Acquisition and Retention of *Xylella fastidiosa* by an Efficient Vector, *Graphocephala atropunctata*

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ABSTRACT

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The blue-green sharpshooter (BGSS), *Graphocephala atropunctata* (Signoret), is an efficient vector of *Xylella fastidiosa* Wells et al, the causal bacterium of Pierce's disease of grapevine. In experiments to assess changes over time in vector transmission efficiency and populations of bacteria in BGSS, 81 of 120 BGSS that were fed for 12 h on infected grapevine acquired the bacterium. Individual infective BGSS were exposed 4 times (1 h, and 7, 21, and 60 days after acquisition) to uninfected grapevines for 3-h inoculation feeding periods to determine the efficiency of transmission to grapevine. After each inoculation efficiency test, some BGSS were tested by culturing for *X. fastidiosa* to determine the bacterial population in the vector heads and bodies. Transmission efficiency increased from 56 to 92% between 1 h and 7 days post-acquisition, and remained high thereafter (91% at 21 days and 76% at 60 days). Bacterial population size in the heads of the inoculative vectors also increased between 1 h and 7 days, but did not consistently increase further. *Xylella fastidiosa* was not recovered by culture from about 40% of the inoculative insects. Populations below the detection limit (100 bacteria) in the BGSS heads were enough for efficient transmission.

Xylem feeding homopterans, such as sharpshooter leafhoppers (Homoptera, Cicadellidae, subfamily Cicadellinae) and spittlebugs (Cercopidae), are vectors of *Xylella fastidiosa,* the causal bacterium of Pierce's disease (PD) of grapevine (*Vitis vinifera* L.) and other plant diseases (4–6,11,15). *Xylella fastidiosa* multiplies and attaches to the food canal (precibarium) and pumping chamber (cibarium) in the foregut of the vector insect (1,2,13). Two unusual transmission characteristics support the hypothesis that the bacteria that are inoculated into a plant come from the foregut of the vector (12). First, adult blue-green sharpshooters (BGSS), *Graphocephala atropunctata* (Signoret), can transmit *X. fastidiosa* immediately after acquiring the bacterium and remain infective until they die, which may be several months later (12,15). Second, infective BGSS are not able to continue to transmit after molting. This loss of infectivity after molting is explained by the loss of the foregut bacteria, all of which are attached to the molted cuticle of the foregut (12). Multiplication and attachment of *X. fastidiosa* in the foregut would account for the long-term infectivity of the adults.

Purcell et al (13) used scanning electron microscopy to locate various aggregations of *X. fastidiosa* in the foregut of the BGSS, the common vector of PD in coastal California. Brlansky and coworkers (1,2) also observed the bacterium in the foregut of two common vectors of *X. fastidiosa* in Florida, *Oncometopia nigrans* (Walker) and *Homalodisca coagulata* (Say). The bacterial cells were not loosely scattered or randomly distributed, and the characteristic locations and appearance of the *X. fastidiosa* aggregations were similar in all three vector species even though the insect species differed in size, geographic distribution, and plant hosts. The bacteria often formed dense aggregations of cells in the precibarium, the cibarial pump, and the entrance to the esophagus. Thus, large numbers of bacteria can accumulate in the foregut of the vector over time. Because *X. fastidiosa* is transmitted to plants from the foregut, it is reasonable to postulate that these aggregations are involved in vector transmission of bacterial cells into the xylem of the plant. If this is so, then vector insects with large numbers of *X. fastidiosa* in the foregut should be more efficient at transmitting the pathogen than insects with smaller populations of bacteria. On the other hand, no bacteria were observed in the cibarial pumps of some transmitting BGSS (13).

The objectives of this research were to determine how the population size of *X. fastidiosa* changed over time in the head of the BGSS, and if bacterial populations in the head were related to the efficiency of transmission of *X. fastidiosa* to grapevine by the BGSS.

