Factors Limiting the Transmission of a Xylem-Inhabiting Bacterium *Clavibacter xyli* subsp. *cynodontis* to Grasses by Insects

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We thank Brad Marder, Reggie Martinez, and Stuart Saunders for research assistance; Stan Kostka, Michael M. Martin, and Steve Tomasino for critical reviews of the manuscript; and Crop Genetics International (Hanover, MD) for partial funding of this research.

Accepted for publication 21 April 1993.

ABSTRACT

Barbehenn, R. V., and Purcell, A. H. 1993. Factors limiting the transmission of a xylem-inhabiting bacterium *Clavibacter xyli* subsp. *cynodontis* to grasses by insects. Phytopathology 83:859-863.

We investigated the potential of phytophagous insects to transmit *Clavibacter xyli* subsp. *cynodontis* to bermudagrass and maize. Neither leaf-chewing nor xylem-sucking insects transmitted the bacterium after feeding on colonized plants. *C. x. cynodontis* was readily transmitted to bermudagrass and maize by mechanical inoculation of their stems, and *Schistocerca nitens* (Orthoptera: Acrididae) transmitted the bacterium to bermudagrass when concentrated cell suspensions were applied to their mouthparts and feeding was restricted to stems. The factors limiting the transmission of *C. x. cynodontis* by chewing insects under natural feeding conditions include the low susceptibility of leaves (vis-à-vis stems) to *C. x. cynodontis* colonization, the small numbers of the bacterium acquired by insects fed colonized foliage, and the retention of relatively few viable bacteria on the mandibles of insects.

Additional keywords: *Cynodon dactylon,* Homoptera, Lepidoptera, *Zea mays.*

Clavibacter xyli subsp. *cynodontis* is a Gram-positive, xylem-limited, coryneform bacterium that causes bermudagrass stunting disease (2,4). Pathological symptoms are observed primarily in ratooned plants or in plants stressed in other ways (2,4). For instance, there is little effect on growth or yield of colonized maize (*Zea mays* L.) under normal growing conditions (8).

Clavibacter xyli subsp. *xyli,* which causes ratoon stunt disease in sugarcane (3), and *C. x. cynodontis* are efficiently transmitted by mechanical inoculation with cutting tools contaminated by colonized xylem sap (3,14). Little work has been done to determine whether phytophagous insects are vectors of *C. xyli.* In tests with aphids (Homoptera), no transmission of *C. x. cynodontis* to maize was found (8). We extended this study by investigating the potential for xylem-sucking and leaf-chewing insects to transmit *C. x. cynodontis* under laboratory conditions and by studying several factors affecting transmission.

The potential of phytophagous insects to transmit *C. x. cynodontis* is also relevant to the safety of the use of genetically engineered *C. x. cynodontis* in crops. This bacterium has been genetically engineered to produce the delta-endotoxin of *Bacillus thuringiensis* and is currently under development for use in maize as a means of protection from lepidopteran insect pests (8,15).

MATERIALS AND METHODS

C. x. cynodontis. Pure cultures of strains BKF1, Ind1, and IndC were originally obtained from colonized bermudagrass (*Cynodon dactylon* (L.) Pers.) in central and southern California. Cultures were maintained in a dark incubator on solid medium at 28 C. The medium, modified from Anderson and Flaherty (1), was made by autoclaving 4.8 g of phytone (soybean) peptone (BBL Labs, Cockeysville, MD), 3.2 g of tryptone (Difco, Detroit, MI), and 8.0 g of Gelrite gellan gum (Scott Labs, West Warwick, RI) in 910 ml of distilled water. After cooling the autoclaved solution to 57 C, we added filtered (0.2 μ) cysteine (0.5 g), glucose (2.0 g), potassium phosphates (dibasic, 0.35 g; monobasic, 1.10 g), magnesium sulfate (2.0 g), cycloheximide (0.025 g), polymyxin B (0.025 g), and colistin (0.005 g) to 95.0 ml of distilled water. When plated on this medium, *C. x. cynodontis* forms circular yellow colonies in 4–6 days (28 C) that contain characteristic pellicles (visible at 30×). The bacterium is also identified by the slightly curved and clubbed shape of the cells (dark-field microscopy, 1,000×) (4).

