Leersia hexandra, an Alternative Host for *Xanthomonas campestris* pv. *oryzae* in Texas

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ABSTRACT

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We surveyed weeds common in canals adjacent to rice (*Oryza sativa*) fields with bacterial leaf blight and found that the perennial weed *Leersia hexandra* (clubhead cutgrass) was an alternative host for *Xanthomonas campestris* pv. *oryzae*. Strains of *X. c. oryzae* isolated from symptomless *L. hexandra* caused bacterial leaf blight symptoms in rice. In artificially inoculated *L. hexandra*, the pathogen multiplied without evidence of disease.

Xanthomonas campestris pv. *oryzae* (Ishiyama) Dye has been identified as the causal agent of bacterial leaf blight of rice (*Oryza sativa* L.) in Texas and Louisiana (13). The pathogen has been found in most rice-growing areas of the world, including Asia (19), Australia (1), West Africa (20), and South and Central America (15,18). The source of primary inoculum in the United States is unknown, but there is evidence from a previous study (13) that seed was not the source. Additionally, the pathogen cannot survive for long periods of time in infected tissue (10,19), and infected plant debris would be an unlikely source of the pathogen because of cultural practices in the United States. In Asia, perennial weeds are considered as one of the most important sources of primary inoculum (19). In Japan, the weeds most frequently implicated are *Leersia oryzoides* (L.) Sw. and *L. sayanuka* Ohwi (8,11,25). At least five species of *Leersia* have been identified in the rice-growing areas of Texas (9).

The objectives of the present study were to develop a medium that would help isolate *X. c. oryzae* from plant tissue, to determine if an alternative host for *X. c. oryzae* existed in Texas, and to determine if strains of *X. c. oryzae* obtained from alternative host(s) could cause symptoms of bacterial leaf blight.

MATERIALS AND METHODS

Isolation and culture maintenance. Symptomless weeds growing on the edge of rice fields and on the banks of canals adjacent to rice fields in Wharton County, Texas, were collected. Some of the fields had symptoms of bacterial leaf blight and others appeared to be free from disease. Rice plants were also sampled from the same fields. Grasses collected included two perennials, *L. hexandra* Sw. (clubhead cutgrass) and

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Rhynchospora corniculata (Lam.) Gray (horned rush), and two annuals, *Leptochola fascicularis* (Lam.) Gray (bearded sprangletop) and *Brachiaria platyphylla* (Griseb.) Nash (broadleaf signalgrass). Samples of leaf tissue (0.1–0.2 g) were dipped in 0.1% (v/v) sodium hypochlorite solution for 2 min, rinsed twice for 1 min each in sterile water, then macerated in 5 ml of potassium phosphate buffer (0.0125 M, pH 7.0). The macerated tissue samples were diluted in buffer and plated onto MXO medium. MXO medium is a modification of MXP medium developed by Claflin et al (3) in which rice starch (8 g/L) and nystatin (300 units per milliliter) are substituted for potato starch and chlorothalonil (Daconil 2787), respectively. The plates were incubated for 3–4 days at 28 C and examined daily for colony formation. Isolated colonies were transferred to nutrient broth-yeast extract agar (NBYA) (24) plates and purified by streak dilution. Strains were maintained on NBYA for daily use. Culture stocks were frozen at −20 C in NBY broth containing 10% glycerol.

