Plasmid, Genomic, and Bacteriocin Diversity in U.S. Strains of *Xanthomonas campestris* pv. *oryzae*

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ABSTRACT

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Twenty-six strains of *Xanthomonas campestris* pv. *oryzae* isolated during a recent outbreak of bacterial leaf blight of rice in the United States were analyzed for their plasmid, genome, and bacteriocin diversity. Twenty of the strains harbored indigenous plasmid(s) and could be divided into three distinct groups. Restriction fragment length polymorphism (RFLP) analyses of genomic DNA revealed hybridization profiles that separated the strains into four groups. Four bacteriocin groups were identified among the strains tested. Five subgroups were identified based on plasmid content, RFLP analyses, and bacteriocin typing.

Xanthomonas campestris pv. *oryzae*, the causal agent of bacterial leaf blight on rice, has been recognized in Asia for many years (26). However, the presence of *X. c. oryzae* in the United States was confirmed only recently (18). A comparison of U.S. and Asian strains of *X. c. oryzae* by analysis of cellular fatty acids, restriction fragment length polymorphisms (RFLP), and reactions to monoclonal antibodies revealed some similarities among strains of distinct geographic origin (18). However, the U.S. strains were less aggressive than the Philippine strains. Although the U.S. strains reacted with two *X. c. oryzae*-specific monoclonal antibodies (Xco-1 and Xco-2), they could be differentiated from the Asian strains by their reaction with monoclonal antibody Xco-5 (1,18). Analysis of *Eco*RI-fragment patterns of genomic DNA and reaction to specific monoclonals allowed for the identification of two distinct groups among the strains (18).

In this study we show the diversity of indigenous plasmid(s), genomic DNA, and bacteriocin production, and sensitivity among strains of *X. c. oryzae*. RFLP analysis with DNA probe pJEL101 (21), a probe for repetitive sequences, was used to detect and assess relationships among strains. Such analyses have been used to assess phylogenetic relationships (biosystemic studies) of other plant pathogens (9,12,14,17).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Twenty-six strains of *X. c. oryzae*, three from Louisiana (provided by C. Rush, Louisiana State University, Baton Rouge) and 23 from Texas (34), were used in this study. Strain designation was based on the origin of the sample. Strains isolated from samples obtained in Wharton, Bazoria, Jackson, Colorado, and LaVaca counties in Texas were designated with the prefix X1-, X4-, X7-, X11-, and X13-, respectively. Strains obtained from Louisiana were designated with the prefix X8-. Cultures of *X. c. oryzae* were maintained routinely on nutrient broth-yeast extract agar (32) at 28 C and preserved at −20 C in NBY broth containing 10% glycerol (15). *Escherichia coli* strain HB101 containing plasmid pJEL101 (21) was grown at 37 C on Luria-Bertani agar or in Luria-Bertani broth containing ampicillin (40 μg/ml) (22). The *E. coli* strain was preserved in glycerol-salts solution at −20 C (22).

Plasmid isolations and analysis. Indigenous plasmid DNA was isolated by a modification of the method of Birnboim and Doly (2). To isolate plasmid DNA on a small scale, bacterial cells were grown in NBY broth (5–10 ml) with constant agitation (250 rpm) for 18 h at 28 C, and then harvested by centrifugation for 10 min at 11,700 *g*. Each culture pellet was resuspended in 1 ml of potassium phosphate buffer (0.0125 M, pH 7.0), transferred to a microcentrifuge tube, centrifuged for 5 min, and resuspended in 0.2 ml of cell lyses buffer (2). The suspension was mixed gently and incubated on ice for 10 min. The plasmid DNA was separated from chromosomal DNA and cell wall debris by adjusting the solution from alkaline (pH at 12.0–12.5) to a neutral condition (pH 7) by adding sodium acetate to a final concentration of 1 M followed by centrifugation as described (2). Plasmid DNA was purified by removal of protein with phenol/chloroform (1:1 by volume) and then with chloroform. Plasmid DNA was precipitated with isopropanol, resuspended in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and digested to completion with *Eco*RI (Promega, Madison, WI) according to the manufacturer's instructions. The DNA fragments were separated by electrophoresis (24 h, 40 V) in 0.7% agarose gel containing Tris-borate buffer (22).

