Bacterial Leaf Scorch of Northern Red Oak: Isolation, Cultivation, and Pathogenicity of a Xylem-Limited Bacterium

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ABSTRACT

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A xylem-limited bacterium associated with scorch in northern red oak (*Quercus rubra*) was consistently isolated and cultured on CS20 medium from August 1982 through January 1983 and from August 1983 through December 1983. The colonies were opalescent white and reached 0.04–0.07 mm in diameter in 2 wk. All triple-cloned isolates failed to grow on nutrient agar, 523, and YDC medium. No colonies developed from sap of symptomless trees. The cells of the leaf scorch bacterium were rod-shaped, measured 0.4–0.75 × 1.05–3.5 μm, and had rippled walls. The cells appeared to divide by binary fission. In double-diffusion serological tests, the bacterium from oak was partially related to the Pierce's disease bacterium but was not related to the phony peach bacterium. Fifty percent of red oak seedlings artificially inoculated with triple-cloned isolates from scorched oak developed scorch, and the bacterium was reisolated from them. Control seedlings inoculated with phosphate-buffered saline remained symptomless, and their xylem yielded no bacteria on CS20 medium.

Leaf scorch of northern red oak (*Quercus rubra* L.) has been observed at the Georgia Experiment Station, University of Georgia, Experiment, for the past 10 yr. Symptoms develop as marginal chlorosis on leaves at the edge of the canopy in early July every year (Fig. 1A). By early August, leaves throughout the crown are affected (Fig. 1B). The chlorotic tissue soon becomes necrotic, and at times a reddish brown band 2–3 cm wide separates healthy and necrotic areas. Tissue remains intact, and leaves remain attached to stems. Dieback of twigs and branches occurs (Fig. 1C), and epicormic sprouts may develop along surviving branches. Hearon et al (8) observed xylem-limited bacteria (XLB) in scorched oak leaves collected in Washington, DC. Attempts to culture the oak bacterium on nutrient agar and JD3 medium, however, were reportedly unsuccessful. Using a modified PW medium, Kostka et al (10) was able to isolate the bacteria from oaks showing leaf scorch and decline symptoms collected from Virginia, Washington, DC, Maryland, Delaware, New Jersey, Pennsylvania, and New York. Bacteria isolated from affected trees were immunofluorescent-positive against antisera to the elm leaf scorch bacterium and Pierce's disease bacterium. The bacteria appeared to play a role in the cause of leaf scorch of oak, but the proof of pathogenicity of the isolated bacterium to fulfill Koch's postulates is still lacking. Therefore, we report here the successful isolation, cultivation, and pathogenicity of an oak leaf scorch XLB and its serological relationship to other phytopathogenic XLB.

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MATERIALS AND METHODS

Isolation of bacteria. CS20 medium (2) was used to isolate the oak leaf scorch bacterium. All ingredients were added and dissolved in the order given in Table 1, then autoclaved for 18 min at 6.8 kg. CS20 liquid medium was prepared as the above without agar. The final pH was 6.6–6.7. CS20 medium differs in composition and preparation from PW (3), PD2 (5), and BCYE (17) media (Table 1), which were formulated primarily for the isolation and culture of phony peach and periwinkle wilt bacteria, Pierce's disease bacterium, and phony peach and plum leaf scald bacteria, respectively. All ingredients for CS20 medium were autoclavable, whereas glutamine and bovine serum albumin fraction V in PW medium, bovine serum albumin fraction V in PD2 medium, and L-cysteine hydrochloride and ferric pyrophosphate in BCYE medium were sterilized through a 0.2-μm membrane filter. In this study, PW, PD2, and BCYE media were not used for isolation of the

Table 1. Composition of the CS20, PW, PD2, and BCYE agar culture media for isolation of *Xylella fastidiosa*

