, building fat reserves for dispersal and egg production, and quickly

achieve a high level of L11 infestation. *D. undecimpunctata* subsp. *howardi* adults became highly infested with L11 after only 12 h of feeding on infested foliage. The level of L11 infestation did not increase with longer feeding periods. In the field, the western corn rootworm feeds for at least 48 h on the larval host plant before dispersing (8). If the same is true for *D. undecimpunctata* subsp. *howardi*, then they will remain on the larval host long enough under natural conditions to become highly infested.

L11-infested *D. undecimpunctata* subsp. *howardi* were able to transmit L11 to noninoculated plants. However, the mean density of L11 in infested insects, measured after the feeding period was over, was not correlated with their success in transmission. Instead, the probability of L11 transmission was inversely proportional to the time since the insects' last exposure to L11, and directly proportional to the length of time that infested insects fed on noninoculated plants. All insects in the transmission experiments were fed noninoculated corn for 18 h before being placed in the transmission test microcosms and, thus, contained densities of bacteria (10 to 100 CFU) that were maintained in our *D. undecimpunctata* subsp. *howardi* populations for at least 7 days after last exposure to L11. Thus, with conservative periods of exposure (24 h), relatively long periods of simulated dispersal (18 h), low levels of insect infestation (10 CFU per insect), and short feeding periods on noninoculated plants (3 h), L11 was still transmitted to at least 50% of exposed plants.

Several processes could lead to declining L11 populations in *D. undecimpunctata* subsp. *howardi* after removal from a source of the bacteria. These include bacterial mortality in the digestive tract or on the body surface, excretion in the frass, or sloughing off from infested body parts. We did not attempt to measure these losses, but on several occasions, we detected L11 in frass samples. Armstrong et al. (3) also examined uptake and transmission of genetically engineered strains of three bacteria, *Enterobacter cloacae*, *Klebsiella planticola*, and *Erwinia herbicola*. Radish leaves were sprayed with a dense inoculum of each strain (10^6 to 10^8 CFU/ml), and variegated cutworms, *Peridroma saucia*, were allowed to feed on those leaves for 24 h. The marked bacteria were later recovered from and found to multiply in the caterpillars' frass, which was implicated as an important route of transmission. In this study as well, frass may be an important route for L11 transmission. However, unlike adult *Lepidoptera* spp., adult *D. undecimpunctata* subsp. *howardi* occasionally burrow directly into the soil and might, therefore, transmit bacteria from their body surfaces to soil and roots. L11 densities in *D. undecimpunctata* subsp. *howardi* after feeding were not positively correlated with transmission success, as we had initially expected. However, if bacteria must be lost (in frass or from body parts) for transmission to occur, then bacterial densities in or on the insects after feeding might not be expected to correlate with transmission success. *D. undecimpunctata* subsp. *howardi* transmission of *Erwinia tracheiophila*, the causative agent of cucurbit wilt, has been well studied. Transmission of that pathogen is usually from infested mouthparts while feeding and through deposition in the frass (6). This suggests that transmission of both pathogenic and nonpathogenic microorganisms can be through similar routes and that much of the research documenting transmission of bacterial pathogens by insects might apply to many nonpathogens as well.

One perceived advantage of using genetically engineered rhizobacteria in the environment is that they are soilborne and therefore unlikely to reach winged adult insects, the most important potential long-range vectors. However, our studies demonstrate that rhizobacteria, which naturally invade the roots and move into the foliage at low densities, may be acquired by insects during feeding and can reach densities sufficient to make subsequent transmission likely. Armstrong et al. (3), in the study described above, also demonstrated that insects could transmit modified bacteria from inoculated to noninoculated plants. In their experiment, the bac-

TABLE 1. Mean number of L11 bacteria on roots of corn provided to larvae and in the foliage of corn provided to adults in the acquisition periods of all trials^a

Trial	Roots		Stems	
	Bacteria	N	Bacteria	N
Reinfestation possible	6.96 ± 0.13	8	4.99 ± 0.49	8
Reinfestation blocked	7.20 ± 0.01	8	2.80 ± 0.33	4
Minimum transmission time replicate 1	7.94 ± 0.07	6	5.00 ± 0.58	4
Minimum transmission time replicate 2	8.71 ± 0.09	7	3.47 ± 0.60	8

^a Bacterial densities are reported as mean ± standard error $\log_{10}(\text{CFU} + 1)/\text{g}$ wet weight of plant tissue; N = sample size.

teria were sprayed directly onto the leaves, so that transmission might have been through direct contact as well as through excretion of frass. Unlike many herbivorous beetles, Lepidopterans do not continue to ingest leaves as adults, which may limit their ability to sustain and transport bacteria during the period of great-mobility.

The movement of pest *Diabrotica* spp. has been well studied. These beetles have been observed to spread more than 80 km in a single year (30) and can disperse great distances by rising up into cold fronts and traveling passively with them (27). In the southeastern United States, *D. undecimpunctata* subsp. *howardi* is a pest on cucurbits, corn, and peanuts (13) and is regularly found in old fields (W. E. Snyder, unpublished data). High rates of dispersal and a wide host range are traits typical of crop pests. The wide host range of *D. undecimpunctata* subsp. *howardi* increases the likelihood that transported bacteria will reach sites favorable for their reproduction. In other experiments, we have found that the grasshopper *Melanoplus femurrubrum* also acquires and transmits L11 (34,35). Since soil bacteria lack inherent dispersal mechanisms, passive transport by herbivorous insects may be an important means for these microorganisms to move through the environment, linking otherwise isolated bacterial populations (31,38). Transmission by insects may help explain the high migration rates reported for some soil bacteria (29). Our results support the hypothesis that dispersal of soilborne bacteria by insects is a common and natural phenomenon.

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