Plasmid pJEL101, a pUC18 clone containing a 2.4-kb fragment of repetitive DNA from a Philippine isolate of *X. c. oryzae* (21), was isolated by the method of Birnboim and Doly (2) and purified by ultracentrifugation in an ethidium bromide-cesium chloride gradient.

Genomic DNA isolation, digestion, and blot hybridization. Total cellular DNA of the bacterium was isolated and purified by a modification (5) of the method of Currier and Nester (11). Total genomic DNA of strains R33 (India) and R48 (Japan) was provided by J. E. Leach (Kansas State University, Manhattan). Genomic DNA of *X. c. oryzae* was digested completely with *Eco*RI, and the DNA fragments separated by gel electrophoresis. DNA fragments were transferred from agarose gels to GeneScreen Plus (New England Nuclear, Boston, MA) membrane by the alkaline transfer method (19). Purified plasmid pJEL101 was labeled with [^{32}P]dATP by nick translation (22). The specific activity of labeled pJEL101 was approximately 5×10^8 cpm/μg. DNA-DNA hybridization was performed as described by the manufacturer of GeneScreen Plus membrane. After hybridization, the membranes were washed at 65 C with constant agitation in a buffer composed of 0.1× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate) (150 mM NaCl, 15 mM sodium citrate) and 0.1% sodium dodecyl sulfate. The washing buffer was changed at 30-min intervals over a period of 2 h. Membranes were exposed to Kodak X-Omat AR film at −80 C in cassettes with intensifying screens.

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Production of bacteriocin. Strains of *X. c. oryzae* were assayed for production of and sensitivity to bacteriocins. All 26 strains were tested as described previously (15). Strains used as indicators included: X1-5, X1-8, X7-2D, and X37-2 (15).

RESULTS

Plasmid isolation and analysis. The DNA fragments of plasmids associated with three groups of *X. c. oryzae* after digestion to completion with restriction enzyme *Eco*RI are shown in Figure 1. Plasmid DNA was isolated from 20 of the 26 strains. With the exception of three strains, each contained one plasmid. The strains were divided into three groups based on the mass of resident indigenous plasmid(s) (Table 1). Group P-I, represented by strain X1-5, consisted of 14 isolates that each contained a single plasmid of 31.7 kb and yielded two *Eco*RI fragments (14.0 and 17.7 kb, respectively). Group P-II, represented by strain X1-8, contained three isolates that harbored a 24.4-kb plasmid. *Eco*RI digestion of this plasmid yielded fragments of 5.7 and 18.7 kb. Group P-III (three strains from Louisiana), represented by strain X8-3, harbored two plasmids: a 16.4-kb (6.2- and 10.2-kb *Eco*RI fragments) and a 15.5-kb plasmid (3.7- and 11.8-kb *Eco*RI fragments).

Genomic diversity among the U.S. strains. The RFLP analysis of 26 strains of *X. c. oryzae* with probe pJEL101 distinguished four unique hybridization groups (Fig. 2). Four of the hybridizing fragments (1.5, 4.1, 7.8, and 16.9 kb) were common to all four groups (Table 2). In groups H-I and H-II, hybridization showed a total of 10 *Eco*RI fragments, ranging from 1.5 to 16.9 kb, that hybridized to the probe. Of these, only two hybridizing fragments (3.8 kb in group H-I and 2.6 kb in group H-II) were unique to their respective group (Table 2). Eleven *Eco*RI DNA fragments from groups H-III and H-IV hybridized to pJEL101. Ten of the hybridizing fragments were in common, with only the 1.7- and 10.5-kb fragments unique to groups H-III and H-IV, respectively (Table 2).