C. x. cynodontis was prepared for the inoculation of insects and plants by pipetting 3.0 ml of sterile succinate-citrate-phosphate (SCP) buffer (6) onto a culture plate colonized by the bacterium, suspending the colonies with a sterile wire loop, and mixing the suspension in a sterile vial. Bacterial density in these suspensions was estimated with a dark-field microscope with a cell counter (1,000×), and the numbers of colony-forming units (cfu) per milliliter (viable bacteria) were counted after serial (1/10) dilution in SCP buffer and culture on solid medium.

Plants and insects. Wheat (*Triticum aestivum* L.) and maize (*Zea mays* cv. Golden Bantam and FR622) were grown from seed. Bermudagrass (cv. California common) was grown from seed and from rooted cuttings from plants free of *C. x. cynodontis.* All plants were grown in plastic pots (10 cm in diameter) in a greenhouse under constant positive pressure with charcoal-filtered air.

Eggs of *Schistocerca nitens* subsp. *nitens* Thunberg (Orthoptera: Acrididae) and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) were acquired from Dow Chemical Co. (Walnut Creek, CA) and the USDA (Albany, CA), respectively. Both species were reared in a greenhouse insectary on wheat seedlings. *Melanoplus sanguinipes* (Fabricius) (Orthoptera: Acrididae) and *Pseudaletia unipuncta* (Haworth) (Lepidoptera: Noctuidae) were reared on wheat or maize, and *Draeculacephala minerva* Ball and *Carneocephala fulgida* Nottingham (Homoptera: Cicadellidae) were maintained on bermudagrass in laboratory colonies. Adult *Diabrotica undecimpunctata* subsp. *undecimpunctata* Mannerheim (Coleoptera: Chrysomelidae) were collected on alfalfa in Alameda and Yolo counties and maintained on maize. Greenhouse insectary temperatures averaged 20 C (night) and 28 C (day) (range: 16–36 C).

Plant assay. Leaf midribs or stem sections (4–6 cm) proximal to the site of inoculation were cut with a sterile razor blade, surface-sterilized in 95% ethanol and 1% (w/v) sodium hypochlorite (45–60 sec each), and rinsed three times in sterile water

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(45–60 sec each). Xylem sap was squeezed with pliers from freshly cut stems and impressed onto the solid medium. Plates were incubated for 6 days or more at 28 C. Colonies of *C. x. cynodontis* were identified by their growth rate, colony morphology, and color with a dissecting microscope (30×). Positive assays were confirmed by examination of the morphology of bacteria with dark-field microscopy (1,000×). A radioimmunoassay was also used to confirm results obtained with *D. u. undecimpunctata*. Xylem from surface-sterilized plant samples was extracted by centrifugation (10,000 rpm, 1 min) in a 1.5-ml centrifuge tube, stored in 10.0 mM phosphate buffer with 0.038 mM sodium azide, and tested at Crop Genetics International (Hanover, MD).

Insect transmission. Five species of insects were tested for their ability to transmit *C. x. cynodontis* from colonized to uncolonized plants (Table 1). Maize (FR632), colonized by one of three California strains of *C. x. cynodontis* (BKF1, Ind1, or IndC), or bermudagrass, colonized by strain BKF1, were used as source plants. We inoculated maize plants by puncturing the bases of their stems with the sharpened eye of a sewing needle containing a suspension of *C. x. cynodontis* (approximately 10^6 cfu/μl of SCP buffer). We inoculated Bermudagrass by cutting through stems with a razor blade that had been dipped in a cell suspension. Forty days or more after inoculation, insects were caged on these plants for various times (Table 1) and were then transferred to uncolonized bermudagrass or maize (FR632 seedlings, three- to four-leaf stage). Insects were removed from the test plants after a feeding period that lasted from 8 h to 13 days (Table 1). Test plants were then sprayed with an aerosol insecticide (acephate or resmethrin) and grown in a greenhouse under constant positive pressure. Test plants were assayed for *C. x. cynodontis* 30 days or more after removal of the insects. Uninoculated plants, which served as controls for *C. x. cynodontis* contamination, and insects fed these plants were also assayed.

