

## Mulberry Leaf Scorch, New Disease Caused by a Fastidious, Xylem-Inhabiting Bacterium

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### ABSTRACT

Kostka, S. J., Tattar, T. A., Sherald, J. L., and Hurtt, S. S. 1986. Mulberry leaf scorch, new disease caused by a fastidious, xylem-inhabiting bacterium. *Plant Disease* 70:690-693.

Mulberry leaf scorch (MLS) symptoms appeared in the Washington, DC, area by mid-July. Leaves showed marginal desiccation with subsequent necrosis of desiccated tissues and development of a chlorotic halo separating necrotic from unaffected, green tissues. Leaves in all stages of symptom development occurred on the same branch. Severely affected leaves abscised prematurely, and limited branch dieback was observed. The disease occurred at epiphytotic levels (125 of 160 trees examined) in Alexandria, VA, and symptomatic trees were observed as far north as southern New York. A gram-negative, xylem-inhabiting bacterium morphologically similar to and serologically related to the Pierce's disease and elm leaf scorch bacteria was isolated from 19 of 22 MLS-affected plants by incubating wood chips in supplemented PW broth or PD-2 broth (5-7 days). Isolation of the MLS bacterium from petioles was achieved on semisolid PD-4 and supplemented PW media (18+ days) with bacteria extracted from petioles by centrifugation or crushing. Each of three isolates of the MLS bacterium was inoculated via root and stem inoculations into four mulberry seedlings. Six months after inoculation, the MLS bacterium was reisolated from 10 symptomatic and one symptomless seedling. No bacteria were isolated from four buffer-inoculated, symptomless control seedlings. Isolates of the MLS bacterium were successfully subcultured on nutrient agar and were morphologically and serologically indistinguishable from isolates grown on supplemented PW or PD-4 media. The MLS bacterium is the least fastidious member of the fastidious, xylem-inhabiting bacteria.

Midsummer leaf scorch is a common problem of deciduous trees in the mid-Atlantic and southern states. Leaf scorch disorders have been attributed commonly to a number of abiotic stresses, particularly drought (15). In 1980, a novel group of fastidious, xylem-inhabiting bacteria (FXIB) were associated with a leaf scorch disease of American elm, sycamore, and red oak (8). The associated bacteria were morphologically similar to and serologically related to the FXIB that causes almond leaf scorch and Pierce's disease (PD) of bunch grapes (8,14). Recently, Koch's postulates were satisfied for the bacterium cultured from leaf scorch-affected sycamores (14).

Paper 2698, Massachusetts Agricultural Experiment Station. This research supported in part from Experiment Station Project 501.

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Accepted for publication 18 February 1986.

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**Isolation and culture.** Stem samples were collected from 22 leaf scorch-affected and 19 symptomless red mulberries in Washington, DC, and Arlington, VA. Sampled plants ranged from seedling (1 m high) to mature trees. Presence of xylem-inhabiting bacteria in collected stem segments was determined by vacuum-flushing stem segments (0.5-1 × 8 cm) with sterile distilled water and examining the extracts by phase-contrast microscopy (6).

For bacterial isolation, stem segments (1 × 15-20 cm) were rinsed in 70% ethanol and flamed. Bark was removed aseptically, and two or three wood chips (0.5 × 1-2 cm) were excised and placed in 10 ml of broth formulations of PD-2 (3) or PW medium (2) supplemented with 0.85 g of (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 2 g of soluble potato starch, 1 g of L-histidine, and 25 mg of cyclohexamide per liter. Cultures were incubated in the dark at 28 C and examined daily for turbidity for up to 7 days, then at 4- to 7-day intervals.

Isolations from petioles were made on semisolid PD-4 (3) or supplemented PW plates with a petiole centrifugation technique or a crushed petiole technique modified from studies on PD and almond leaf scorch (3,4). Leaves were collected from symptomatic and symptomless trees; petioles were excised and disinfested for 2 min in a 0.5% sodium hypochlorite solution, then rinsed twice in sterile distilled water. Sap was extracted by placing petiole segments (1.0-1.5 cm) in 0.5 ml of a sterile, phosphate-buffered citrate magnesium solution (PBCM) (3) and centrifuged for 2 min at 15,600 × g in an Eppendorf Microfuge Model 5412. Bacteria were resuspended by vortexing and 0.05-ml aliquots were placed as droplets on semisolid media. The crushed petiole extraction consisted of disinfesting petioles as before and aseptically cutting them into segments. Segments were crushed aseptically with a pliers, and the droplet adhering to the petiole segment was blotted onto semisolid media. All isolates were subsequently maintained through biweekly transfer on semisolid PD-4.