MATERIALS AND METHODS

Plants and insects. All grapevine plants were seedlings of cv. Pinot noir, a variety that is highly susceptible to PD (10), developing symptoms under our greenhouse conditions within 6–8 wk of infection. Three grapevine plants were inoculated with a strain of *X. fastidiosa* that was isolated from a vineyard near Yountville, California and designated as YVPD. After 2–4 mo, when these vines were 30–60 cm tall and had begun to exhibit symptoms of PD, they were used as inoculative sources of *X. fastidiosa* to infect the BGSS used in these experiments. Insects were caged in groups of 40 on these inoculative grapevines for 12 h to acquire *X. fastidiosa.* The noninfected grapevines used to test for transmission by BGSS or to maintain the BGSS were 8–15 cm tall Pinot noir seedlings. Insects were caged singly on these plants such that they had access to the entire seedling. BGSS nymphs were collected from residential ornamentals on a street in Berkeley, California, where we consistently had found less than 1% of adult BGSS to be infected with *X. fastidiosa.* The nymphs were reared on California mugwort (*Artemisia douglasiana* Besser) and transferred every 2–3 days to new plants. As the nymphs molted to adults, they were removed daily to grapevine plants to be screened for *X. fastidiosa* and held until an experiment began.

Culture methods. The medium used to culture *X. fastidiosa* (PWG) was a modification of PW medium (3). The PWG medium substituted 9 gm of Gelrite (Kelco Div of Merck and Co., Inc., San Diego, CA) for the agar used in PW, and the amount of 20% (w/v) bovine serum albumin solution was reduced from 30 to 15 ml per liter. Insects or plant samples (0.1–0.2 gm of stem or petiole tissue) were surface sterilized by successive 1-min immersions in 90% ethanol, then 2% hypochlorite, followed by 3 rinses in at least 100 ml of sterile water. The plant samples were chopped aseptically with a razor blade on a sheet of sterile paper and then homogenized in 2 ml of sterile phosphate-buffered saline using a Polytron (Brinkman Instruments, Inc., Westbury, NY) homogenizer. Previous experimentation determined that the viability of *X. fastidiosa* cells was unaffected by 2 min of homogenization (B. L. Hill, *unpublished data*). The Polytron generator probe (model PT-DA3012/2) was sterilized between samples by operating it for 15 sec in 95% ethanol and rinsed twice by operating in two bottles of sterile water. No carryover of viable bacteria on the homogenizer between samples was detected in three previous experiments designed to test the technique. In each run of samples, positive controls were processed, followed by negative controls as a routine quality control to ensure no carryover of bacteria between samples. Two 10-fold dilutions of homogenized plant samples were made with phosphate-buffered saline, and 2 droplets (20 μl each) were spread on culture plates to allow duplicate counting of cfu for each dilution. If the insect or plant sample had at least 100 cultivable *X. fastidiosa* cells, and if they were randomly distributed by homogenization, then the probability of detecting one or more cfu in a droplet by culture was 87% (Poisson distribution). On this basis the theoretical lower threshold of detection was approximately 100 cultivable *X. fastidiosa* cells per insect sample or, in plant samples, 1×10^3 cultivable *X. fastidiosa* cells per gram of plant tissue.

