A Fastidious, Xylem-Limited Bacterium Infecting Ragweed

L. W. Timmer, R. H. Brlansky, R. F. Lee, and B. C. Raju

First author, associate professor, and second and third authors, assistant professors, Agricultural Research and Education Center, University of Florida, Lake Alfred 33850; and fourth author, chief plant pathologist, Yoder Brothers, Inc., Box 68, Alva, FL 33920.
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ABSTRACT

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A fastidious, ragweed xylem-limited bacterium (RgXLB) was isolated that grew well on media developed for the phony peach (PP) bacterium, but not on media for the Pierce's disease (PD) bacterium. Xylem vessels of ragweed (*Ambrosia artemesiifolia*) mechanically inoculated with the RgXLB became heavily colonized. Infected ragweed was stunted compared to healthy controls, but showed no other symptoms. The disease was designated ragweed stunt. The RgXLB was transmitted from ragweed to ragweed by the sharpshooters, *Oncometopia nigricans* and *Homalodisca coagulata*. Plum became infected following mechanical and insect vector inoculations, periwinkle only following mechanical inoculations, and grape, peach, and citrus were not infected. The RgXLB was reisolated from infected plum and periwinkle, but the plants did not develop symptoms. Bacteria from ragweed and from culture were small and rod-shaped (0.3–0.5 × 1.5–3.0 μm) with a rippled cell wall typical of other xylem-limited bacteria (XLB). The cibaria and precibaria of *O. nigricans* were colonized by the RgXLB in a manner similar to that of other XLB. In enzyme-linked immunosorbent assay, the RgXLB reacted more strongly with antiserum to the plum leaf scald (PLS) bacterium than to antiserum to PD bacterium. In reciprocal tests, the PLS bacterium reacted more strongly with an antiserum prepared to the RgXLB than did the PD bacterium. The RgXLB appears to be closely related to, but pathologically distinct from, the PLS and PP bacteria and more distantly related to other XLB based on cultural characteristics, host range, and serological tests.

Additional key words: ragweed stunt, rickettsialike bacteria.

Fastidious, Gram-negative, xylem-limited bacteria (XLB) are well established as the causal agents of Pierce's disease of grape (PD), almond leaf scorch (ALS), alfalfa dwarf (AD), phony peach (PP), and plum leaf scald (PLS) (6,12,16,17,19,20,25,28). Pierce's disease, ALS, and AD are caused by the same organism (8,12,19) and PLS and PP also have common etiologies (11,28). In addition, XLB have been implicated as causal agents of periwinkle wilt (18), of elm, oak, and sycamore leaf scorch (13), and of several other diseases (9,21).

Fastidious, xylem-limited bacteria have been recovered from trees affected by citrus blight (young tree decline) and may be implicated as a causal agent of the disease (10,14,15). Our search for xylem-limited bacteria in citrus groves affected with blight yielded a bacterium capable of infecting ragweed (3,26). In the present study, this bacterium was isolated and cultured and the pathogenicity, host range, serology, cytology, and vector relations of this organism were studied. An abstract of a portion of this work has been published (26).

MATERIALS AND METHODS

Plant material and greenhouse conditions. Ragweed (*Ambrosia artemisiifolia* L.), periwinkle [*Catharanthus roseus* (L.) G. Don. 'Little Pinkie'], and other nonwoody plants were grown from seed and used at about 2–3 mo of age. Plum (*Prunus cerasifera* Ehrh. 'Myrobalan'), peach [*Prunus persica* (L.) 'Lovell'], and rough lemon (*Citrus jambhiri* Lush.) were grown from seed. Pathogen-free grapes [*Vitis vinifera* L. 'Chardonnay,' 'Mission,' and 'St. George'] and citron (*C. medica* L. 'Etrog') were propagated by rooting cuttings in a mist bed. Woody plants were used at about 6 mo of age.

Plants in inoculation tests were maintained in the greenhouse at 15–35 C or in the screenhouse at 5–35 C. Caged plants during insect transmission trials were kept in a greenhouse at 18–26 C and received 6 hr of supplemental light per day.

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Isolations and media. For isolations, stems of ragweed and other plants were surface disinfested by soaking in 0.5% NaOCl for 2 min and in 70% ethanol for 1 min and then rinsing in sterile, deionized water. Xylem fluid was squeezed aseptically from the stems using forceps or pliers and blotted directly onto the surface of the media.