Isolates were tested for their ability to grow on the following common microbiological media: nutrient agar, King's medium B agar, potato-dextrose agar,

During a survey of shade trees for the above-mentioned diseases, numerous red mulberries (*Morus rubra* L.) were observed that showed leaf scorch symptoms. In this paper, we describe 1) the symptoms and distribution of mulberry leaf scorch (MLS) in the eastern United States, 2) the isolation and culture of a bacterium from diseased trees, 3) the pathogenicity of three isolates of the bacterium, and 4) morphological similarities and serological relatedness of the MLS bacterium to other FXIB. Preliminary reports have been published (11,12).

### MATERIALS AND METHODS

#### Symptomatology and distribution.

Leaf scorch-affected mulberries in the Washington, DC, area were monitored monthly throughout the 1982 and 1983 growing seasons to follow symptom development. In August 1983, a natural population of red mulberries along 3 km of the George Washington Memorial Parkway in Alexandria, VA (a Washington, DC, suburb), was surveyed to determine the incidence of MLS. Also in August 1983, mulberries growing in both rural and urban roadsides and natural sites were surveyed from northern Virginia through the eastern mid-Atlantic states to the northern range of red mulberry in southern New England to determine disease distribution.

yeast-dextrose calcium carbonate agar (YDC), D-1 agar, and crystal violet polypectate agar (CVP). All cultures were incubated in the dark at 28 C. Isolates were examined by phase-contrast microscopy ( $\times 1,000$ ) for rod-shaped cells similar to other FXIB. Selected isolates were tested for Gram stain and catalase and oxidase reactions.

**Serology.** The serological relationship of bacteria isolated from mulberries to the PD and elm leaf scorch (ELS) bacteria was tested by indirect immunofluorescent antibody staining (IFAS) (7,8) with antisera prepared against whole-cell preparations of the PD and ELS bacteria (S. Kostka, unpublished). Cultures of the PD bacterium and the ELS bacterium were treated in the same manner. Heat-fixed bacteria reacted with rabbit preimmune serum before treatment with FITC-conjugated goat-antirabbit globulin served as controls.

**Pathogenicity test.** Three isolates obtained in September 1982 from scorch-affected mulberries in the Washington, DC, area were selected for pathogenicity tests. Bacterial colonies grown for 7-10 days on PD-4 were washed from the agar and suspended by vortexing in sterile PBCM. Cells were diluted to  $1 \times 10^8$ - $10^9$  cells per milliliter with a bacterial counting chamber (Hausser Scientific, Blue Bell, PA). In January 1983, 12 greenhouse-grown, potted mulberry seedlings approximately 50 cm tall were inoculated. Each isolate was inoculated into four seedlings with three inoculation techniques.

Seedlings were unpotted, and a major root was surface-disinfested with 70% ethanol, then severed with a sterile pruning shears. The severed root was immediately connected via a segment of latex tubing to a 1-ml pipette containing 1 ml of bacterial inoculum. Seedlings were

reotted with a pipette connected to the root and watered. After reotting, a surface-disinfested stem about 0.4-0.5 cm in diameter was excised from near the apical point of the plant and a latex tubing reservoir attached. Approximately 0.5 ml of inoculum was placed in the reservoir and taken into the stem by the receding embolism. In addition, two sites along the stem were surface-disinfested with 70% ethanol and two incisions made to the xylem with a sterile scalpel. Inoculum (0.02-0.03 ml) was placed in each of the two stem incisions with a hypodermic syringe. Four control seedlings were inoculated with the same techniques. Control inoculum consisted of sterile PBCM washings of uninoculated PD-4 agar plates. Inoculum uptake was rapid via all inoculation routes: within minutes for the xylem incision and cut stem techniques and within 24 hr via the severed root. Plants were maintained under greenhouse conditions and watered sparingly through April 1983.

**Electron microscopy.** Symptomatic and symptomless leaves were collected from an inoculated mulberry seedling for ultrastructural studies. Primary and secondary veins were excised from nonnecrotic tissue adjacent to scorched tissues and from analogous areas in symptomless leaves. Samples were prepared and examined as described previously (8).

In conjunction with isolation studies, negative stain preparations were made of cultured bacteria (14).