	Concentration (g/L)			
Constituents	**CS20**[a]	**PW**	**PD2**	**BCYE**
Soy peptone (Scott Laboratories)	2.0	...[b]	...	...
Bacto tryptone (Difco)	2.0	...	4.0	...
Phytone peptone (BBL)	...	4.0	...	...
Trypticase peptone (BBL)	...	1.2	...	...
Soytone (Difco) or phytone (BBL)	...	...	2.0	...
Yeast extract	...	...	...	10.0
Hemin chloride (Sigma)	0.005	0.01	0.01	...
Dextrose	1.0	...	...	...
ACES Buffer (Sigma)	...	...	...	10.0
$(NH_4)_2PO_4$	0.8	...	...	...
KH_2PO_4	1.0	1.0	1.0	...
K_2HPO_4	...	1.2	1.5	...
$MgSO_4 \cdot 7H_2O$	0.4	0.4	1.0	...
Ferric pyrophosphate, soluble	...	...	...	0.25
L-glutamine	6.0	...	...	...
L-histidine·HCl	1.0	...	...	...
L-cysteine $HCl \cdot H_2O$	...	...	...	0.40
Glutamine	...	4.0	...	...
Trisodium citrate	...	...	1.0	...
Disodium succinate	...	...	1.0	...
Potato starch, soluble (J. T. Baker)	2.0	...	...	...
Activated charcoal (Norit SG)	...	...	...	2.00
Bovine serum albumin fraction V (Sigma)	...	6.0	2.0	...
Bacto agar (Difco)	12.0	...	15.0	17.0
Granulated agar (BBL)	...	12.0	...	...
Phenol red	0.01	0.02	...	...

[a] Concentration for CS20 medium was g/1,100 ml of deionized distilled water.
[b] Not included.

oak leaf scorch bacterium.

Nutrient agar, yeast extract-dextrose-calcium carbonate (YDC), and 523 media were used to determine if growth of OLS isolates would occur. In 1 L of water, YDC agar medium (18) contained 10 g of yeast extract, 20 g of dextrose, 20 g of calcium carbonate, and 15 g of agar, whereas 523 agar medium (9) contained 10 g of sucrose, 8 g of casein acid hydrolysate, 4 g of yeast extract, 2 g of K_2HPO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, and 15 g of agar.

Every month from August 1982 through January 1984, 30–40 first- to second-year twig segments 0.6–0.9 cm in diameter and 5–7.6 cm long were randomly sampled from each of two scorched oak trees, placed in 95% ethanol for 3 min, and flamed. A surface-sterilized segment was then cut from the center of each twig with alcohol-flamed pruners, and the freshly cut end was squeezed with pliers. The sap that appeared was immediately withdrawn with a sterile capillary Pasteur pipet and placed on CS20 agar in a petri plate. Plates were enclosed in plastic bags and incubated at 30 C. The same number of twigs was collected from two symptomless trees in October and November 1982, and the isolation procedures described above were followed. Colonies that developed were observed with a dissecting microscope (50×) weekly for 1 mo. Isolation rates were expressed as the numbers of positive isolations divided by the total number of isolations attempted.

Table 2. Pathogenicity tests of five bacterial isolates associated with oak leaf scorch

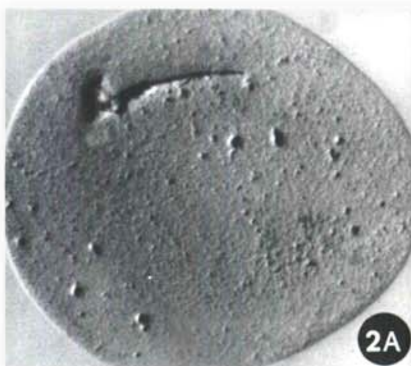
	Number of seedlings			
Isolate	Injected[a]	Survived	Scorched[b]	Positive in bacterial reisolation
OLS2	5	4	0	0
OLSIIF9	5	4	3	3
OLSIB28	4	3	1	1
OLS4	5	4	2	2
OLS14	8	7	5	5
PBS[c] (control)	3	2	0	0

[a] Northern red oak seedlings were injected on 16 March 1984.
[b] Scorch developed from mid-September to mid-October 1984.
[c] Phosphate-buffered (0.1 M) saline.

