APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1995, p. 1020–1026
0099-2240/95/$04.00+0

Vol. 61, No. 3

Genetic Diversity among *Xanthomonas campestris* Strains Pathogenic for Small Grains

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Received 8 August 1994/Accepted 3 January 1995

A collection of 51 *Xanthomonas campestris* strains from throughout the world was studied to detect and assess genetic diversity among pathogens of small grains. Isolates from barley, bread wheat, bromegrass, canary grass, cassava, maize, orchard grass, rice, rough-stalked meadow grass, rye, timothy, and triticale were analyzed by pathogenicity tests on bread wheat cv. Alondra and barley cv. Corona, indirect immunofluorescence, and restriction fragment length polymorphism (RFLP). Three probes were used for the RFLP analysis. They were an acetylaminofluorene-labelled 16S+23S rRNA probe from *Escherichia coli* and two ^{32}P-labelled restriction fragments from either plasmidic (pBSF2) or chromosomal (pBS8) DNA of *X. campestris* pv. manihotis. Strains clustered in 9 and 20 groups with the rRNA probe and the pBSF2 DNA probe, respectively. Strains of *X. campestris* pv. graminis, *X. campestris* pv. phleipratensis, and *X. campestris* pv. poae are shown to

TABLE 1. Origins and descriptions of bacterial strains

Strain[a]	Identification	Host	Year of isolation	Country where isolated	Isolated by:
NCPPB1159	*X. campestris* pv. manihotis	*Manihot esculenta*	1941	United States	W. Burkholder (New York)
NCPPB1585	*X. campestris* pv. oryzicola[b]	*Oryza sativa*	1964	Malaysia	A. C. Hayward (United States)
NCPPB1837	*X. campestris* pv. phleipratensis[b]	*Phleum pratense*	1966	United States	J. Wallin (United States)
NCPPB1944	*X. campestris* pv. cerealis[b]	*Bromus inermis*	1941	United States	J. Wallin (United States)
NCPPB1945	*X. campestris* pv. undulosa[b]	*Triticum aestivum*	1943	Canada	W. Hagborg (Canada)
NCPPB2389	*X. campestris* pv. hordei[b]	*Hordeum vulgare*	1970	India	G. S. Shekhawat (India)
NCPPB2612	*P. syringae* pv. atrofaciens[b]	*Triticum aestivum*	1972	New Zealand	J. Wilkie (New Zealand)
NCPPB2700	*X. campestris* pv. graminis[b]	*Dactylis glomerata*	1973	Switzerland	T. Egli (Switzerland)
NCPPB2821	*X. campestris* pv. undulosa	*Triticum turgidum*	1966	Canada	W. Hagborg (Canada)
NCPPB2822	*X. campestris* pv. secalis[b]	*Secale cereale*	1966	Canada	W. Hagborg (Canada)
NCPPB3230	*X. campestris* pv. poae[b]	*Poa trivialis*	1978	Switzerland	J. Herzog (Switzerland)
NCPPB973	*X. campestris* pv. translucens[b]	*Hordeum vulgare*	1933	United States	C. S. Reddy (United States)
UPB397	*X. campestris* pv. undulosa	*Phalaris canadiensis*	1988	Uruguay	H. Maraite (Belgium)
UPB410	*X. campestris* pv. undulosa	*Triticum aestivum*	1988	Argentina	J. Colin (Belgium)
UPB412	*X. campestris* pv. undulosa	*Triticum aestivum*	1988	Argentina	J. Colin (Belgium)
UPB426	*X. campestris* pv. undulosa	*Triticum aestivum*	1988	Argentina	J. Colin (Belgium)
UPB480	*X. campestris* pv. undulosa	*Triticum durum*	1988	Pakistan	H. Maraite (Belgium)
UPB482	*X. campestris* pv. undulosa	*Triticum aestivum*	1988	Pakistan	H. Maraite (Belgium)
UPB513	*X. campestris* pv. undulosa	*Triticosecale*	1987	Mexico	E. Duveiller (Mexico)
UPB522	*X. campestris* pv. undulosa	*Triticum aestivum*	1987	Mexico	E. Duveiller (Mexico)
UPB545	*X. campestris* pv. translucens	*Hordeum vulgare*	1987	Mexico	E. Duveiller (Mexico)
UPB599	*X. campestris* pv. undulosa	*Triticum aestivum*	1987	Mexico	E. Duveiller (Mexico)
UPB600	*X. campestris* pv. undulosa	*Secale cereale*	1987	Mexico	E. Duveiller (Mexico)
UPB605	*X. campestris* pv. undetermined	*Triticum aestivum*	1988	Brazil	C. Bragard (Belgium)
UPB631	*X. campestris* pv. undulosa	*Triticum aestivum*	1988	Mexico	C. Bragard (Belgium)
UPB633	*X. campestris* pv. undulosa	*Hordeum vulgare*	1988	Mexico	C. Bragard (Belgium)
UPB644	*X. campestris* pv. undulosa	*Triticum aestivum*	1988	Zambia	C. Bragard (Belgium)
UPB645	*X. campestris* pv. undulosa	*Triticum aestivum*	1989	Mexico	C. Bragard (Belgium)
UPB659	*X. campestris* pv. undulosa	*Triticum aestivum*	1988	Mexico	C. Bragard (Belgium)
UPB663	*X. campestris* pv. undulosa	*Triticum aestivum*	1989	Ethiopia	C. Bragard (Belgium)
UPB664	*X. campestris* pv. undetermined	*Triticum aestivum*	1988	Bolivia	C. Bragard (Belgium)
UPB670	*X. campestris* pv. undetermined	*Triticum aestivum*	1988	Bolivia	C. Bragard (Belgium)
UPB675	*X. campestris* pv. translucens	*Secale cereale*	1989	South Africa	J. Smith (South Africa)
UPB676	*X. campestris* pv. translucens	*Secale cereale*	1989	South Africa	J. Smith (South Africa)
UPB680	*X. campestris* pv. undulosa	*Triticum durum*	1989	South Africa	J. Smith (South Africa)
UPB681	*X. campestris* pv. undulosa	*Triticum aestivum*	1989	South Africa	J. Smith (South Africa)
UPB684	*X. campestris* pv. hordei	*Hordeum vulgare*	1984	Iran	A. Alizadeh (Iran)
UPB685	*X. campestris* pv. cerealis	*Triticum aestivum*	1984	Iran	A. Alizaden (Iran)
UPB686	*X. campestris* pv. holcicola	*Zea mais*	1970	Australia	M. Moffet (Australia)
UPB721	*X. campestris* pv. cerealis	*Bromus* sp.	1984	Japan	K. Miyagima (Hokkaido, Japan)
UPB727	*X. campestris* pv. undulosa	*Triticosecale*	1989	Ethiopia	C. Bragard (Belgium)
UPB728	*X. campestris* pv. undulosa	*Triticum durum*	1989	Ethiopia	C. Bragard (Belgium)
UPB729	*X. campestris* pv. undulosa	*Triticum durum*	1989	United States	C. Bragard (Belgium)
UPB733	*X. campestris* pv. undulosa	*Triticosecale*	1989	Peru	C. Bragard (Belgium)
UPB753	*X. campestris* pv. undulosa	*Triticum aestivum*	1990	Brazil	Y. R. Mehta (Brazil)
UPB755	*X. campestris* pv. undulosa	*Triticum aestivum*	1990	Brazil	Y. R. Mehta (Brazil)
UPB756	*X. campestris* pv. undulosa	*Triticum aestivum*	1990	Brazil	Y. R. Mehta (Brazil)
UPB757	*X. campestris* pv. undulosa	*Triticum aestivum*	1990	Brazil	Y. R. Mehta (Brazil)
UPB758	*X. campestris* pv. undulosa	*Triticum aestivum*	1990	Brazil	Y. R. Mehta (Brazil)
UPB763	*X. campestris* pv. translucens	*Hordeum vulgare*		United States	D. Sands (United States)
UPB876	*X. campestris* pv. undulosa	*Triticum aestivum*	1991	Madagascar	A.-P. Ferauge (Belgium)
UPB882	*X. campestris* pv. undulosa	*Triticum durum*	1991	Yemen	A.-P. Ferauge (Belgium)