## RESULTS

**Symptomatology and distribution.** Symptoms of MLS appeared in mid-July (1982 and 1983) in the Washington, DC, area as a marginal leaf desiccation and curl. Although a major portion of the

entire leaf lamina may have been desiccated, only slight discoloration of the tissues occurred initially (Fig. 1). Once tissues became desiccated, the lamina took on a water-soaked appearance and necrosis developed, first in the desiccated tissues adjacent to the unaffected tissues, then outward in the leaf lamina to the margin.

Advanced symptoms were characterized by a marginal, undulating leaf necrosis bordered by a distinctive chlorotic halo (Fig. 2). Symptoms progressed apically, and leaves in all stages of symptom development were observed on the same branch. Severely scorched leaves abscised prematurely, leaving tufts of symptomless leaves at the branch apex. Branch die-back was observed in affected trees, but no mortality of individual trees was noted.

One hundred sixty mulberries ranging in size from 2-m-tall saplings to mature trees were surveyed along 3 km of the George Washington Memorial Parkway in Alexandria, VA. Mulberries were common understory plants in sites containing leaf scorch-affected sycamores and red oaks. Of this population, 125 trees (78%) expressed characteristic MLS symptoms. Symptoms were severe in all affected plants.

The disease ranged from northern Virginia and the District of Columbia, east throughout the Delmarva Peninsula, and north through Maryland, Delaware, eastern Pennsylvania, and New Jersey to New York City and New Rochelle, NY (Fig. 3). The disease was widespread throughout much of the northeastern range of red mulberry and occurred in both urban and nonurban trees. The disease was not apparent in escape populations of red mulberry in the southern New England states.

**Isolation and culture.** Vacuum-flushing of stem segments proved inadequate for determining the presence of xylem-inhabiting bacteria in stem segments collected from scorching trees. Bacteria were difficult to identify in extracts examined by phase-contrast microscopy because of excessive crystalline, cellular debris. Bacteria were identified by repeated examination of

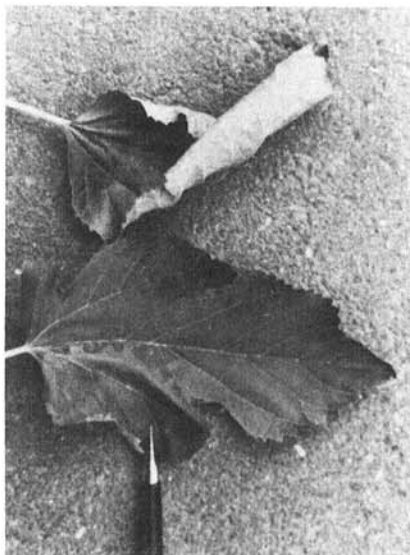


Fig. 1. Initial symptoms of mulberry leaf scorch. Note marginal tissue desiccation of leaf on right (indicated by the pointer) and the severe leaf curl in the leaf on the left.

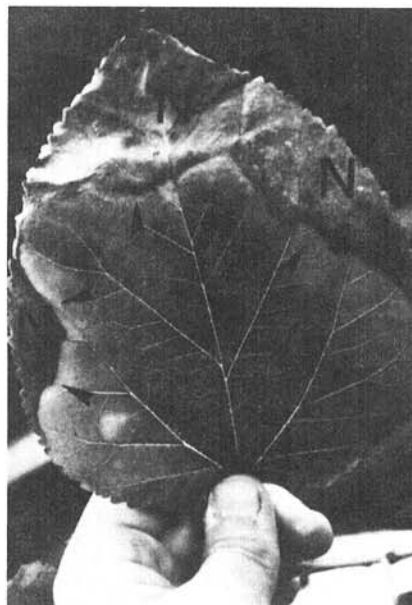


Fig. 2. Marginal necrosis (N) and bounding chlorotic halo (arrows) of advanced symptoms of mulberry leaf scorch.



Fig. 3. Distribution of mulberry leaf scorch in the northeastern coastal states.

extracts from nine of 12 samples. Because of the difficulty in observing bacteria in the extracts, the vacuum-infiltration technique was discontinued.

Bacteria were readily isolated from stem segments of affected trees by incubating wood chips in supplemented PW broth. Turbidity commonly developed in cultures within 5–7 days. Bacteria were isolated from 19 of 22 leaf scorch-affected mulberries and from 3 of 19 symptomless trees in the Washington, DC, area. The MLS bacterium was cultured from stems of affected trees within 7 days in simultaneously inoculated broth cultures of supplemented PW and PD-2 media.