To investigate whether inoculated insects are able to transmit *C. x. cynodontis* when feeding on plant tissues that are most susceptible to *C. x. cynodontis* colonization, we transferred insects from colonized bermudagrass to uncolonized bermudagrass stems. Adult male and female *S. nitens* ($n = 41$) were starved for 18 h and then fed colonized bermudagrass. While they fed, insects were removed from these plants and placed singly in clear plastic cages that covered the exposed stems of uncolonized bermudagrass ($n = 41$). Stems were exposed by the gentle removal of leaves and leaf sheaths along an 8- to 12-cm length. The insects were observed continuously and were removed after they had taken approximately five to 10 bites. The feeding sites were marked with plastic tape. The time between feeding on colonized and on uncolonized plants was defined as the residence time of *C. x. cynodontis* on an insect. Xylem sap was assayed from stems proximal to the site of feeding after 42 days. *C. x. cynodontis* source plants ($n = 2$) and positive control plants ($n = 4$) were also assayed.

To investigate whether insects inoculated with large numbers of *C. x. cynodontis* are able to transmit the bacterium when feeding on leaves, we treated their mandibles before placing the insects on uncolonized bermudagrass. Third-instar *S. nitens* nymphs ($n = 16$) were chilled at −20 C for 2–3 min. Using a dissecting microscope, we applied cell suspensions (0.7 μl, 4.8×10^7 cfu/μl) to the mandibles of each insect with a pipette (Gilson, 0–20 μl), and the time of application was recorded. Nymphs were placed singly in cages on bermudagrass. The insects were removed after making five to 10 bites, and the time was recorded. The leaves ($n = 3$) or stems ($n = 3$) of positive control plants were cut with a sterile razor blade that was dipped repeatedly in the cell suspension. Uninoculated plants ($n = 16$) served as controls for *C. x. cynodontis* contamination. Plants were assayed after 21 days.

This experiment was repeated with fourth-instar *S. nitens*. Insects were treated with 0.7 μl of the cell suspensions at concentrations of 20, 2×10^4, or 2×10^7 cfu/μl ($n = 10$ insects per treatment). Plants were assayed for *C. x. cynodontis* after 24 and 108 days.

To investigate whether insects inoculated with large numbers of *C. x. cynodontis* are able to transmit the bacterium when feeding on stems, we fed treated insects uncolonized bermudagrass stems. Cell suspensions (1 μl, 6.7×10^7 cfu/μl) were coated on the mandibles of adult males that had been starved for 24 h, and the time of treatment was recorded. The treated *S. nitens* were then caged singly on bermudagrass stems and observed continuously. Insects ($n = 5$) were removed after they began feeding, and the time was recorded. The stems of positive control plants ($n = 3$) were cut with dissecting scissors after the blades were dipped in the cell suspension. The stems of negative control plants ($n = 15$) were cut with scissors that had been dipped in SCP buffer. Plants were assayed after 21 days, and uncolonized plants were reassayed after 67 days.