Characterization of bacterial strains. Presumptive strains of *X. c. oryzae* isolated from rice and *L. hexandra* were subjected to standard biochemical tests used for identification of the pathogen (23). Five strains of each of the three groups identified were subjected to further characterization. Media composition and conditions for the biochemical tests as well as for analysis of cellular fatty acids were as previously described (13,16,17). Fatty acid profiles were computer-matched to information contained in the TSBA aerobic library (Microbial ID, Inc., Newark, DE, version 3.0 and Texas-Xco version 3.0 [13]). Absorbance spectra and thin-layer chromatography of pigment extracts were conducted as previously described (12, 13). The sensitivity of strains to lytic phage TXO-1 was determined on NBYA overlayed with 3 ml of NBY soft agar (0.75%) seeded with the strain, spotted with 10 μl of a suspension of 10^9 plaque-forming units per milliliter, and incubated at 28 C for 18 hr. Plaque formation indicated sensitivity. For determination of bacteriocin production, the strains were grown on NBYA for 48 hr at 28 C, exposed to chloroform vapors, and overlayed with soft NBY agar seeded with the indicator strain. For determination of bacteriocin sensitivity, the strains were used as indicators in the overlays described above. Strains used as indicators included X1-5, X1-8 (13), X7-2D (Jackson County strain), and X37-2 (LaVaca County strain). Plates were incubated at 28 C and observed for zones of inhibition after 18 hr. Strains in bacteriocin group B-I show activity against strains X1-8 and X37-2, strains in group B-II show activity against strains X1-8 and X7-2D, and strains in group B-III show no activity against the indicator strains but are sensitive to bacteriocins produced by group B-I and B-II strains. Strains of *X. c. oryzae* obtained from two different fields and *L. hexandra* obtained from canals adjacent to the fields were examined for sensitivity to lytic phage TXO-1, bacteriocin production and sensitivity, and pathogenicity in rice. Hypersensitive reaction on tobacco (*Nicotiana tabacum* L. 'Hicks') was determined on 40-day-old plants by the method of Gitaitis et al (5).

Pathogenicity tests. Forty-day-old rice plants of the susceptible cultivar Lemont were wound-inoculated by the double-needle method (13,19). The method was used to introduce 10^2 cfu per inoculation site into the three uppermost leaves of each of three plants. Inoculum was prepared from cultures grown for 18 hr at 28 C on a rotary shaker (250 rpm) in NBY broth that did not contain glucose. Cells were concentrated by centrifugation, resuspended in sterile distilled water, and diluted to an optical density of 1.0 at 425 nm, which is equivalent to 1×10^9 cfu per milliliter. Control plants were wound-inoculated in the same manner with sterile water instead of inoculum. Plants were incubated for 14 days in a growth chamber with a 13-hr light period at 28 C followed by an 11-hr dark period at 22 C and with constant relative humidity of 80%. Plants were observed for symptom development and results were recorded.

Bacterial growth and pathogenicity studies in *L. hexandra*. Plants of *L. hexandra* were obtained from the banks of an irrigation canal in a county where bacterial leaf blight had not been observed. The plants were transplanted to trays (35 × 29 × 8 cm), cut back, and allowed to grow to obtain a uniform leaf size. Samples of plant tissue were assayed for the presence of *X. c. oryzae*, and no evidence of the pathogen was found. Plants with an average leaf length of 10 cm were inoculated by the leaf-clipping method (19) with a rifampin-resistant (100 μg/ml) derivative of bacterial strain X1-5 (X1-5R) and incubated in a growth chamber as described above. The bacterial inoculum was prepared as described above. Six leaves were sampled at 2-day intervals for 22 days. Each sample was macerated in 5 ml of phosphate buffer, diluted, and plated onto NBYA amended with nystatin (300 units per milliliter) and rifampin (75 μg/ml). Plates were incubated at 28 C and colonies were counted after 72 hr. Symptoms were recorded at the same time samples were taken.

Isolation of lytic bacteriophage. Rice plant tissue with bacterial leaf blight symptoms was collected from seven different counties in Texas and one parish in Louisiana. The plant material (1–2 g) was macerated in phosphate buffer, filtered (Whatman No. 1), and centrifuged at 8,000 *g* for 10 min at 4 C. One-half of the supernatant was filtered through a 0.22-μm (pore size) filter (Millipore Corp., Bedford, MA), and the other half was treated with chloroform (5% final concentration). Then, 0.5 ml of the filtered or chloroform-treated (aqueous phase) samples was added to 25 ml of a log-phase culture of strains X1-5, X1-8, and X8-1A (13) growing in NBY broth and incubated at 28 C on a rotary shaker (200 rpm). After 16 hr, the cultures were centrifuged, the supernatants were divided equally, and the samples were either filtered (0.22-μm filter) or treated with chloroform as described above. The samples were serially diluted and the plaque-forming units per milliliter determined by the soft