Cellular morphology and ultrastructure. Log-phase cells of two isolates (OLS4 and OLS14) were used for electron microscopic observation. For negative staining, a drop of culture (10^8–10^9 cells/ml) from each isolate was pipetted on a Formvar-coated copper specimen grid. After 5 min, the excess culture broth was removed with filter paper, and a drop of 5% aqueous ammonium molybdate containing 0.1% bovine serum albumin fraction V was placed on the coated grid for 1 min, then removed with filter paper.

For ultrathin sections, both isolates were grown in CS20 broth for 7–10 days. Cells were centrifuged at 15,600 *g* for 15 min, and the pellet was then covered with a few drops of melted 2% Bacto agar. The solidified agar, with the pelleted cells attached, was removed from the centrifuge tube and fixed with 2% glutaraldehyde and post-fixed with 2% osmium tetroxide for 45 min each. It was then stained with 2% uranyl acetate in 10% ethanol for 5 min before dehydration in an ethanol series (50, 75, 95, and 100% for 5 min

Fig. 1. Oak leaf scorch symptoms on **(A, B, and C)** naturally infected trees and **(D)** inoculated seedling. **(A, B, and D)** Leaf symptoms; **(C)** dieback and epicormic sprouting along the large branches.



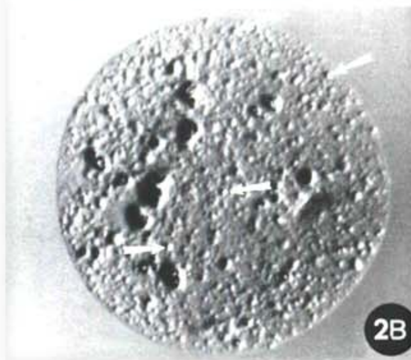


Fig. 2. Isolation of oak leaf scorch bacteria on CS20 agar medium: **(A)** With sap from symptomless tree, no colonies developed. **(B)** With sap from a scorched tree, colonies (arrows) developed after 10 days of incubation.

each). The agar pellet was embedded in Spurr mixture (15) at 68 C overnight after two 30-min propylene oxide/Spurr mixture (1:1) treatments. Sample blocks were sectioned with a diamond knife and ultramicrotome (Sorvall MT-1), and sections were stained with lead citrate and examined with a Zeiss EM-10A transmission electron microscope.

Pathogenicity tests. Red oak seedlings (60–80 cm tall) were inoculated with five isolates on 16 March 1984, using the method of Sherald et al (14). Briefly, inoculation was performed by removing 30 seedlings from pots, severing one large root of each seedling, and connecting the severed root with a short piece of Tygon tubing to a 10-ml pipet containing 10 ml of bacterial suspension ($1-3 \times 10^8$ cells/ml). Seedlings were repotted with the pipet reservoirs connected to the roots. Most of the inoculum was absorbed in 3–4 days. Plants were maintained in a shade house, watered regularly, and observed biweekly for symptoms. Bacteria were reisolated from inoculated seedlings by squeezing sap from petioles onto CS20 agar.

Serology. Indirect immunofluorescence of OLS and *Xanthomonas campestris* pv. *campestris* B24 cells was performed using immunoglobulin G (IgG) produced in response to crude ribosomes of the phony peach bacterium (PP-1), IgG to membranes of Pierce's disease bacterium (PD-2), or ribosomes to *X. c.* pv. *campestris* B24 using fluorescein isothiocyanate (FITC)-conjugated anti-rabbit goat IgG (GIBCO, Grand Island, NY). Cultured cells were obtained from CS20 agar by removing colonies with a loop and suspending them in phosphate-buffered saline (PBS). The serum IgG was separated by column chromatography using Sephadex A-500 (6). A block test (7) was used to determine the optimum dilutions of IgG and antirabbit IgG. Staining was performed as described (13), and slides were examined with a Zeiss standard incident-light fluorescence microscope. Bacteria were observed under a Planapo 100× oil-immersion objective. *X. c.* pv. *campestris* B24 used for the indirect immunofluorescence test was originally isolated from cabbage and grown in 523 medium.