Experimental design. Altogether, 120 individual BGSS were tested in three independent experimental replicates with 40 BGSS each. The second and third replicates were started 4 and 7 days after the first. The BGSS were prescreened before use to ensure that they were not infected with *X. fastidiosa*. For prescreening, they were caged in groups of five insects for 3 days on grapevine seedlings. The prescreening grapevine plants were held for 3 mo, then examined for PD symptoms and tested by culture for the presence of *X. fastidiosa*. If *X. fastidiosa* had been detected in a prescreening grapevine, the experimental results from the five insects that had been caged on this plant would have been omitted. After prescreening, the noninfected BGSS were caged (in the replicate groups of 40) overnight for a 12-h acquisition assess period (AAP) to acquire *X. fastidiosa* on the symptomatic grapevine plants. After feeding on the infected grapevine plants, the BGSS were individually tested four times: 1 h, 7 days, 21 days, and 60 days after the 12-h AAP. Each of these tests consisted of 2 parts. *(1)* The BGSS were fasted for 1 h, then caged individually on uninfected grape seedlings for an inoculation access period (IAP) of 3 h to determine the inoculation efficiency, i.e., the percentage of BGSS that transmitted *X. fastidiosa* to these test seedlings. *(2)* Thirty individual insects (10 from each of the three replicates) were surface sterilized, the head severed from the body, and heads and bodies tested separately by culture to determine the population sizes of *X. fastidiosa* in the foreguts and bodies of the BGSS. The heads were tested by culture separately because they contain the foregut from which *X. fastidiosa* can be transmitted to plants, whereas the bodies contain the midgut and hindgut from which no transmission of *X. fastidiosa* can occur. The 1-h fasting period before being placed on the test grapevine seedlings was to uniformly motivate the insects to feed during the 3-h IAP. Altogether, 120 individual insects were tested immediately after acquisition feeding for inoculation efficiency, and then 30 were sacrificed for culture. After 7 days, the remaining 90 BGSS were tested for inoculation efficiency, and another 30 were sacrificed for culture. The remaining BGSS were again tested at day 21 and at day 60. Between tests, the BGSS were individually maintained on grapevine seedlings and the insects were transferred to fresh plants twice each week (a total of more than 600 holding plants) to maximize survival of the insects. These grapevine holding plants, as well as the inoculation efficiency test plants, were held for 3 mo and monitored for PD symptoms to test for the transmission of *X. fastidiosa*. If symptoms were not decisive, the plants were tested by bacterial culture for *X. fastidiosa* to determine whether transmission had occurred, and the transmission results were recorded separately for each insect. Insects that did not transmit to any plants and were negative for *X. fastidiosa* by culture were scored as not having acquired the bacterium, and only insects that could be confirmed as having acquired the bacterium were included in calculations of transmission efficiency and bacterial population sizes in heads and bodies.

The choice to use a 12-h AAP and a 3-h IAP was based on previous correlations of the percentage of transmission by BGSS with the length of the IAP, as well as on correlations of the percentage of BGSS that acquired *X. fastidiosa* with the length of the AAP (12). A 12-h AAP was selected because it was an interval long enough to ensure that most of the insects would acquire *X. fastidiosa*, but not long enough for extensive multiplication of *X. fastidiosa* to occur in the head of the BGSS. A 3-h IAP was selected because it would result in approximately 50–70% transmission. Longer IAPs should result in most insects transmitting, which would preclude efficiency comparisons.

RESULTS

During the 12-h AAP, 81 of the 120 insects (68%) acquired *X. fastidiosa*, thus becoming infective (able to transmit the bacteria to plants). This was somewhat less than the approximately 83% that was expected based on the previous study of Purcell and Finlay (12). The observed transmission efficiencies during a 3-h IAP were higher than projected but consistently less than 93%, so that efficiency comparisons were possible. *Xylella fastidiosa* was transmitted to each of the more than 600 holding plants on which the individual infective insects were maintained for 3- to 4-day intervals during the time between the tests. This high transmission rate attests to the validity of using a 3-day IAP in prescreening BGSS on grape to confirm the lack of infectivity of field-collected insects.

The transmission efficiency of the 81 insects that acquired *X. fastidiosa* did not vary significantly among the three independent experimental replicates ($P > 0.05$, paired comparisons; test of differences between 2 proportions) (8) when replicates were compared at 7, 21, and 60 days. The results from the three replicates were combined, and the pooled results are presented here. Transmission efficiency after a 12-h AAP and 1 h fasting was about 56% (Table 1) and increased significantly ($Z = 4.95$, $P < 0.01$, test of differences between 2 proportions) (8) to 92% by day 7. Transmission efficiency did not change significantly thereafter ($P > 0.05$), although by day 60 only 17 infective insects survived.