The following media were tested for isolation and culture of the ragweed xylem-limited bacterium (RgXLB): PD-2, a medium developed for the isolation and culture of the PD bacterium (7); buffered charcoal-yeast extract agar (BCYE) (27) and BCZE (25) developed for isolation and culture of the PP and PLS bacteria; PW media, originally developed for isolation of the periwinkle wilt bacterium, but also successful for culture of the PP and PLS bacteria (5). All isolation plates and cultures were incubated in the dark at 28 C.

Insect transmission. In initial tests, field-collected adults of the sharpshooters, *Oncometopia nigricans* (Walker) and *Homalodisca coagulata* (Say), were used for transmission studies. For further tests, greenhouse-reared *O. nigricans* were used. These were reared by placing field-collected *O. nigricans* in a single cage with grape, periwinkle, rough lemon, and elderberry (*Sambucus canadensis* L.) plants and allowing them to breed and lay eggs. Eggs collected from the plants were placed on moist filter paper in petri dishes in the laboratory. As nymphs hatched, they were placed in a cage with healthy periwinkle, elderberry, and water primrose (*Jusaiaea peruviana* L.) and reared to adulthood. It was presumed that no transovarial passage would occur with the RgXLB because it does not occur with the other XLB and vectors lose their ability to transmit XLB after molting (9,22).

In transmission tests, 10–20 adults were allowed a 10- to 30-day acquisition access period on ragweed infected with the RgXLB. They were then given about a 30-day inoculation access period on healthy plants. An equal number of control plants, which were not exposed to insects, were maintained in all transmission experiments.

After the inoculation access period, the surviving insects were dissected and prepared for scanning electron microscopy (SEM) as described below. The number of insects in which the pump organs had been colonized by bacteria was determined in most experiments.

Mechanical inoculation. Bacterial cultures for inoculations were

grown for 7–10 days on BCYE or PW medium. Bacteria were scraped from plates in a small volume of sterile phosphate-buffered saline (PBS) (NaCl, 8 g; KH_2PO_4, 0.2 g; Na_2HPO_4, 1.15 g; KCl, 0.2 g per liter of water, pH 7.4). A sample for counting was diluted in PBS, a drop of Tween-20 was added, the mixture was vortexed vigorously for 1–2 min, and the XLB was counted in a bacterial cell counter (Hauser Scientific, Blue Bell, PA 19422). The original suspension was diluted to 10^8–10^9 cells per milliliter. The main stem of plants was inoculated by pin-pricking droplets of the inoculum into the xylem with a fine needle at six to eight sites per stem. In some cases the procedure was repeated up to four times at 3- to 4-wk intervals in attempts to attain infection. An equal number of control plants was inoculated with PBS in each mechanical transmission experiment.

Detection of bacteria. Inoculated and control plants were examined periodically for the presence of bacteria, beginning 3 mo after inoculation. Xylem extracts of stems were squeezed onto microscope slides, dried, stained 2–3 min with 0.1% methylene blue and rinsed with water. Slides were mounted in Aqua-Mount (Lerner Laboratories, New Haven, CT 06513) and observed at ×400 using phase contrast optics for the presence of clumps of long, narrow, rod-shaped bacteria. Plants were discarded if no infection was detected after 10–12 mo, but infected plants were maintained for up to 2 yr to observe symptom development.

Infection was confirmed or questionable samples were checked for bacteria by direct immunofluorescence (1) using antisera to PD, PP, or RgXLB labeled with fluorescein or tetramethylrhodamine isothiocyanate. In most mechanical transmission tests, infection was confirmed by reisolation of the bacterium on PW or BCYE media as described above.

Effect on the host. Eight ragweed seedlings were inoculated with the RgXLB by field-collected *O. nigricans* to determine the symptoms associated with infection. Eight control seedlings were caged without insects during the inoculation access period. Plants were observed regularly for symptoms and growth was measured. The height of the tallest shoot on each plant was measured 3, 5, 7, 9, and 11 mo after inoculation. The average leaf length of the top five fully expanded leaves on each plant was determined at 9 and 11 mo after inoculation. At each recording date, plants were cut back leaving four stems each about 40 cm tall, and the fresh weight of the shoots removed was determined.

Serology. Antisera to the PD, PP, PLS, and RgXLB were prepared as described previously (24). For enzyme-linked immunosorbent assay (ELISA), the double-sandwich technique of Clark and Adams (4) was used. Gamma-globulin was purified and conjugated with alkaline phosphatase by previously described methods (23,24). Plant materials and bacterial cultures were prepared for ELISA tests as described previously (23).