The membrane protein complex (MPC) of isolates OLS4, OLS14, PP-1, and PD-2 was extracted individually in 0.2 M LiCl at 45 C for 2 hr with constant shaking (1). The extracted cells were filtered through cheesecloth, and the filtrate was then centrifuged twice to pellet the MPC. The MPC was used as an antigen to inject New Zealand white male rabbits. Three graded injections (200, 400, and 600 μg of protein, respectively) were performed at 10-day intervals. Rabbits were bled from the marginal ear vein 1 and 2 wk after the last injection. Ouchterlony double-diffusion tests of the MPC were conducted in a 0.75% agarose medium containing 1% trypan blue.

Table 3. Indirect fluorescent-antibody staining of oak leaf scorch (OLS) cells from agar culture

		Immunoglobulin G to:		
Cells	Month isolated	PP-1 crude ribosomes	PD-2 membranes	*Xanthomonas campestris* pv. *campestris* B24 ribosomes
PP-1		+++[a]	+++	–
PD-2		++	++++	–
OLS12	August 1982	++	++++	–
OLS2	October 1982	++	++++	–
OLS4	October 1982	++	+++	–
OLS14	November 1982	++	+++	–
OLS15	November 1982	++	+++	–
OLSI(B)-3	December 1982	++	+++	–
OLSII(F)-17	December 1982	++	+++	–
X. c. pv. *campestris* B24		–	–	+++

[a] – = No fluorescence (cells not visible), ++ = green fluorescence, +++ and ++++ = bright green fluorescence.

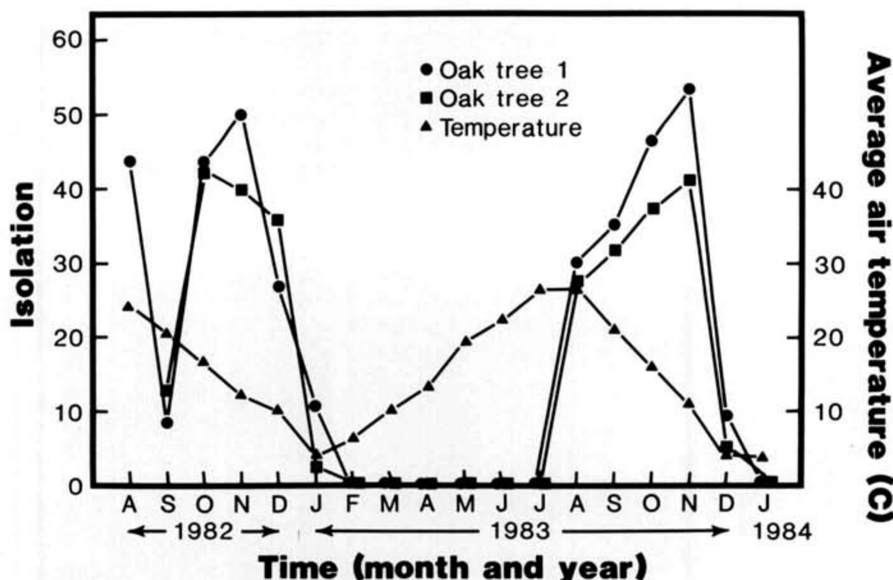


Fig. 3. Percentage of twig samples from which oak leaf scorch bacteria were isolated on CS20 agar medium from August 1982 through January 1984, with air temperatures during same period.