TABLE 1. Plasmid, hybridization, and bacteriocin groups of U.S. strains of *Xanthomonas campestris* pv. *oryzae*

Strain	Plasmid group	Hybridization group	Bacteriocin group
X1-, 5, 7	P-I	H-III	B-I
X7-, 2D, 3E, 5A; X11-, 1A, 1B, 2D, 4D, 5A, 5B; X13-, 2E, 3A, 5C	P-I	H-IV	B-I
X1-, 6, 8, 10	P-II	H-II	B-III
X8-, 1A, 3, 4	P-III	H-I	B-IV
X4-, 1B, 1C, 2C, 3D, 4D, 8C	ND[a]	H-I	B-III

[a] No plasmid detected by method used in this study.

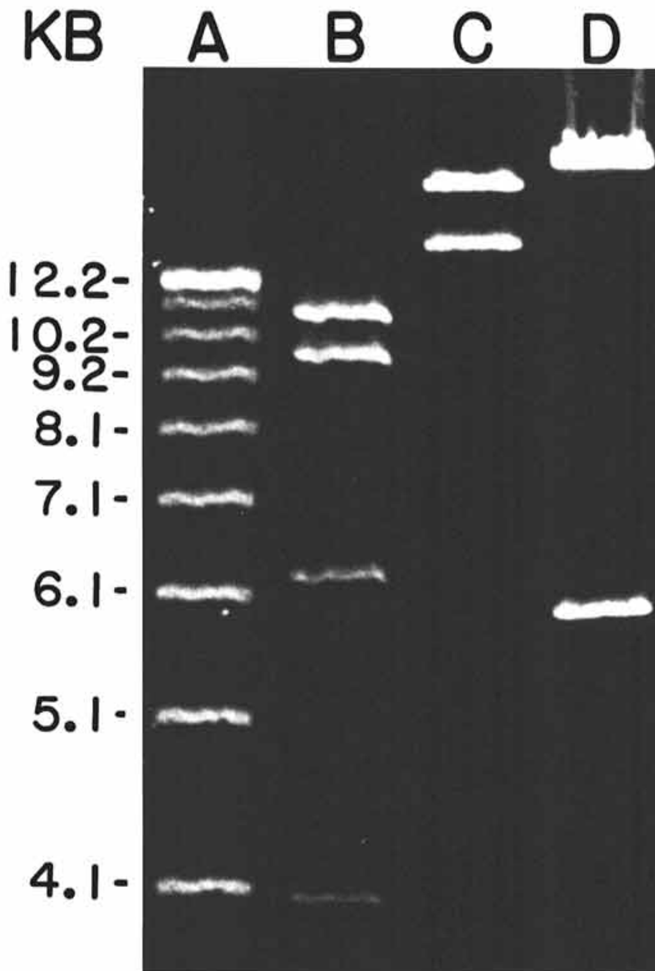

Fig. 1. Agarose gel electrophoresis of *Eco*RI-digested plasmid DNA from each plasmid group of *Xanthomonas campestris* pv. *oryzae*. Lane A, 1-kb DNA ladder; lane B, group P-III (strain X8-3 from Louisiana); lane C, group P-I (strain X1-5 from Texas); lane D, group P-II (strain X1-8 from Texas). Molecular size standards ranging from 4.1 to 12.2 kb are shown to left.