The number of bacteria that were cultured from the heads and bodies of the infective BGSS (Fig. 1) was highly variable for each of the four time-intervals examined. Altogether, 30 insects were examined at the end of each post-acquisition interval; however, only the 81 infective insects were included in Fig. 1. The numbers that were infective and consequently included in Fig. 1 for the head and body cultures were 15, 21, 23, and 17 for 1 h, 7 days, 21 days, and 60 days, respectively. There was

TABLE 1. Efficiency of transmission of *Xylella fastidiosa* to grapevine by blue-green sharpshooters during 3-h inoculation access periods

	Post-acquisition interval			
	1 h	7 days	21 days	60 days
Percent[z] transmissions	56 a	92 b	91 b	76 b
Number of infective vectors	81	66	45	17
Number of transmissions	45	61	41	13

[z] Percentages followed by same letter are not significantly different ($P < 0.05$) by test of differences between two proportions (8).

some mortality during the 60 days of the experiments, and five infective insects were lost before being cultured. The proportion of infective insects with detectable numbers of bacteria increased between 1 h and 7 days, but thereafter there was no obvious pattern of increase in bacterial populations per insect, nor was there an increase in the percentage of insects with detectable bacteria. At day 7 and day 21, about 90% of the insects in Fig. 1 were transmitting, even though about 40% of them had bacterial populations that were not detectable by culture. One hour after the AAP, 56% transmitted, but about 80% of these insects had nondetectable bacterial populations.
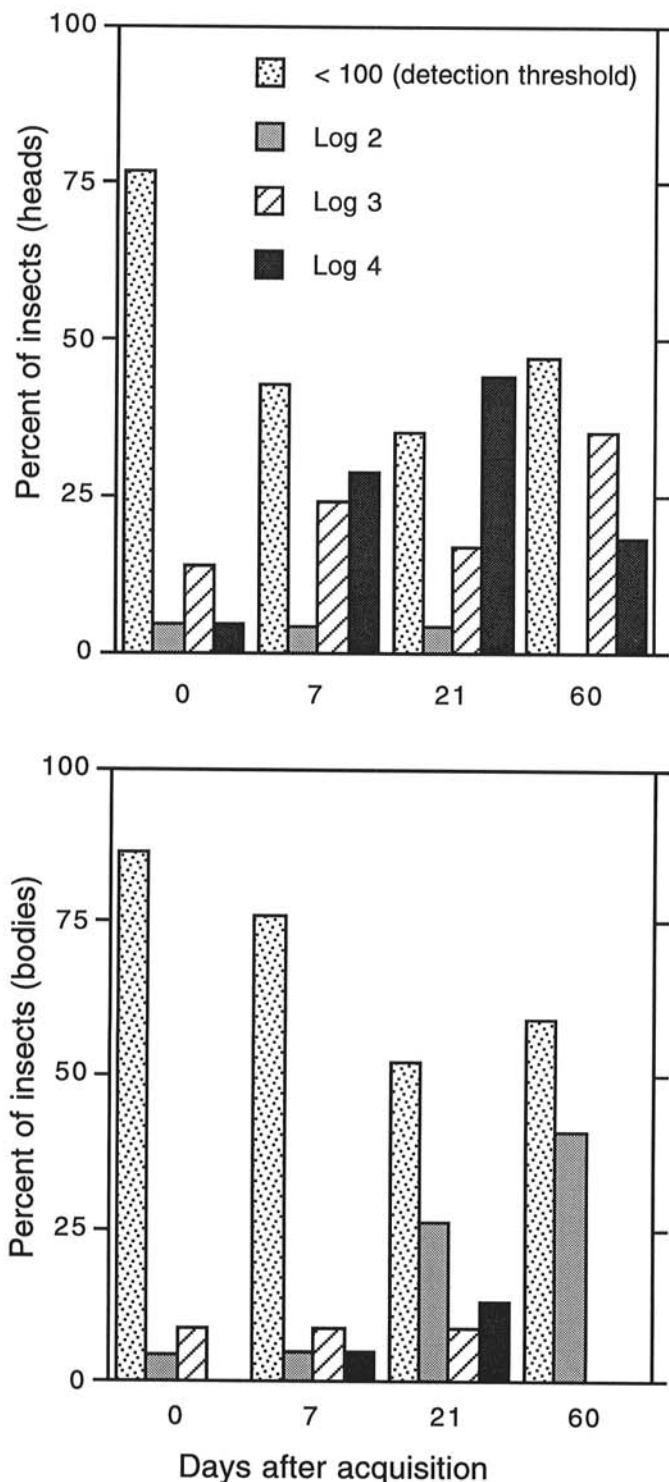
Large populations of bacteria in the insect head did not ensure transmission; the insects that failed to transmit included individuals with large populations of bacteria in the head. Five infective insects did not transmit during the 3-h IAP to test plants on day 7, and four failed to transmit on day 21 even though they transmitted to all their intermediate holding plants. Bacteria were not recovered from four of these nine insects but were recovered at population sizes of 10^3, 10^4, and 10^5 from 1, 3, and 1 insects, respectively.