Bacterial growth was detected in crushed or centrifuged petiole isolations after 18 days of incubation on both PD-4 and supplemented PW agar. Colonies on PD-4 agar were white, convex, with entire margins, and about 0.5–0.8 mm in diameter. Darkened areas were observed at one or both ends of the organism under phase-contrast microscopy. On supplemented PW agar, colonies were also 0.5–0.8 mm in diameter but were buff-white and more appressed to the medium. Bacteria stained gram-negative and were catalase-positive and oxidase-negative. Bacterial cells from agar and broth culture measured 0.5  $\mu\text{m}$  wide by 1–3  $\mu\text{m}$  long using phase-contrast microscopy and an eyepiece micrometer. Growth was not observed on potato-dextrose agar, King's medium B agar, D-1 agar, YDC agar, or CVP agar. Colonies developed on nutrient agar after 2 wk of incubation, and cells remained viable through three serial transfers. Colony development time on PD-4 agar was normally 5 days. Isolates subcultured on PD-4 from nutrient agar cultures produced bacterial colonies and cells indistinguishable from those in the original PD-4 cultures. The bacterium grown on nutrient agar or PD-4 was gram-negative, catalase-positive, and oxidase-negative.

Isolations from leaf scorch-affected mulberries in all localities where the disease was observed (Fig. 3) consistently

**Table 1.** Isolation of fastidious, xylem-inhabiting bacteria from leaf scorch-affected mulberries in localities surveyed outside the Washington, DC, area in 1982 and 1983

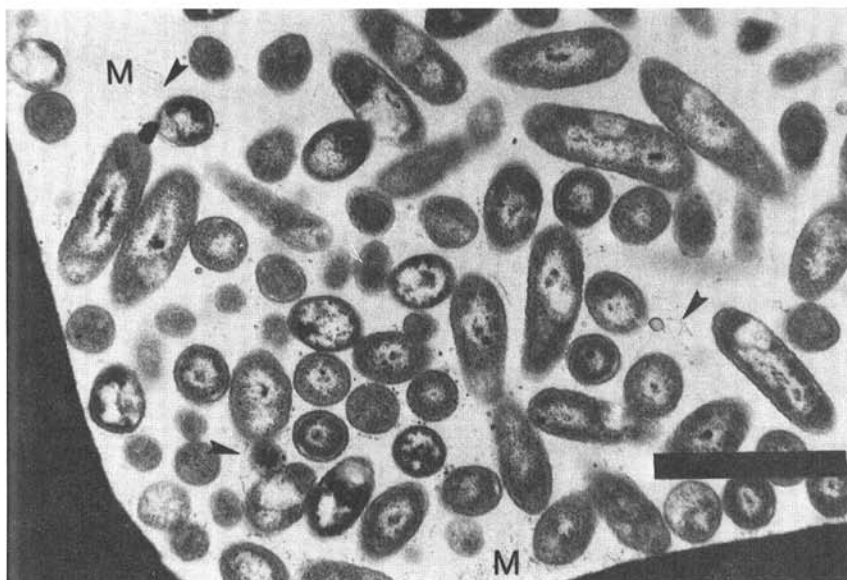
| Locality         | Ratio<br>(no. isolations/<br>no. trees sampled) |
|------------------|---|
| Baltimore, MD    | 1/1   |
| Kent Island, MD  | 1/1   |
| Ocean City, MD   | 1/1   |
| Elkton, MD       | 2/2   |
| Lewes, DE        | 2/2   |
| Dover, DE        | 4/4   |
| New Castle, DE   | 1/1   |
| Wilmington, DE   | 3/3   |
| Berwyn, PA       | 2/2   |
| Masonville, NJ   | 1/1   |
| Hedding, NJ      | 2/2   |
| New York, NY     | 2/2   |
| New Rochelle, NY | 3/3   |
| Total            | 25/25   |

yielded FXIB (Table 1). In addition to isolation of the bacterium in the metropolitan Washington, DC, area (northern Virginia and Maryland suburbs), the bacterium was also isolated from 25 MLS-affected trees sampled in Maryland, Delaware, Pennsylvania, New Jersey, and New York (Fig. 3, Table 1).