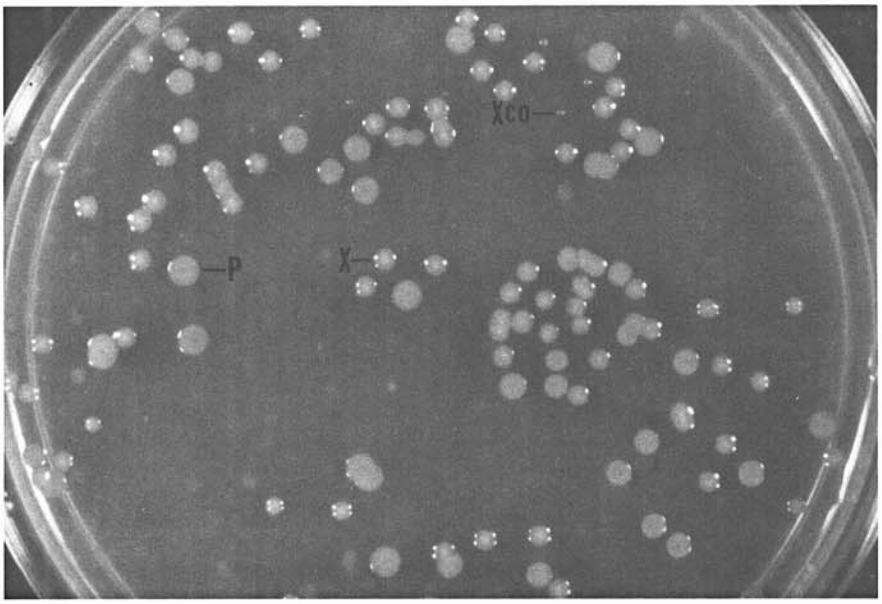

Fig. 1. Colonies of *Xanthomonas campestris* pv. *oryzae* (Xco), *Xanthomonas* sp. (X), and *Pseudomonas paucimobilis* (P) isolated from leaf tissue of *Leersia hexandra* on MXO medium.

agar overlay method (7), with the propagating strain used as the host. Single plaques were removed from the overlay and propagated on the homologous host. The procedure was repeated twice. For preparation of high-titer lysates (10^9–10^{10} plaque-forming units per milliliter), purified phage was added to a log-phase culture at a multiplicity of infection of 10^{-2}, and the culture was allowed to grow for 18 hr at 28 C on a rotary shaker (250 rpm). The culture was centrifuged, the supernatant filter was sterilized, and the plaque-forming units per milliliter were determined. Phage stocks were stored in 10% glycerol at −20 C. The above procedure was used to isolate lytic phage TXO-1 used in this study.

RESULTS

Identification of the alternative host. During the 1988 growing season, samples of grasses were collected from fields and irrigation canals adjacent to rice fields with or without bacterial leaf blight. Only *L. hexandra* collected adjacent to diseased fields harbored populations of *X. c. oryzae* (average population 10^3 cfu/g of leaf tissue). In addition, two other groups of yellow-pigmented bacteria were consistently isolated from *L. hexandra*. The three groups of yellow-pigmented bacteria were identified as *X. c. oryzae* (group I), *Pseudomonas paucimobilis* Holmes et al (group II), and *Xanthomonas* sp. (group III) (Table 1). The *Xanthomonas* sp. and *P. paucimobilis* were consistently recovered from *L. hexandra* in the presence or absence of *X. c. oryzae*. Colonies of the *Xanthomonas* sp. and *P. paucimobilis* were visible on the semiselective medium after 48 hr. *Xanthomonas* sp. appeared as large (2 mm) pale-yellow colonies surrounded by a clear zone indicating amylase activity, *P. paucimobilis* appeared as dark-yellow colonies with no evidence of amylase activity, and *X. c. oryzae* appeared after 4 days as small (1 mm) pale-yellow colonies with weak amylase activity (Fig. 1).