We repeated this experiment on 4 days using adult male and female *S. nitens* treated in random order with one of three cell suspensions: 10^5 cfu/μl, 10^6 cfu/μl, and 10^8 cfu/μl. The total

TABLE 1. Insect transmission of *Clavibacter xyli* subsp. *cynodontis* from colonized to uncolonized plants

Insect order: family Species	Insects (no.)	Plants (no.)	Test plant	Aquisition period[a]	Test plant inoculation period	Colonized test plants (no.)
Orthoptera: Acrididae						
Melanoplus sanguinipes[b]	117	37	Maize	3–8 days	3–5 days	0
M. sanguinipes (control)	64	21	Maize	...	3–5 days	0
Coleoptera: Chrysomelidae						
Diabrotica undecimpunctata	282	47	Maize	1–12 days	1–12 days	0
D. undecimpunctata (control)	10	1	Maize	1 day	1 day	0
Lepidoptera: Noctuidae						
Pseudaletia unipuncta[c]	206	27	Maize	8–24 h	1–6 days	0
P. unipuncta[d]	52	5	Maize	24 h	8–24 h	0
P. unipuncta (control)	59	7	Maize	...	8–144 h	0
Homoptera: Cicadellidae						
Carneocephala fulgida	123	28	Bermudagrass	1–15 days	6–9 days	0
C. fulgida (control)	36	8	Bermudagrass	...	7–9 days	0
C. fulgida	37	8	Maize	4–15 days	6–9 days	0
C. fulgida (control)	48	8	Maize	...	6–9 days	0
Draeculacephala minerva	31	4	Maize	14 days	1–13 days	0
D. minerva (control)	14	2	Maize	...	1–7 days	0
D. minerva	35	6	Bermudagrass	10–14 days	2–7 days	0
D. minerva (control)	30	5	Bermudagrass	...	2–7 days	0

[a]Colonized bermudagrass and maize were used as source plants for *C. x. cynodontis*.
[b]Second, third, and fourth instars.
[c]Second and third instars.
[d]Third, fourth, and fifth instars.

numbers of insects that fed in each experiment were 21, 18, 15, and 12, respectively. Ten to 27 negative control plants were prepared per experiment. Positive control plants ($n = 3$) also were prepared in the first and final experiments. Plants inoculated by *S. nitens* served as positive controls for *C. x. cynodontis* viability in the other experiments. Each plant was assayed after 36–45 days and again after 103–107 days. Frequencies of transmission by insects treated with three levels of the bacterium were compared with a test of independence (G-test) on a 2 × 3 table (13). The patterns of transmission on each day (experimental blocks) appeared similar, and therefore blocks were combined for analysis. Data from negative controls were not included in the analysis. Residence times of *C. x. cynodontis* on the mandibles of *S. nitens* that transmitted ($n = 10$) or failed to transmit the bacterium ($n = 43$) were compared with the Mann-Whitney U-test (16).

Abundance of *C. x. cynodontis* on insect mandibles. To establish the maximum number of *C. x. cynodontis* retained on the mandibles of treated *S. nitens,* we chilled adult females ($n = 10$) (−20 C, 5–10 min) and coated their mandibles with 1 μl of a cell suspension (5.7 × 10^6 cfu/μl). We anesthetized insects after 30 min by placing them in a container of CO_2 for 1 min. The mandibles were dissected, placed in 100 μl of SCP buffer, and vortexed for 15 s at high speed. Suspensions of bacteria were serially diluted and plated on solid medium. The *C. x. cynodontis* colonies were identified and counted.

The abundance of *C. x. cynodontis* was measured on the mandibles of *S. nitens* that fed on colonized bermudagrass ($n = 11$). Adult males that had been starved for 24 h were placed singly in cages over the stolon of a colonized plant. The insects were allowed to feed for 1–4 min and were then anesthetized in CO_2. The abundance of *C. x. cynodontis* on their mandibles was assayed as described. Nearby leaves of similar quality (age and general appearance) from each plant ($n = 6$) were collected, and concentrations of the bacterium in leaves eaten by *S. nitens* were estimated. Each leaf was weighed, surface-sterilized, and ground in 500 μl of SCP buffer with a mortar and pestle on ice. Leaf homogenates were serially diluted and plated on solid medium.