RESULTS

Isolation and cultivation of OLS bacteria. No colonies developed from sap of symptomless trees (Fig. 2A), but colonies of the bacteria were visible (50×) after 10–14 days of incubation on CS20 (Fig. 2B). The colonies were opalescent white and reached 0.04–0.07 mm in diameter in 2 wk. Twelve isolates derived from single cells three times in succession did not grow on nutrient agar, 523, or YDC medium. The OLS bacterium was isolated from twigs from August 1982 through January 1983 and from August 1983 through December 1983 (Fig. 3).

Cellular morphology and ultrastructure. Cells of OLS bacteria were rod-shaped, 0.4–0.75 μm wide and 1.05–3.5 μm long, and appeared to divide by binary fission. The cell walls were rippled (Fig. 4).

Pathogenicity tests. Five of 27 seedlings inoculated with bacteria and one of three control seedlings that received only phosphate-buffered saline died without developing leaf scorch (Table 2). Reisolation of XLB from the dead seedlings was not attempted because death was attributed to lack of water rather than to inoculum. Eleven of the remaining 22 inoculated seedlings developed scorch about 6 mo after inoculation (Fig. 1D). Bacteria were cultured from all 11 scorched seedlings, whereas none was cultured from 13 symptomless seedlings, including two PBS-injected trees.

Serology. Cells from seven isolates of OLS bacteria gave a strong fluorescence with IgG produced in response to crude ribosomes of PP-1 or with IgG produced in response to membranes of PD-2 (Table 3). Cells of OLS bacteria failed to

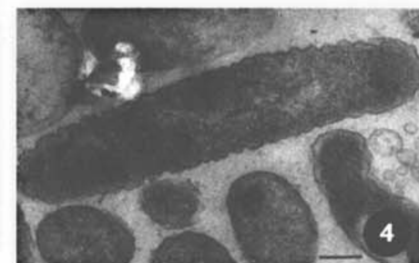


Fig. 4. Electron photomicrograph of oak leaf scorch bacteria showing ultrathin sectioned cells with rippled walls. Scale bar = 0.2 μm.

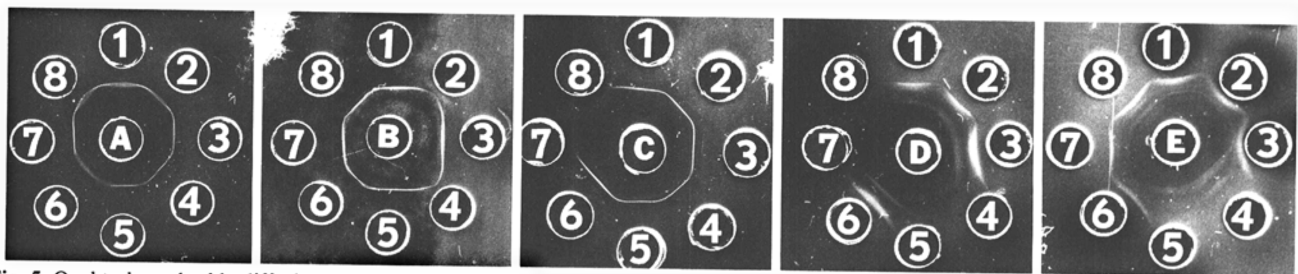


Fig. 5. Ouchterlony double-diffusion patterns of membrane protein complexes (MPCs) of different strains of xylem-limited bacteria. Center wells contained antisera to MPCs of strains: **(A and C)** OLS14, **(B)** OLS4, **(D)** PD-2, and **(E)** PP-1. Outer wells contained MPCs of strains: **(A)** OLS14 in 1, 3, 5, and 7, OLS4 in 2, OLS12 in 4, OLS15 in 6, and OLSIIF9 in 8; **(B)** OLS4 in 1, 3, 5, and 7, OLS12 in 2, OLS14 in 4, OLS15 in 6, and OLSIIF9 in 8; **(C)** OLS14 in 1, 2, 3, and 6, PD-2 in 4 and 5, and PP-1 in 7 and 8; **(D)** PD-2 in 1, 2, 3, and 6, OLS4 in 4 and 5, and OLS14 in 7 and 8; and **(E)** PP-1 in 1, 2, 3, 6, 7, and 8 and OLS4 in 4 and 5.

fluoresce when reacted with IgG produced in response to ribosomes of *X. c.* pv. *campestris* B24; reciprocal tests were also negative (Table 3).