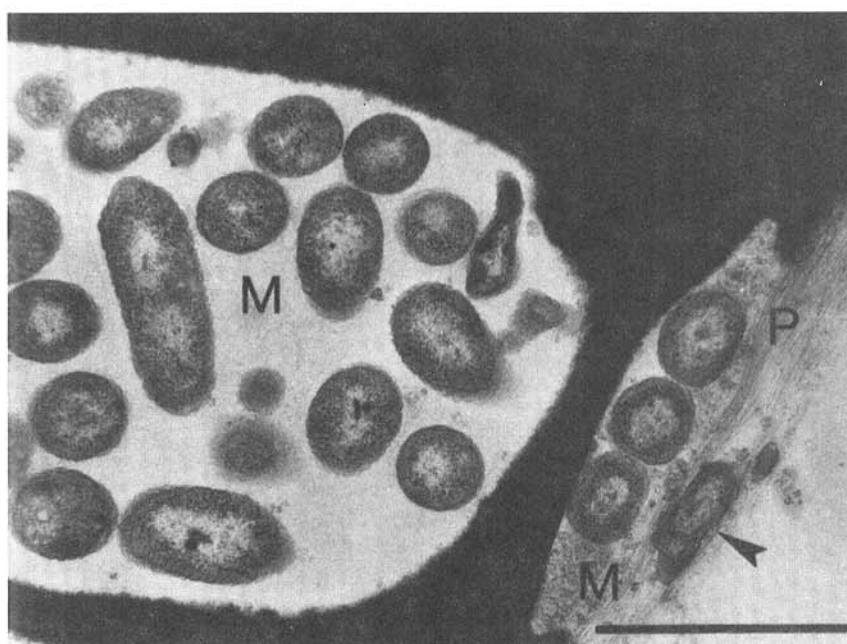
**Serology.** Bacterial isolates fluoresced strongly when reacted with antisera to the ELS or PD bacteria in the indirect IFAS test. Similarly, the ELS and PD bacteria reacted strongly with either antiserum. No reaction was obtained when isolates were reacted with preimmune sera. Isolates from mulberry that were subcultured on nutrient agar also

fluoresced in indirect IFAS when reacted with the immune bacterial antisera.

**Pathogenicity tests.** Characteristic MLS symptoms developed in three of 12 inoculated seedlings 3 mo after inoculation (Table 2). Isolations were made from the three symptomatic seedlings, and the MLS bacterium was recovered from each. The reisolated organisms fluoresced in indirect IFAS tests using antisera to the ELS and PD bacteria. Symptoms developed in 10 of the 12 inoculated seedlings 6 mo after inoculation (Table 2). The MLS bacterium was reisolated from the 10 symptomatic seedlings and from one symptomless bacteria-inoculated seedling. No bacteria were recovered



**Fig. 4.** Transmission electron micrograph of transverse and longitudinal sections of bacteria in a vessel of a symptomatic leaf from an inoculated plant. Bacteria appear embedded in a lightly staining matrix (M). Nodulated tubular structures surround the bacteria and extend into the matrix (arrows). Scale bar = 1  $\mu\text{m}$ .



**Fig. 5.** Electron micrograph of the cross section of a xylem vessel with bacteria lodged in the pit (P) and appressed, possibly degenerative, bacterial cells (arrows). Note variations in matrix staining (M). Scale bar = 1  $\mu\text{m}$ .

from four symptomless, buffer-inoculated, control seedlings.

**Electron microscopy.** The MLS bacterium was observed in the xylem of symptomatic leaves collected from an inoculated mulberry seedling (Figs. 4 and 5). Cells were 0.3–0.4  $\mu\text{m}$  wide by 1–2  $\mu\text{m}$  long and had a rippled cell wall. Bacteria were embedded in a lightly staining matrix (Figs. 4 and 5) that contained nodulated strands surrounding the bacteria (Fig. 4). Variations in matrix staining occurred between adjacent vessels (Fig. 5). Appressed, more densely staining cells, possibly degenerative forms of the bacterium, were observed (Fig. 5). Cells lodged in pits were compressed and surrounded by granular material and fibrous strands.

Negatively stained bacteria from cultures showed the rippled cell wall topography characteristic of FXIB. Cultured bacteria were morphologically similar to cells found in xylem vessels (Fig. 6). No morphological dissimilarity was noted between isolates grown on PD-4 or nutrient agar.