This experiment was repeated twice with adult females ($n =$ 16 and 14). Changes in the above protocol included chilling insects before dissection (−20 C, 10 min), sampling two leaves from each of three plants used, and homogenizing leaves in 2.0 ml of SCP buffer with a Brinkman Polytron homogenizer using a PT-2 bit (Brinkman Instruments, Westbury, NY). The mandibles of untreated insects ($n = 3$) were dissected and used as controls in each experiment.

The abundance of *C. x. cynodontis* on the mandibles of larval Lepidoptera that had been fed colonized bermudagrass was measured in two experiments with sixth-instar ($n = 7$) and fourth-instar ($n = 11$) *H. zea* larvae. Larvae were placed singly in cages on colonized bermudagrass and allowed to feed for 1–8 min. The mandibles of chilled larvae (0 C, 3 min) were dissected, and bacteria were extracted in 50 μl of SCP buffer. Serially diluted samples were plated on solid medium. Control larvae ($n = 3$) were fed uncolonized wheat. *C. x. cynodontis* concentrations in homogenized leaves ($n = 2$) from each plant eaten by larvae were measured as described.

Mechanical transmission. To investigate the efficiency of colonization of bermudagrass when leaves are inoculated, we cut a single leaf per plant with sterile dissecting scissors that had been dipped in one of two cell suspensions (3 × 10^6 or 3 × 10^8 cfu/μl; $n = 28$ per treatment). Leaves of negative control plants were cut with scissors that had been dipped in SCP buffer ($n = 10$). The stems of positive control plants ($n = 3$) were inoculated with the more concentrated cell suspension. The plants were assayed after 35 days and reassayed after 91 days.

To compare the efficiency of colonization of *C. x. cynodontis* through different plant tissues, we inoculated leaves or stems of bermudagrass and maize (cv. Golden Bantam). Bermudagrass leaves ($n = 30$) or stems ($n = 30$) were cut with dissecting scissors that were dipped repeatedly in a cell suspension (7.0 × 10^6 cfu/μl of SCP buffer). The plants were assayed after 26 days and reassayed after 48 days. Leaves from 13-day-old maize plants were inoculated with scissors that had been dipped in a cell suspension (5.6 × 10^6 cfu/μl). Cuts (5-mm) were made to laminae ($n = 19$ plants), midribs ($n = 20$ plants), and sheaths (near the bases of laminae; $n = 15$ plants). The maize stems ($n = 8$) were inoculated with a sewing needle eye that contained 2.3 μl of the cell suspension and that was inserted near the soil level. Xylem sap from the midribs of leaves one to two nodes above the site of inoculation was cultured after 31 days and used to assay the plants. We retested plants after 55 days by assaying the xylem sap from stem bases.

The relationship between the concentration of *C. x. cynodontis* in the inoculum and the frequency of colonization was established for stem-inoculated bermudagrass. Stems were cut with scissors that were dipped repeatedly in a cell suspension (0–10^4 cfu/μl; $n = 15$ per treatment). The plants were assayed after 39 days and reassayed after 107 days.

RESULTS

Insect transmission. None of the leaf-chewing insects (*M. sanguinipes, D. undecimpunctata,* and *P. unipuncta*) or xylem-sucking insects (*C. fulgida* and *D. minerva*) transmitted *C. x. cynodontis* when transferred in groups from colonized to uncolonized plants (Table 1). *S. nitens,* transferred individually to bermudagrass stems immediately after feeding on colonized bermudagrass leaves, also failed to transmit *C. x. cynodontis.* The average residence time of the bacterium on *S. nitens* mandibles was 45.3 min (range: 4–131 min). All *C. x. cynodontis* source plants and positive control plants were colonized.