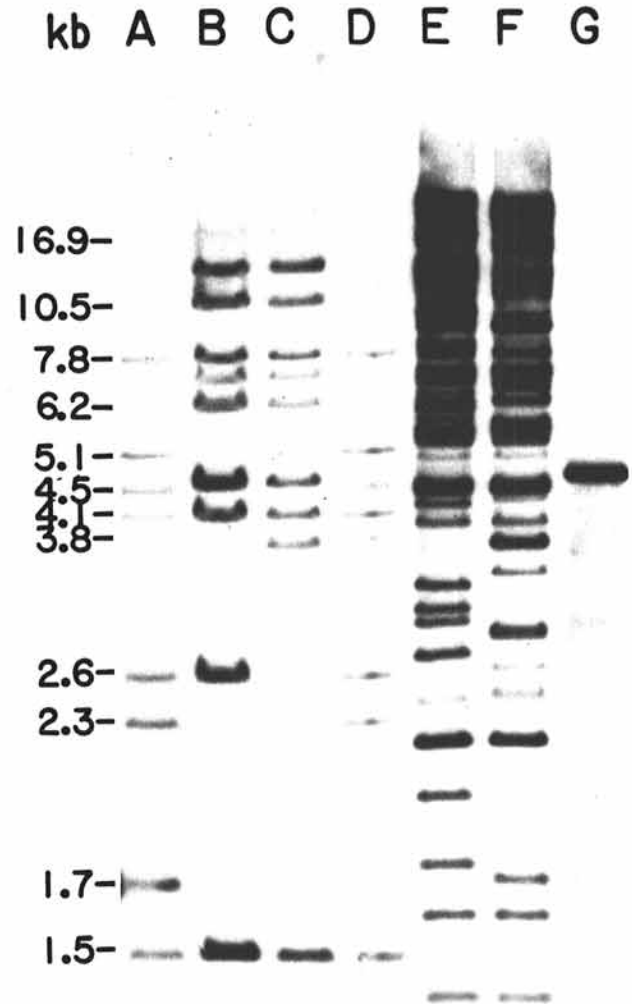

Fig. 2. Southern blot hybridization of representative U.S. and Asian strains of *Xanthomonas campestris* pv. *oryzae*. Lane A strain X1-5 (group H-III); lane B, strain X1-8 (group H-II); lane C, strain X8-3 (group H-I); lane D, strain X7-2D (group H-IV); lane E, strain R33 (India); lane F, strain R48 (Japan); and lane G, probe pJEL101. DNA fragment size standards are shown to left.

Bacteriocins. Each strain tested could be placed into one of four groups based on bacteriocin production and sensitivity patterns. Strains in bacteriocin group B-I showed activity against strains X1-8 and X37-2, whereas strains in group B-II showed activity against strains X1-8 and X7-2D. Group B-I contained 14 strains that produced bacteriocin and is typified by strain X1-5 (Table 1). Zones of bacterial growth inhibition were visible after 24–48 h. The zones were clear and measured 2–3 mm from the edge of the bacterial colony to the edge of the indicator growth. Group B-II contained one strain (X37-2), which is also in plasmid group P-1. No detectable production of bacteriocin was observed by group B-III strains; however, group B-III strains (nine strains) were indicators for the group B-I and B-II bacteriocin producers (Table 1). Group B-IV consisted of three strains from Louisiana that did not produce a detectable bacteriocin with the indicators used and were insensitive to the bacteriocins produced by any of the strains tested.

DISCUSSION

Characterization of indigenous plasmids, RFLP analysis of genomic DNA, and bacteriocin typing of U.S. strains of *X. c. oryzae* indicated that diversity does exist among the strains. The strains were placed into groups based on the mass of indigenous plasmid(s), RFLPs observed in the genomes, and bacteriocin production and sensitivity patterns.

Twenty of the 26 U.S. strains tested harbored one or more indigenous plasmid(s). Although the presence of plasmid DNA has been reported in Asian strains of *X. c. oryzae* (6,21,27), this is the first report of indigenous plasmid(s) in U.S. strains of *X. c. oryzae*. Indigenous plasmids have been observed in all genera of plant pathogenic bacteria (7,10), and the phenotypic function of the plasmids has been well established in *Agrobacterium tumefaciens* (31), *A. rhizogenes* (24), *Pseudomonas savastanoi* (8,25), *P. syringae* (28), and *X. c. vesicatoria* (3,29,30). However, the role of most plasmids in phytopathogenic bacteria is still unknown. Plasmid-encoded bacteriocin production was evaluated in the U.S. strains of *X. c. oryzae* by the elimination of the resident plasmids. Plasmid-cured strains produced the same bacteriocins identified in the parent strains, suggesting that the structural gene(s) for bacteriocin production are chromosomal (G.-W. Xu and C. F. Gonzalez, *unpublished*). The correlation between the presence of plasmid(s) and the variability of pathogenicity, or race specificity, and ecological fitness remain to be determined.