## DISCUSSION

MLS is a newly reported disease caused by an FXIB. The bacterium is morphologically similar to and serologically related to two other FXIB; the ELS bacterium and the PD bacterium. Although the MLS bacterium was isolated in/on supplemented PW, PD-2, or PD-4 media with several isolation techniques, growth was most rapid when wood chips were incubated in broth (5–7 days). When bacteria from crushed petioles or centrifuged petioles were used as inoculum on semisolid supplemented PW or PD-4 media, colonies were evident at 18 days. The wood chip/broth isolation technique is rapid and suitable for making isolations from large numbers of samples. Contamination of cultures by other wood-inhabiting or epiphytic bacteria was not a problem. Fewer than

**Table 2.** Symptom development and recovery of bacteria from mulberry seedlings inoculated with isolates of the mulberry leaf scorch-associated bacterium

| Treatment      | No. trees treated | April 1983        |                    | July 1983         |                    |
|----------------|-------------------|-------------------|--------------------|-------------------|--------------------|
|                |                   | Symptomatic trees | Bacterial recovery | Symptomatic trees | Bacterial recovery |
| Isolate MS1    | 4                 | 1                 | 1/1 <sup>a</sup>   | 4                 | 4/4                |
| Isolate M3     | 4                 | 2                 | 2/2                | 4                 | 4/4                |
| Isolate M4     | 4                 | 0                 | 0/0                | 2                 | 3/4                |
| Sterile buffer | 4                 | 0                 | 0/0                | 0                 | 0/4                |

<sup>a</sup>Number of isolations/number of seedlings sampled.

20% of all isolations became contaminated with other bacteria and were normally discarded after 24 hr.

On the basis of growth studies, the MLS bacterium is less fastidious than the sycamore leaf scorch bacterium (14), the oak leaf scorch bacterium (1), or the ELS bacterium (10; S. Kostka, *unpublished*) but is comparable to the PD bacterium (5), which to date is the least fastidious of this group of bacteria. The MLS bacterium is unique among the other cultured FXIB because of its ability to grow on a common microbiological medium. Isolates grown on nutrient agar or semisolid PD-4 are indistinguishable morphologically, biochemically, and serologically. Growth on nutrient agar indicated that the MLS bacterium is the least fastidious FXIB yet discovered.

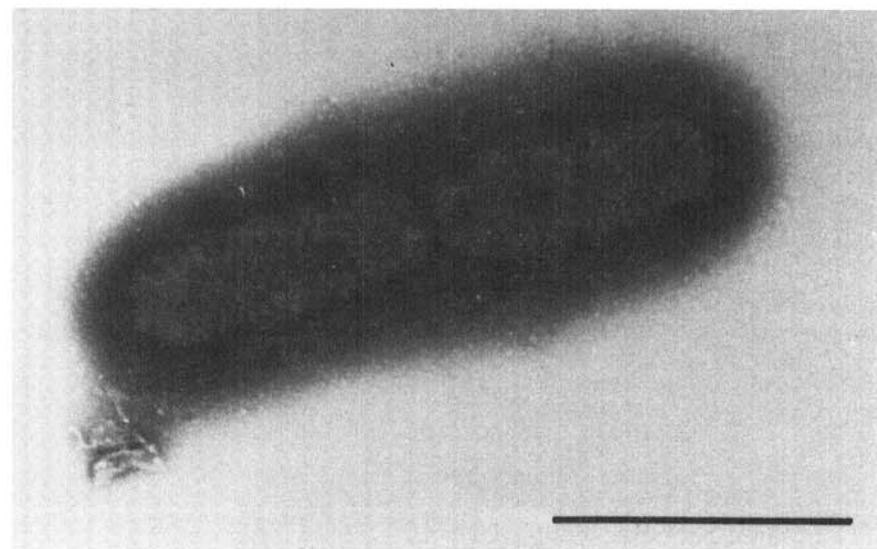
In 1983, MLS occurred at epiphytic levels in northern Virginia and the District of Columbia. Disease incidence was high in all localities where the disease was observed. In addition to the northern range, similar symptoms were observed in New Orleans and Baton Rouge, LA, though isolations of the bacterium were not attempted (S. Kostka, *unpublished*).

Bacteria serologically related to the ELS and PD bacteria were consistently isolated from symptomatic trees throughout the range of the disease. The recovery of bacteria from three symptomless trees in northern Virginia and the District of Columbia may be attributable to presymptomatic infections.

This study shows that MLS is the most geographically widespread disease caused by an FXIB in the eastern United States. Further, this is the northernmost report of a disease caused by a member of this group of phytopathogenic bacteria. Leaf scorch symptoms caused by FXIB may be due to bacterial restrictions of water translocation (9) and/or pathogen-produced toxins (13). We do not know whether one or both of these factors are involved in MLS.

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**Fig. 6.** Electron micrograph of negatively stained mulberry leaf scorch bacteria grown on supplemented PW agar showing typical rippled wall topography. Scale bar = 1  $\mu\text{m}$ .