C. x. cynodontis was not transmitted to bermudagrass by *S. nitens* nymphs feeding on leaves, even when their mandibles were freshly coated with the bacterium. The average residence time of the bacterium on their mandibles was 19.2 min (range: 2–43 min). The plants that were mechanically inoculated with *C. x.*

TABLE 2. Transmission of *Clavibacter xyli* subsp. *cynodontis* to bermudagrass by treated *Schistocerca nitens* and by scissors[a]

Concentration of cell suspension (cfu/μl)	Means of inoculation (plant tissue inoculated)[b]					
	S. nitens (leaves)		Scissors (leaves)		Scissors (stems)	
	+	−	+	−	+	−
10^7	0	26	0	6	6	0
10^4	0	10	0	3	3	0
10	0	10	0	2	0	3

[a] Cell suspensions were applied to the mandibles of insects with a pipette, and then insects were caged singly on plants. Scissors were dipped in cell suspensions. Data from two experiments using 10^7 cfu/μl were combined.

[b] + = Transmission; − = no transmission.

TABLE 3. Transmission of *Clavibacter xyli* subsp. *cynodontis* to bermudagrass by treated *Schistocerca nitens*[a]

Concentration of cell suspension (cfu/μl)	+[b]	−	Transmission (%)
10^7–10^8	10	15	40.0
10^6	2	21	8.7
10^5	2	21	8.7
0[c]	0	67	0

[a] Cell suspensions were applied to the mandibles of insects with a pipette, and then insects were caged singly on bermudagrass stems. Results of experiments repeated on 5 days were combined.

[b] + = Transmission; − = no transmission.

[c] Control plants were mechanically inoculated with succinate-citrate-phosphate buffer.

TABLE 4. Abundance of *Clavibacter xyli* subsp. *cynodontis* on the mandibles of insects and in the bermudagrass leaves on which they fed[a]

Insect species	Insects (no.)	Colony-forming units: On mandibles[b]	Colony-forming units: In leaves (no./mg)	Residence time (min)[c]
Schistocerca nitens (male)	10	$2.6 \times 10^3 \pm 1.5 \times 10^3$	$1.4 \times 10^5 \pm 0.8 \times 10^5$	8.4 ± 1.6
S. nitens (female)	8	$8.6 \times 10^3 \pm 3.9 \times 10^3$	$2.3 \times 10^5 \pm 1.0 \times 10^5$	13.6 ± 1.1
S. nitens (female)	4	$1.7 \times 10^3 \pm 3.0 \times 10^3$	$1.8 \times 10^4 \pm 0.8 \times 10^4$	18.0 ± 1.4
Helicoverpa zea	11	0	$5.6 \times 10^3 \pm 2.7 \times 10^3$	7.2 ± 0.4
H. zea	6	0	$1.7 \times 10^5 \pm 1.1 \times 10^5$	17.8 ± 3.7

[a] Data are presented as mean ± SE.
[b] *C. x. cynodontis* was extracted from dissected mandibles in succinate-citrate-phosphate buffer, and colonies were counted after dilution plating.
[c] The time between the end of feeding on a colonized plant and the dissection of mandibles.

cynodontis through stem cuts were colonized, but plants that were inoculated through leaves remained uncolonized. No *C. x. cynodontis* was detected in negative control plants. We obtained similar results when this experiment was repeated with a range of *C. x. cynodontis* concentrations (Table 2). The residence time of the bacterium averaged 15.8 min (range: 1–47 min).

Transmission by insects occurred only after the mandibles of *S. nitens* were coated with high concentrations of *C. x. cynodontis* (10^5–10^8 cfu/μl) and their feeding was restricted to short periods on bermudagrass stems. The results of five experiments were similar and are summarized in Table 3. The frequency of colonization increased at the highest bacterial concentrations (10^7–10^8 cfu/μl) ($P < 0.025$; G-test). The residence times of *C. x. cynodontis* on insects that transmitted (41.1 min ± 7.2 standard error [SE]) and those that failed to transmit the bacterium (49.0 min ± 4.1 SE) were not significantly different ($P = 0.47$; Mann-Whitney U-test). All positive control plants treated in the first and fifth experiments were colonized.