RFLP analysis provides a sensitive means for the detection of genomic differences within or among species of plant pathogenic bacteria. The *X. campestris* is comprised of 125 pathovars based on pathogenicity to particular host (4). Lazo *et al.* (20) reported that pathovars of *X. campestris* can be distinguished by RFLP analysis. When cloned DNA fragments from *X. c. citri* were used as probes, hybridization to genomic DNA revealed profiles that appeared to be highly conserved and unique for each of the pathovars tested. RFLP analyses also have been used to distinguish pathovars of *P. syringae* (12), and strains of *X. c. citri* (17). In this study, RFLP analysis was used to compare the U.S. strains of *X. c. oryzae*. Four groups were identified. The hybridization profiles obtained appear to be due to the presence of sequences in the genomic DNA, because pJEL101 does not hybridize with plasmid DNA from groups P-I, II, or III. The patterns obtained for the U.S. strains of *X. c. oryzae* are clearly different from those observed for the strains of Asiatic origin. In a more extensive comparison of genomic DNA of strains of *X. c. oryzae* from diverse geographic origins (Philippines, Japan, Thailand, India, Burma, Colombia, Australia, Bangladesh, Sri Lanka, and the United States), it was found that all strains with the exception of the U.S. strains contained the repetitive cloned sequence in pJEL101 in high copy number (21). These results indicate that the pathovar evolved as a distinct clonal population. Also, it provides additional evidence that the strains of *X. c. oryzae* now identified in the United States are genetically unique and were not introduced.

Production and sensitivity to bacteriocins have been used to type various phytopathogenic bacteria (13,16,33). We have found that the U.S. strains of *X. c. oryzae* could be categorized into bacteriocin producer, indicator, and nonproducer groups that are either bacteriocin sensitive or insensitive. Mew *et al.* (23) reported that 22 Asiatic strains produced bacteriocinlike compounds that were heat stable and sensitive to trypsin and protease and were not inducible by UV and mitomycin C treatment. The bacteriocins produced by the U.S. strains are produced in broth culture and appear to be low molecular weight proteins (C. F. Gonzalez, *unpublished*).

In a previous study, we determined that strains of *X. c. oryzae* obtained from rice plants and *Leersia hexandra*, an alternative host for *X. c. oryzae*, growing in the adjacent canal at one location, belonged to bacteriocin group B-I, whereas strains obtained from a different location belonged to group B-III (15). In this study, four of the groups comprised unique populations based on plasmid content, and bacteriocin typing (Table 1). All strains of plasmid group P-I were in bacteriocin group B-I and were either in hybridization groups H-III or H-IV, whereas strains in P-II were in groups B-III and H-II. The three strains from Louisiana belong to groups P-III, B-IV, and H-I, whereas strains with no detectable plasmid were in groups B-III and H-I. This indicates that distinct subgroups exist among the U.S. strains of *X. c. oryzae* and that plasmid, genomic, and bacteriocin diversity exist among the U.S. strains. Further studies will establish if isolates obtained in subsequent cropping years resemble those obtained in this study or if the population will be more diverse. Although no direct correlations presently exist between the more complex RFLP patterns observed in the Asian strains and the less complex pattern observed in the less aggressive U.S. strains, the hybridization groups established in this study will useful in following the genetic evolution of U.S. strains.

TABLE 2. DNA-DNA hybridization profiles of the U.S. strains of *Xanthomonas campestris* pv. *oryzae*

	Group			
	H-I	H-II	H-III	H-IV
Representative strain	X8-3	X1-8	X1-5	X7-2D
No. strains in group	9	3	2	12
*Eco*RI-fragments homologous to pJEL101[a]	1.5[b]	1.5	1.5	1.5
	3.8	2.6	1.7	2.3
	4.1	4.1	2.3	3.8
	6.2	6.2	3.8	4.1
	7.1	7.1	4.1	4.5
	7.8	7.8	4.5	5.1
	10.5	10.5	5.1	7.8
	13.4	13.4	7.8	10.5
	16.9	16.9	11.6	11.6
			16.9	16.9

[a]Plasmid pJEL101 is a pUC18 clone containing a 2.4-kb fragment from a Philippine isolate of *X. c.* pv. *oryzae*.
[b]Molecular sizes expressed as kb.

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