DISCUSSION

Transmission efficiency was not related to population sizes of *X. fastidiosa* in BGSS heads, in which the minimum number of bacteria that ensured efficient transmission was below the detection threshold of about 100 cultivable cells. Large populations of bacteria in the insect head did not ensure transmission. Also, low bacterial population sizes apparently did not limit transmission efficiency because approximately 90% of the infective insects transmitted at days 7 and 21 even though about 40% of these infective BGSS had bacterial population sizes below detection levels. This result is consistent with previous observations using scanning electron microscopy (13) that some insects transmitted *X. fastidiosa* but had no visible bacteria in their heads.

Although other kinds of bacteria have not been observed in this region, *X. fastidiosa* can attach to and multiply in the pumping chamber and food canal of the foregut. This is a very turbulent environment with high flow velocity during feeding. Moreover, *X. fastidiosa* seems to attach to foregut locations where the flow velocities during xylem sap intake are highest. Based on the amount of food intake over a 24-h period, the daily average flow velocity of xylem sap at the food meatus (canal from the stylet tip to the cibarium) is estimated at more than 8 cm per sec, and peak velocities should be much higher during active feeding (13). Microscopy of transmitting insects indicated that bacterial cells are embedded in a matrix of extracellular material with staining characteristics and appearance similar to the matrix surrounding *X. fastidiosa* in xylem elements in plants (13). Brlansky et al (1) speculated that the matrix may protect the bacteria from being swept away by the high velocity of the stream of xylem sap during feeding. The matrix surrounding the bacterial cells may also aid in extracting nutrients from the xylem sap (7). The bacterial cells all had a polar attachment and were closely associated, resembling a shag carpet. Further evidence for multiplication was that some cells appeared to be dividing, and in the BGSS the aggregates were generally larger and denser 4 and 12 days following acquisition feeding than they were 1 day post-acquisition (13). In the precibarium, aggregations of bacteria were present in the epipharynx and hypopharynx, both anterior and posterior to the precibarial valve (1). In the cibarial pump, aggregates were attached in the food meatus and in the groove on the floor of the cibarium connecting the meatus with the esophagus. The bacteria also aggregated in the apodemal groove in the pump diaphragm. In the BGSS, aggregates of *X. fastidiosa* were also found anterior to the esophagus, adjacent to the cibarium (13).

The adaptations of *X. fastidiosa* for attachment to the foregut of xylem feeders may be important or even essential for vector transmission. The actual mechanism by which cells of *X. fastidiosa* are introduced into the xylem of plants from the vector is not known. If the cellular aggregations posterior to the cibarial valve were necessary for transmission, then it is reasonable to assume that as the number of bacteria in the foregut increases, the efficiency of transmission would also increase. The absence of a relationship between transmission efficiency and populations of *X. fastidiosa* in the foregut suggests that bacteria in the cibarium are probably superfluous to vector transmission. The occurrence of bacteria in aggregates suggests that occasional losses of aggregated cells from the surface of the insect foregut is responsible for inoculation of the plant, but such losses from the vector may not be dependent on aggregate size. The bacteria that are transferred to the plant probably reside in the food canal of the stylets

Fig. 1. *Xylella fastidiosa* recovered by culture (dilution plating) from heads and bodies of infective blue-green sharpshooters. Numbers of insects cultured were 15, 21, 23, and 17 at 0, 7, 21, and 60 days after acquisition, respectively.

or the precibarium anterior to the precibarial valve. The number of cells in the stylets would be much less than the number in the cibarium because the surface area and cross-sectional dimensions of the feeding stylets would not support aggregations as large as those in the pump chamber. The lack of a perceptible latent period (12) further supports the hypothesis that small numbers of bacteria in the food canal anterior to the cibarial valve are sufficient for transmission.