Electron microscopy. Sharpshooters that had fed on infected ragweed were prepared for SEM using the procedures reported previously (3). Cibaria and diaphragms were dissected from sharpshooter heads after critical-point drying, mounted on SEM stubs, sputter-coated with 10 nm of gold paladium, and examined for bacteria in a JEOL JSM 35 scanning electron microscope. Cibaria were further dissected by separating the epipharnyx and hypopharnyx halves. The precibarial areas of these halves were mounted, sputter-coated, and examined for bacteria by SEM.

Infected and healthy ragweed stems and bacterial colonies from agar plate culture were prepared for SEM and transmission electron microscopy (TEM) by using the procedures reported previously (1). Ultrathin sections for TEM were made on a Huxley LKB ultramicrotome (LKB Instruments, Inc., Rockville, MD 20352), stained with uranyl acetate and lead citrate, and viewed with a Philips 201 electron microscope. Samples for SEM were mounted, sputter-coated, and viewed as described above.

TABLE 1. Mechanical inoculation of plants with isolates of the ragweed xylem-limited bacterium (RgXLB), the plum leaf scald (PLS), and the phony peach (PP) bacteria

Expt. no.	Isolate no.	Times inoc.[a]	Plant inoc.	(No. positive)/(no. tested)[b] Microscopy	Reisolation
I	RgXLB-1	2	Ragweed	5/6	4/5
II	RgXLB-2	2	Ragweed	3/3	3/3
	RgXLB-2	2	Plum	3/3	3/3
	RgXLB-2	2	Periwinkle	2/3	1/2
	RgXLB-2	2	Grape	0/3	...[d]
	RgXLB-2	2	Peach	0/3	...
III	RgXLB-1	3	Ragweed	5/5	5/5
	PP-5[c]	3	Ragweed	2/5	1/2
	PLS-39[c]	3	Ragweed	0/5	...
IV	RgXLB-3	4	Ragweed	2/9	...
	PP-1	4	Ragweed	2/9	...

[a] Inoculated by pin-pricking six to eight drops containing 10^8–10^9 bacteria per milliliter into the stem of each plant the number of times indicated. Inoculations were separated by about 3–4 wk.
[b] Number of plants infected per number of plants tested as determined by microscopic examination of xylem extracts for typical bacteria or by reisolation on PW media. No bacteria were detected in an equal number of control plants examined in each experiment.
[c] Isolates obtained from W. J. French, Univ. Florida, Monticello 32344.
[d] Not determined.

RESULTS

Isolation, culture, mechanical inoculation, and reisolation. Attempts to isolate the RgXLB on PD-2 media were not successful. Small (<0.5 mm in diameter) colonies formed in 4–5 days in the droplets of xylem fluid placed on the agar medium, but they failed to develop further. No colonies formed when the initial colonies were transferred to fresh PD-2 plates. The RgXLB was first successfully isolated on BCYE on which 1- to 2-mm-diameter, white to opalescent colonies formed in 6–8 days. The bacterium also has been isolated on BCZE, but only occasional, small colonies

TABLE 2. Transmission of the ragweed xylem-limited bacterium to various plants by the sharpshooters, *Oncometopia nigricans* and *Homalodisca coagulata*

Expt. no.	Insects: Species	Source[a]	No.	Feeding time (days)[b]: Acq.	Inoc.	Pump organs (no. positive/no. examined)[c]	No. infected/no. inoculated[d]: ragweed	plum	grape	periwinkle	citrus
I	*O. nigricans*	Field	26	30	30	...[e]	8/8	...	...	...	...
II	*O. nigricans*	Reared	19	15	30	10/12	2/2	1/1	0/1	0/1	0/3
III	*O. nigricans*	Reared	18	18	30	7/8	...	1/1	0/1	0/1	0/3
IV	*O. nigricans*	Reared	10	10	40	1/5	1/2	1/1	0/1	0/1	...
V	*H. coagulata*	Field	12	15	30	6/11	2/2	...	...	...	...
	Totals						13/14	3/3	0/3	0/3	0/6

[a] Field-collected adults or adults reared from eggs in the laboratory and greenhouse on healthy plants.
[b] Acquisition access and inoculation access periods in days.
[c] Number of sharpshooters with cibaria colonized by bacteria divided by the number of insects examined by scanning electron microscopy after inoculation feeding.
[d] Typical bacteria detected in xylem extracts by phase contrast microscopy. No bacteria were detected in an equal number of control plants not exposed to sharpshooters.
[e] Not determined.

formed. In our experience, the PW medium has been the most reliable for isolation of the RgXLB. Generally, colonies were slightly larger, formed more rapidly, and occurred in a higher percentage of the droplets of xylem fluid blotted onto the media.