Formation of a precipitating band indicated that the OLS bacterial isolates were serologically homogenous (Fig. 5A and B). When antiserum against MPC of OLS14 was used to react with PD-2 and PP-1, an identity reaction occurred between OLS14 and PD-2, whereas no reaction occurred between OLS14 and PP-1 (Fig. 5C). When antiserum against the MPC of PD-2 was used, no reaction was observed between PD-2 and OLS4 or OLS14 (Fig. 5D). When antiserum against the MPC of PP-1 was used, no reaction occurred between PP-1 and OLS4 (Fig. 5E).

DISCUSSION

Several strains of XLB associated with the leaf scorch of oak were isolated from diseased trees and maintained in axenic culture on CS20 medium. These strains all shared the following features with other XLB: 1) requirement for a specific enriched medium such as CS20, 2) slow growth rate, and 3) rippled cell walls. Based on the consistent isolation from diseased trees from August 1982 through January 1983 and from August 1983 through December 1983, the pathogenicity test, and the reisolation of the bacterium from inoculated seedlings, we concluded that this leaf scorch disease is caused by a xylem-limited bacterium. And this bacterium could well belong to one of the strains of *Xylella fastidiosa* as reported by Wells et al (16) even though none of our strains was included in their study. In addition to sharing the three features mentioned above with other strains of *X. fastidiosa*, the cells of OLS strains were single (occasionally filamentous), nonmotile, aflagellate straight rods. The average cell size of our OLS strains is $0.4–0.75 \times 1.05–3.5\ \mu m$, which appears to be larger than that of the *X. fastidiosa* strains ($0.25–0.35 \times 0.9–3.5\ \mu m$) as reported by Wells et al (16).

The OLS cell ultrastructure in vivo was similar to that reported by Hearon et al (8) in which the bacterium measured $0.3–0.4 \times 1–2\ \mu m$. Often, small, irregular to spindle-shaped bodies with rippled walls and dense contents defined by Hearon et al (8) were observed in veins from diseased oaks. Mollenhauer and Hopkins (12) described similar bodies as compressed and degenerating forms of the Pierce's disease bacterium.

Successful isolations from naturally infected trees occurred only between August and December or January, with the highest percentage of recovery in November. No bacterium was isolated from February to July. Scorch usually developed in early July before the bacterium was recovered from the twigs. From August to November, we speculate that the bacterial population increases and moves acropetally, resulting in severe xylem blockage that in turn enhances the scorch symptom. From November to January, the bacterial population may decrease because of the cold temperature. Bacterial populations may gradually increase in late January and move from underground tissues to aboveground tissues, yet the bacterium could not be isolated until August. Further investigation is necessary to elucidate this hypothesis.

Even though it shares several features with other XLB, the OLS bacterium differed serologically from the single strains of Pierce's disease and phony peach bacteria that were tested. Our results indicate that the OLS bacterium was partially related to Pierce's disease bacterium but not related to phony peach bacterium. Whether the OLS bacterium is related to other XLB is unknown. The MPCs extracted from the OLS bacterium and from other XLB appear to be useful in differentiating among them.

Since the discovery in 1978 that XLB causes Pierce's disease of grapevines (4), XLB have been found in several other tree disease syndromes, e.g., leaf scorch of almond (4,7), elm (8), sycamore (14), and mulberry (11). Therefore, it may be worthwhile to reinvestigate the possible role of XLB in association with other tree scorch diseases, especially with the isolation technique we described in this report and the simple enriched medium formulated for their growth.

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