Abundance of *C. x. cynodontis* on insect mandibles. Ten percent of the colony-forming units applied to the mandibles of *S. nitens* was recovered and remained viable after a residence time of 30 min on the mouthparts (5.7×10^5 cfu ± 3.5×10^5 [SE] recovered per 5.7×10^6 cfu applied). The abundance of *C. x. cynodontis* on the mandibles of *S. nitens* that fed on colonized bermudagrass leaves averaged 4.3×10^3 cfu per insect in three experiments (Table 4). No *C. x. cynodontis* was detected on the mandibles of negative control insects.

No *C. x. cynodontis* was recovered from the mandibles of *H. zea* larvae that fed on colonized bermudagrass (Table 4). However, an unidentified coccoid bacterium was abundant in most *H. zea* samples. Dilution plating of samples separated colonies sufficiently to rule out interference between different colonies as an inhibiting factor in the detection of *C. x. cynodontis.*

Mechanical transmission. The efficiency of colonization of bermudagrass leaves by *C. x. cynodontis* by mechanical inoculation was relatively low. Seven percent of the plants inoculated with 10^6 cfu/μl were colonized, and 0% of the plants inoculated with 10^8 cfu/μl were colonized 35 days after inoculation. Two of three positive control plants were colonized, and all negative control plants remained uncolonized. After 91 days, *C. x. cynodontis* was detected in 14% of the plants inoculated with *C. x. cynodontis* (10^8 cfu/μl), but no additional plants inoculated with 10^6 cfu/μl were colonized.

Results presented in Table 2 suggest that *C. x. cynodontis* colonizes bermudagrass stems more efficiently than it colonizes leaves. Results of an expanded experiment conducted to verify this observation showed that 0% of leaf-inoculated plants were colonized 26 days after inoculation, whereas 100% of the stem-inoculated plants were colonized. After 48 days, 20% of the leaf-inoculated plants were colonized. Some of the 30 leaf-inoculated plants were accidentally discarded, but of the 16 previously uncolonized plants remaining, two additional plants were found to be colonized after 103 days. We obtained similar results in mechanical-inoculation experiments on maize. *C. x. cynodontis* efficiently colonized plants through the stem and leaf sheath but rarely through the leaf blade (midrib or lamina) (Table 5).

The frequency of colonization of bermudagrass through

TABLE 5. Colonization of maize by *Clavibacter xyli* subsp. *cynodontis* following mechanical inoculation of stems and leaves[a]

Inoculation site	31 days +	31 days −	55 days +	55 days −
Stem	7	1	7	1
Leaf				
Sheath	12	3	14	1
Midrib	0	19	0	20
Lamina	0	19	1	19
Lamina (control)	0	9	0	9
Stem (control)	0	8	0	8

Column headers: Time from inoculation to assay[b]

[a] Plants were inoculated with scissors that had been dipped in a cell suspension (5.6×10^6 cfu/μl).
[b] + = Transmission; − = no transmission.

mechanical inoculation of stems depended on the concentration of *C. x. cynodontis* in the inoculum: 0, 15.4, 60, and 100% of the plants were colonized at 0, 10, 10^2, and 10^3 and 10^4 cfu/μl, respectively.

DISCUSSION

The results of our studies demonstrate that a wide variety of leaf-chewing and xylem-sucking insects are not normally vectors of *C. x. cynodontis.* Our findings extend the work of Prunier et al (8), which showed that aphids are not vectors of this bacterium. Although it is conceivable that some untested species of insects are vectors of *C. x. cynodontis,* we identified several factors suggesting that insects in general are poor vectors: 1) leaf tissues have a low susceptibility to colonization by *C. x. cynodontis,* 2) insects feeding on colonized foliage acquire relatively small numbers of *C. x. cynodontis,* and 3) insect mouthparts retain relatively small numbers of viable *C. x. cynodontis,* even when large numbers are applied. Insect transmission of *C. x. cynodontis* to bermudagrass occurred only when unusually high concentrations of *C. x. cynodontis* were applied directly to their mandibles and when inoculation feeding was limited to a brief period on stems.