The RgXLB, once isolated in pure culture, grew well on BCYE, BCZE, and on the PW medium, but not on PD-2. Growth of the bacterium has been slightly more vigorous on PW medium than on BCYE or BCZE.

When the RgXLB from pure culture was mechanically inoculated into healthy ragweed seedlings, the bacterium multiplied in xylem but was not detectable in a high percentage of stems until 3–5 mo after inoculation. By 6–7 mo postinoculation, the bacterium had spread throughout much of the plant and was readily detectable by phase-contrast microscopy of smears of xylem fluid. The bacterium was reisolated on PW media from nearly all of the inoculated plants from which isolations were attempted (Table 1).

Transmission. In a high percentage of the cases, the RgXLB was transmitted from infected to healthy ragweed by using field-collected *O. nigricans* and *H. coagulata* (Table 2). *O. nigricans* reared from eggs, which were free of any bacterium that might have been carried by field-collected insects, also transmitted this bacterium efficiently. The RgXLB was also transmitted by *O.*

TABLE 3. Effect of insect inoculation with the ragweed xylem-limited bacterium on the growth of ragweed (*Ambrosia artemisiifolia*)[a]

Growth parameter[b]	Inoc.	Postinoculation growth at					
		3 mo	5 mo	7 mo	9 mo	11 mo	Avg
Shoot height (cm)	+	94*[c]	118*	106	111	93*	104*
	−	104	131	114	125	113	117
Leaf length (cm)	+	...[d]	...	...	10.2*	10.1	10.2*
	−	...	...	...	12.0	11.6	11.8
Fresh wt (g)	+	76	92	53	66*	60*	70*
	−	70	92	64	87	110	85

[a] Eight ragweed inoculated using *Oncometopia nigricans* and eight comparable uninoculated control plants. Bacteria were detectable in three inoculated plants at 3 mo postinoculation and in all inoculated plants by 7 mo postinoculation.

[b] Height of the tallest shoot on each plant, length of the top five fully expanded leaves, and fresh weight of the new shoot growth that emerged after the previous cutting and recording date.

[c] *Significantly less than the uninoculated control according to Student's *t*-test ($P = 0.05$).

[d] Not determined.