Morphological characterization of the three groups determined them to be gram-negative rods with a single polar flagellum. Results of biochemical tests (Table 1) were consistent with reactions for the genera *Xanthomonas* (group I and III) and *Pseudomonas* (group II). On the basis of profiles of the methyl esters of fatty acids from whole-cell extracts, we identified groups I, II, and III as *X. c. oryzae, P. paucimobilis*, and *Xanthomonas* sp., respectively. Strains of *X. c. oryzae* contained predominantly C16 and C17 fatty acids and a lesser amount of C18 fatty acids, whereas the *Xanthomonas* sp. had a more complex profile that included 42 fatty acids, with C15 fatty acids accounting for 35% of the total composition. The group III fatty acid profiles had nine peaks, with C14, C16, and C18 representing 8, 24.4, and 65.1%, respectively, of the total composition. Pigment extracts from groups I and III had an absorption maximum at 443 nm, whereas the spectra of group II had absorbance peaks at 445 and 472 nm. The pigment extracts from groups I and III chromatographed to an R_f value of 0.44–0.47, whereas the group III extract banded at R_f value of 0.75.

Strains in group I elicited a weak hypersensitive response in tobacco, whereas those in groups II and III induced no response. The control, strain HS191 of *P. syringae* pv. *syringae* van Hall (6), induced a strong hypersensitive response in tobacco. During the 1989 growing season, additional strains of *X. c. oryzae* were isolated from *L. hexandra* (Wharton County, Texas) growing in a canal adjacent to fields that appeared to be free from bacterial leaf blight. The pathogen was isolated from the leaf tissue at 10^3 cfu/g. Groups II and III bacteria were also isolated from the tissue.

Further analysis of bacterial strains. On the basis of biochemical (Table 1) and fatty acid analyses of bacterial strains isolated from diseased rice fields and from *L. hexandra* growing adjacent to rice fields, we concluded that *X. c. oryzae* was present in both plants. The relationship between strains from rice and those from *L. hexandra* was not clear, however. Strains of *X. c. oryzae* obtained from rice plants in field 1 and *L. hexandra* growing in the adjacent canal were typed to bacteriocin group B-I, whereas strains isolated from rice field 2 and *L. hexandra* found in the adjacent canal were typed to bacteriocin group B-III. Strains from both fields as well as those from the *L. hexandra* in adjacent canals were sensitive to phage TXO-1 and pathogenic on the susceptible rice cultivar Lemont. However, none of the group II or III strains obtained from rice or *L. hexandra* caused disease in rice. Strains of *X. c. oryzae* isolated from symptomless *L. hexandra* during the 1989 growing season typed to bacteriocin group B-III, were sensitive to phage TXO-1 and caused bacterial leaf blight symptoms in rice.

Growth and pathogenesis in *L. hexandra*. The initial bacterial population introduced by the leaf-clipping inoculation technique of 1×10^4 per gram of leaf tissue increased to 1.3×10^5 per gram of leaf tissue by day 4. The population

Table 1. Morphological and biochemical characteristics of gram-negative, yellow-pigmented bacterial groups isolated from *Leersia hexandra*

Test	Group I: *Xanthomonas campestris* pv. *oryzae*	Group II: *Pseudomonas paucimobilis*	Group III: *Xanthomonas* sp.
Morphology			
Shape	Rod	Rod	Rod
Flagella	Single polar	Single polar	Single polar
Colonies	Mucoid, 96 hr	Mucoid, 48 hr	Dry, 48 hr
Growth at:			
25 C	+[a]	+	+
28 C	+	+	+
36 C	+	+	+
41 C	−	+	−
Oxidase test	−	+	−
Oxidative metabolism	+	+	+
Catalase activity	+	+	+
Hydrolysis of:			
Aesculin	+	+	+
Casein	+	−	+
Gelatin	−	−	+
Potato starch	+	−	+
Rice starch	+	−	+
Growth on sole carbon source as:			
Fructose	+	+	+
Glycerol	−	+	+
Trehalose	+	+	+
D-xylose	+	+	+
Acetoin production	−	+	−
Indole production	−	−	−
Urease production	−	−	−
K_2TeO_3 reduction	−	−	−
Nitrate reduction	−	−	−
H_2S from L-cysteine	+	+	+
Pectinase activity	−	−	+
Phenylalanine deaminase activity	−	−	−
Tolerance to triphenyl tetrazolium chloride at:			
0.02%	−	+	+
0.10%	−	−	−

[a] + = Positive, − = negative.

was 4×10^5 per gram of leaf tissue on day 8, and this level was maintained through day 22. During the 22-day period, no symptoms were observed in *L. hexandra*.