We estimate that the numbers of viable *C. x. cynodontis* retained on the mandibles of insects fed colonized bermudagrass, e.g., *S. nitens* (10^3 cfu), are too low to be transmitted to uncolonized plants. A transmission frequency of 8% was observed when approximately 10^4 cfu were present on the mandibles of *S. nitens* that fed on stems (10^5 cfu applied; 10% retained). Insects that had 10^3 cfu on their mandibles and that fed on stems, therefore, would be expected to have a frequency of transmission of less than 8%. When the insects feed on leaves, in which there is a low probability of colonization even at much higher *C. x. cynodontis* levels, a negligible frequency of transmission would be expected. In addition, uninterrupted feeding may further reduce the probability of transmission as a result of the ingestion and dilution of the inoculum on the mouthparts.

Unlike other xylem-limited bacteria (e.g., *Erwinia* spp.) that survive for extended periods in insect vectors (5,9), *C. x. cynodontis* appears to have a low tolerance for the pre-oral cavities

of leaf-chewing insects. No *C. x. cynodontis* was recovered from the mandibles of *H. zea*, and 10% of the bacterial population applied to the mandibles of *S. nitens* was recovered after a 30-min period. These results suggest that mortality, and possibly ingestion in the case of *S. nitens*, is a factor limiting transmission by some insects.

Similarly, although both leafhopper species tested in this study feed on the xylem of grasses (10) and are efficient vectors of the xylem-limited bacterium *Xylella fastidiosa* (9,12), neither leafhopper transmitted *C. x. cynodontis*. Viable *C. x. cynodontis* was recovered from *C. fulgida* (range: $0-10^5$ cfu per insect) and *D. minerva* (range: $0-10^3$ cfu per insect) after they fed on colonized plants (11; B. Hill and A. H. Purcell, *unpublished*). Our results suggest that such levels of *C. x. cynodontis* are generally too low to effect transmission, and it is uncertain whether *C. x. cynodontis* in these species is present in the saliva that is introduced into the xylem (9).

The residence time of *C. x. cynodontis* on insect mouthparts was not shown to be an important factor in limiting transmission. However, our experiments were designed to minimize residence times with the use of pretrial fasting and rapid manual transfer. Under natural conditions, movement and other activities minimized in our studies could reduce the viability of *C. x. cynodontis* on insect mouthparts at the start of inoculation feeding by greatly increasing residence times. Under natural conditions, transmission may be limited as a result of any or all of the above factors.

There are two clear similarities between the factors limiting the transmission of *C. x. cynodontis* and the transmission of another xylem-limited bacterium, *Erwinia amylovora*. The frequency of colonization of apple leaves inoculated with *E. amylovora* is higher through leaf midribs than through leaf laminae (7). The results of insect- and mechanical-inoculation experiments of bermudagrass or maize with *C. x. cynodontis* showed that the bacterium also has a low probability of colonization of leaf laminae compared to colonization of stems. Secondly, the introduction of small numbers of viable bacteria into host plants by *Aphis pomi* limits transmission (7). Our study showed that the numbers of *C. x. cynodontis* acquired by leaf-chewing insects is generally too low to colonize bermudagrass. Further work is needed on the mechanisms affecting the colonization of different plant tissues by xylem-limited bacteria.

The factors we studied provide a mechanistic basis for understanding the rarity of *C. x. cynodontis* transmission by insects in laboratory experiments or in large-scale field trials (8,14). Our results suggest that the spread of genetically engineered *C. x. cynodontis* from colonized maize by phytophagous insects is unlikely.

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