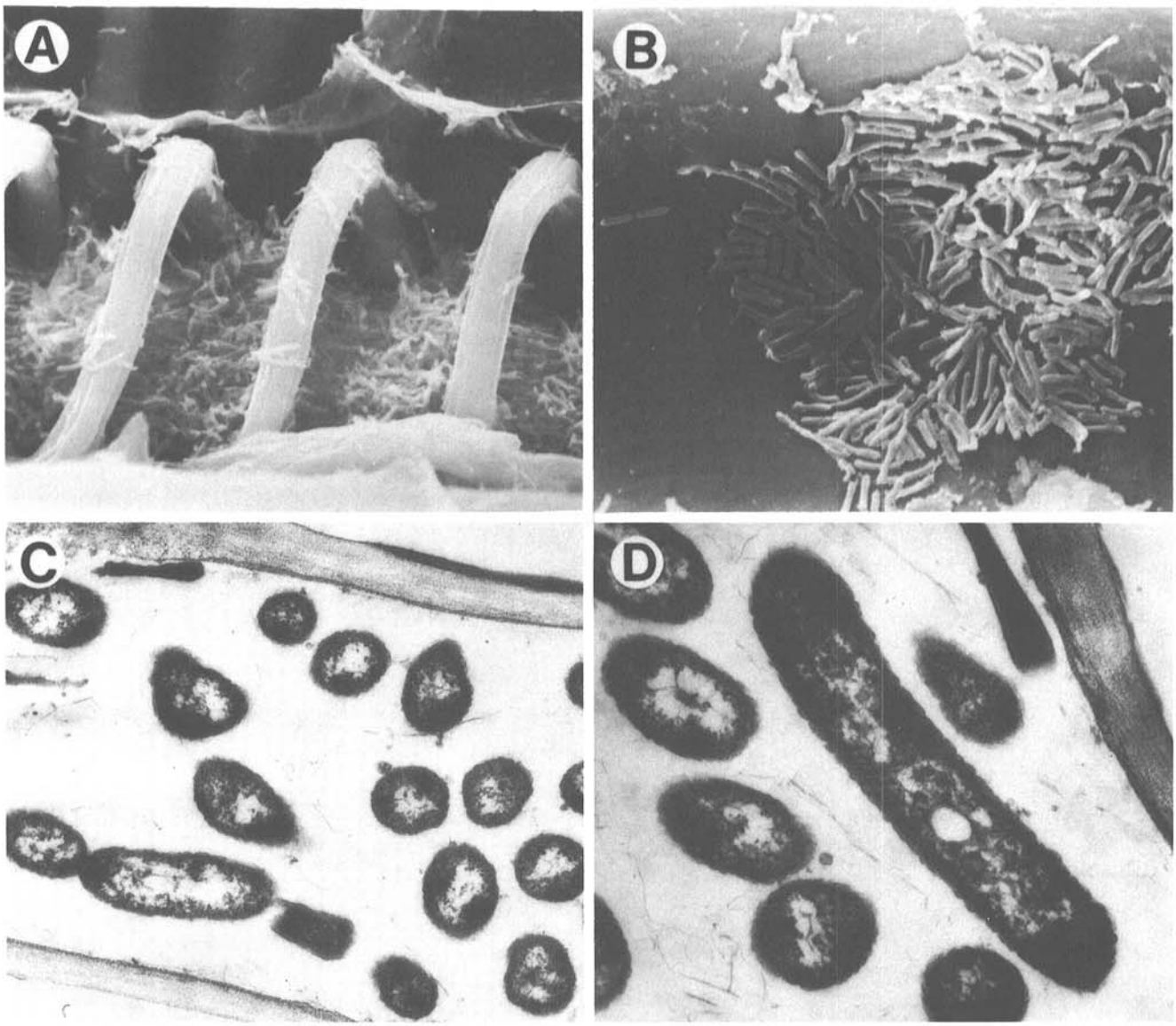

Fig. 1. The ragweed xylem-limited bacterium (RgXLB) in *Ambrosia artemisiifolia*. **A and B,** Scanning electron microscopy of the bacterium in the lumen of a xylem vessel at **A,** ×2,000, and **B,** ×3,200. **C and D,** Transmission electron microscopy of the bacterium in the xylem vessels at **C,** ×30,100, and **D,** ×42,500.

nigricans from ragweed to plum, but not to grape, periwinkle, or to citrus in three experiments (Table 2).

The cibaria of *O. nigricans* and *H. coagulata* were colonized by the RgXLB after the sharpshooters fed on infected ragweed. The cibaria of over 50% of the *O. nigricans* and *H. coagulata* allowed acquisition access periods of 15 days or longer were heavily colonized by the RgXLB. Only one of five cibaria of *O. nigricans* was colonized in an experiment where the acquisition access period was 10 days (Table 2).

When recently recovered isolates of the RgXLB (RgXLB-1, RgXLB-2) were used for pin-prick inoculation, a high percentage of the ragweed became infected (Table 1, Expts. I-III). However, when an isolate, RgXLB-3, which had been maintained in culture for about 6 mo and transferred every 2 wk, was used for inoculation, only two of nine plants became infected, even though they had been inoculated four times (Table 1, Expt. IV).

The RgXLB also infected most of the plums and periwinkles that had been mechanically inoculated. Periwinkle became infected following mechanical inoculation, but not when inoculated using *O. nigricans*. Peaches, grapes, and citrus were not infected by the RgXLB whether inoculated mechanically or by sharpshooters (Tables 1 and 2).

Ragweed became infected when inoculated mechanically with the PP bacterium, but the percentage of the plants infected was low whether ragweed was inoculated with a recently recovered isolate (PP-5) or with one which had been in culture for more than 6 mo (Table 1, Expts. III and IV). We were unable to infect ragweed with a recently recovered PLS isolate (Table 1, Expt. III). None of the control plants in any of the insect transmission or mechanical transmission experiments became infected with the RgXLB.