Enzyme-linked immunosorbent assay (ELISA) tests have been used to detect *X. fastidiosa* in field studies of sharpshooter vectors (14,16). There are some important limitations to the use of ELISA in epidemiological studies. The threshold for the detection of *X. fastidiosa* using ELISA is greater than 10^4 cells in the reaction well on the ELISA plate (9). This is two orders of magnitude above the numbers of bacteria in the foregut that are adequate for efficient vector transmission. In addition to these false negatives, vectors may lose their infectivity when molting to adults, but still retain bacteria in the midgut or hindgut in numbers that could be detected by ELISA. Therefore, field studies that use ELISA techniques to determine the presence and extent of infective vectors in a location where PD transmission is being assessed could fail to detect *X. fastidiosa* in infectious insects or could detect the bacterium from insects that are no longer infectious.

LITERATURE CITED

1. Brlansky, R. H., Timmer, L. W., French, W. J., and McCoy, R. E. 1983. Colonization of the sharpshooter vectors, *Oncometopia nigricans* and *Homalodisca coagulata,* by xylem-limited bacteria. Phytopathology 73:530-535.
2. Brlansky, R. H., Timmer, L. W., and Lee, R. F. 1982. Detection and transmission of a gram-negative, xylem-limited bacterium in sharpshooters from a citrus grove in Florida. Plant Dis. 66:590-592.
3. Davis, M. J., French, W. J., and Schaad, N. W. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr. Microbiol. 6:309-314.
4. DeLong, D. M., and Severin, H. H. P. 1950. Spittle-insect vectors of Pierce's disease virus I. Characters, distribution, and food plants. Hilgardia 19:339-355.
5. Freitag, J. H., and Frazier, N. W. 1954. Natural infectivity of leafhopper vectors of Pierce's disease virus of grape in California. Phytopathology 44:7-11.
6. Hewitt, W. B., Frazier, N. W., and Freitag, J. H. 1949. Pierce's disease investigations. Hilgardia 19:207-264.
7. Hopkins, D. L. 1989. *Xylella fastidiosa:* A xylem-limited bacterial pathogen of plants. Annu. Rev. Phytopathol. 27:271-290.
8. Loether, H. J., and McTavish, D. G. 1988. Descriptive and Inferential Statistics. Allyn and Bacon Div. of Simon and Schuster, Boston, MA.
9. Nomé, S. F., Raju, B. C., Goheen, A. C., Nyland, G., and Docampo, D. 1980. Enzyme-linked immunosorbent assay for Pierce's disease bacteria in plant tissues. Phytopathology 70:746-749.
10. Purcell, A. H. 1974. Spatial patterns of Pierce's disease in the Napa Valley. Am. J. Enol. Vitic. 25:162-167.
11. Purcell, A. H. 1990. Homopteran transmission of xylem-inhabiting bacteria. Pages 243-266 in: Advances in Disease Vector Research, Vol. 6. Springer-Verlag, New York.
12. Purcell, A. H., and Finlay, A. H. 1979. Evidence for noncirculative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. Phytopathology 69:393-395.
13. Purcell, A. H., Finlay, A. H., and McClean, D. L. 1979. Pierce's disease bacterium: Mechanism of transmission by leafhopper vectors. Science 206:839-841.
14. Raju, B. C., Goheen, A. C., and Frazier, N. W. 1983. Occurrence of Pierce's disease bacteria in plants and vectors in California. Phytopathology 73:1309-1313.
15. Severin, H. H. P. 1949. Transmission of the virus of Pierce's disease by leafhoppers. Hilgardia 19:190-202.
16. Yonce, C. E., and Chang, C. J. 1987. Detection of xylem-limited bacteria from sharpshooter leafhoppers and their feeding hosts in peach environs monitored by culture isolation and ELISA techniques. Environ. Entomol. 16:68-71.