DISCUSSION

Bacterial leaf blight of rice was first confirmed in the United States in 1987 in the Gulf Coast area of Texas (13). Strains of the pathogen isolated in the United States were related to, but distinct from, *X. c. oryzae* strains of Asian origin. Most notably, the U.S. strains appeared to be less aggressive than the Philippine strains (13). The origin of the U.S. strains has been in question. In previous studies, *X. c. oryzae* did not survive for extended periods in field residue and survived less than 10 days in infected leaf tissue (10,19). Because most rice producers practice crop rotation and direct-seeding techniques, infected rice stubble would be of minimal importance in survival of the pathogen in the United States.

Evidence that infected seed is important in transmission of the pathogen is inconclusive (2,4,14). Hsieh and Buddenhagen (10) reported that *X. c. oryzae* cannot survive long when the relative humidity and temperature are high. They found that bacterial populations were reduced by 99% when seed was soaked for 24 hr in water and were not detectable after 5 days. Kauffman and Reddy (14) found that the pathogen was viable up to 2 mo on seed stored at 25–35 C. Singh and Rao (21) reported that strains from India survived for up to 11 mo on seed. In Texas during 1987, bacterial leaf blight developed in fields planted with approximately 15 different seed sources (13). In several cases, rice fields that did not develop bacterial leaf blight were planted from the same seed lots as fields that did (13). In many cases, the fields were less than 1.5 km apart and were exposed to the same weather conditions. Thus, rice seed did not appear to be the original source of inoculum for disease. Distribution of bacterial leaf blight within fields and weather conditions associated with disease development in 1987 provide evidence that an alternative host(s) was the source of inoculum. Extensive and prolonged storms resulted in localized flooding and caused damage to leaves that may have facilitated spread of *X. c. oryzae* from an alternative host. In most fields, the disease appeared first at the edges and later spread into the field.

In temperate rice-growing areas, perennial weed hosts have been reported as the primary means by which the bacterium survives the winter (8,11,19). In Japan, for example, the bacterium survives the winter in the rhizosphere of *L. sayanuka*. In the spring, *X. c. oryzae* multiplies on foliage and becomes the primary inoculum for nearby rice plantings (8,11).

The perennial grass *L. hexandra* appears to be an alternative host for the pathogen *X. c. oryzae* and to provide a source of inoculum when conditions are conducive for disease development. In India, *L. hexandra* not only acts as an alternative host but also causes water-soaked lesions when inoculated with Asian strains of *X. c. oryzae* (22). The failure of the U.S. strains to cause symptoms in *L. hexandra* may reflect the low aggressiveness of the strains. The pathogen is able to survive in the alternative host at populations of 10^3 cfu/g of tissue under conditions that are not conducive to disease development in rice. This was observed during the 1989 growing season, when no symptoms were seen in rice fields adjacent to canals containing *L. hexandra* that harbored *X. c. oryzae*.

The role of the group II and III strains in the development of bacterial leaf blight is unknown at this time. As has been observed previously (13), however, they are found in association with *X. c. oryzae* in rice tissue. Both group II and III strains were consistently present at a population of 10^3–10^4 cfu/g of seed in 15 rice seed samples examined in our laboratory (Gonzalez, *unpublished*). To date, however, we have no evidence that U.S. strains of the pathogen are transmitted by seed. The consistent presence of both the *Xanthomonas* sp. and *P. paucimobilis* in rice tissue and seed is evidence that these bacteria are part of the natural bacterial flora. This association of the group I, II, and III strains can now be further extended to include the asymptomatic host, *L. hexandra*. Although Lozano et al (15) have identified *P. paucimobilis* as the causal agent of brown blotch on rice in South America, there is no evidence that strains obtained in a previous study (13) or in this study cause any symptoms in rice. The presence of the nonpathogenic strains of *Xanthomonas* sp. (group II), however, could be a source for potential false-positive identification in seed and plant samples if the methods used do not differentiate pathogenic and nonpathogenic bacteria of the same genus that are found in the same ecological niche.

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