Symptomatology. No obvious symptoms occurred on ragweed inoculated mechanically or with insect vectors. Infection reduced plant height, leaf length, and fresh weight of shoots compared to healthy controls (Table 3). Reductions in shoot fresh weight were substantial by 11 mo after inoculation, but relatively minor prior to that time.

Plums and periwinkle, which were infected by the RgXLB, showed no symptoms of leaf scald or wilt and appeared to be as vigorous as noninoculated controls. Inoculated peaches, grapes, and citrus showed no symptoms and bacteria were never recovered from these species (Table 1).

Serology. In ELISA tests, the RgXLB in infected ragweed stem tissue reacted with antisera prepared to other XLB. Absorbance values at 405 nm were 0.41 using antiserum to a pure culture of the PD bacterium, 0.60 using antiserum to a pure culture of the PP bacterium, and 0.72 using antiserum to the PP bacterium isolated in pure culture from root xylem. Using the same antisera, healthy ragweed gave absorbance values of 0.01, 0.01, and 0.02, respectively.

In ELISA tests the RgXLB bacteria from culture reacted most strongly with the homologous antiserum, nearly as strongly with the antiserum to the PLS bacterium, and to a lesser extent with the antiserum to the PD bacterium (Table 4). The PP and PLS bacteria reacted more strongly with the antiserum to the RgXLB than with antisera to the PD bacterium. Pierce's disease, PP, and PLS bacteria had a higher ELISA readings in homologous than in heterologous systems and *Pseudomonas syringae* did not react with any of the antisera.

Electron microscopy. The RgXLB was examined in infected ragweed, in culture, and in the insect vectors by SEM and TEM. In the plant, the bacterium was confined to xylem vessels that were often heavily colonized and plugged by bacteria (Fig. 1A) or lined with bacteria attached to the vessel walls (Fig. 1B). In sectioned material viewed with TEM, the RgXLB was typical of other Gram-negative, xylem-limited bacteria. Bacteria averaged 0.3–0.5 μm wide and 1.5–3.0 μm long and had a rippled wall composed of a

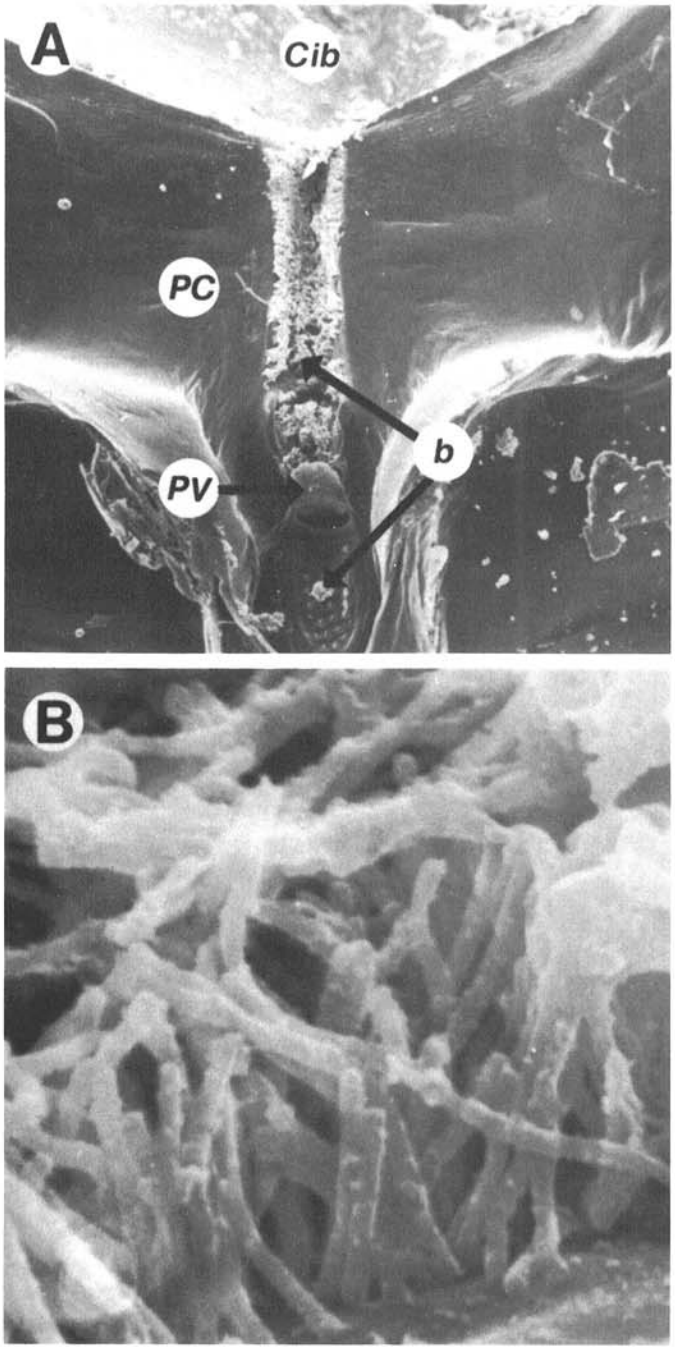

Fig. 2. Scanning electron microscopy of the ragweed xylem-limited bacterium in the pump organs of *Oncometopia nigricans*. **A,** Bacteria (b) in the precibarium (PC) above and below the precibarial valve (PV) (×240). Cib = cibarium. **B,** Bacteria attached to the groove in the cibarium (×2,000).

TABLE 4. Serological comparison of pure cultures of the ragweed xylem-limited bacterium (RgXLB), plum leaf scald bacterium (PLSB), the phony peach bacterium (PPB), the Pierce's disease bacterium (PDB), and *Pseudomonas syringae* by enzyme-linked immunosorbent assay

	$A_{405\ nm}$		
Bacterium[x]	RgXLB[y]	PLSB	PDB
RgXLB	1.04 a[z]	0.99 a	0.43 b
PDB	0.40 c	0.26 c	1.42 a
PPB	0.84 b	0.91 b	0.31 c
PLSB	0.87 b	0.96 ab	0.31 c
P. syringae	<0.01 d	<0.01 d	<0.01 d

[x] Bacteria from pure culture used as antigen at 10^8 cells per milliliter.

[y] Antisera prepared against pure cultures of the RgXLB, the PLSB and the PDB.

[z] Values are the averages of three determinations. Means in columns followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

cytoplasmic membrane, R-layer, and outer cell wall (Fig. 1C and D). Bacteria from culture examined by SEM and TEM had characteristics similar to those bacteria observed within sections from infected plants.

In the sharpshooter vectors, the RgXLB colonized the cibaria in a manner similar to that observed with other XLB such as PD, PP, PLS, and PW bacteria (2,22) (Fig. 2). Bacteria were present in the precibarium above and below the precibarial valve (Fig. 2A), in the cibarium (Fig. 2B), and in apodemal groove of the diaphragm.

DISCUSSION

Koch's postulates have been fulfilled for the RgXLB found previously in ragweed (3,26). Since the RgXLB causes no diagnostic symptom of disease other than stunting we propose the name ragweed stunt for this disease.

The RgXLB was similar to all of the other plant pathogenic, xylem-limited Gram-negative bacteria in that it was confined to xylem vessels in its plant host; colonized the cibaria of its sharpshooter vectors as do other XLB (2,22); was fastidious in its nutrient requirements, grew on the same media as some other XLB; and formed similar colonies. The cell size and cell wall structure of the cell was similar to those of other Gram-negative XLB and the RgXLB was serologically related to all other XLB that were tested.

The RgXLB appeared more closely related to the PP and PLS bacteria than to the PD bacterium. It did not grow on the PD-2 media, did not infect grape, and reacted less strongly with antiserum to the PD bacterium than to antisera of the PLS and PP bacteria. It grew well on media, which supported the growth of the PP and PLS bacteria, and in ELISA, the PLS, PP, and RgXLB reacted nearly as well in heterologous as in homologous systems. The RgXLB infected plums and the PP bacterium infected ragweed. However, the RgXLB was biologically distinguishable from the PP and PLS bacteria since it produced no symptoms in plum and did not infect peach. The PLS bacterium did not infect ragweed and the PP bacterium did not thrive in this host. While closely related to PLS and PP bacteria morphologically and serologically, the RgXLB is pathologically distinct from these bacteria. A new genus and species is being erected to accommodate the PLS and PP bacteria (J. M. Wells and B. C. Raju, *personal communication*) and the RgXLB should be considered as a pathovar of that species.

The RgXLB was originally recovered from sharpshooters collected in a blight-affected citrus grove (3). We (*unpublished*) have since recovered the bacterium from sharpshooters from other blighted citrus groves. However, at present we have no evidence that the bacterium is capable of infecting citrus or that it plays any role in citrus blight.

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