[a] The strain numbers as they were received from the National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom, and the Unité de Phytopathologie Bacterial Collection, Université Catholique de Louvain, Louvain-la-Neuve, Belgium.

[b] Pathotype or neopathotype strain.

immunofluorescence of 24-h-old pure cultures was done with rat monoclonal antibody AB3-B6 (0.44 μg/ml) directed against *X. campestris* pv. translucens and related pathovars (6, 10) and with mouse anti-rat immunoglobulin M Marm4 (IMEX, UCL, Woluwé, Belgium). For each strain, the experiment was repeated three times. Strains UPB513 and NCPPB2613, a *Pseudomonas syringae* pv. atrofaciens strain, were used as positive and negative controls, respectively. In positive reactions, fluorescein isothiocyanate caused bacterial cell walls to appear green.

RFLP analysis, restriction digests, electrophoresis, and blotting. Genomic DNA was extracted from cultures grown overnight in medium O (peptone, 10 g; Casamino Acid, 1 g; yeast extract, 1 g [pH 7.2]) according to the method of Boucher et al. (3).

Different restriction endonucleases were tested in preliminary assays of strains UPB513, NCPPB973, NCPPB1944, NCPPB2389, and NCPPB2821. *Bam*HI, *Bgl*II, *Eco*RI, and *Xho*I were chosen as restriction enzymes. The choices were based on the diversity of the fragment patterns.

For each strain, 5 μg of DNA was digested with the different restriction endonucleases chosen, according to the manufacturer's instructions (Eurogentec, Liège, Belgium). Electrophoresis of DNA was carried out in a 0.7% agarose gel with TBE buffer (0.13 M Tris, 0.15 M boric acid, 3 mM EDTA, 12 mM ethidium bromide) at 3 V/cm for 14 h. The standard set Raoul I (Appligene, Illkirch, France) and DNA of *X. campestris* pv. manihotis NCPPB1159 were included as controls. DNA was transferred either to nylon membranes (Hybond N+; Amersham), according to the manufacturer's specifications, or to nitrocel-

TABLE 2. Pathogenicity on barley and wheat, indirect immunofluorescence, and RFLP analysis of *X. campestris* pathovars

Strain	Pathovar	Host	Pathogenic on[a]:		Immuno-	RFLP type no.		Total no.
							pBSF2[c]	

lulose membranes (Schleicher and Schuell BA85), as described by Grimont and Grimont (13).

Hybridization procedure. Three probes were used: (i) a 16S+23S rRNA probe from *E. coli* (Eurogentec), (ii) a 13-kb *Hin*dIII restriction fragment (pBSF2) (22) from a 44-kb plasmid from *X. campestris* pv. manihotis CFBP1851, and (iii) a restriction fragment from chromosomal DNA (pBS8) selected previously (22) in a genomic library of strain CFBP1851. The first probe was labelled with acetylaminofluorene (AAF), while the two others were labelled with [^{32}P]dCTP by random priming (Multiprime; Amersham, Les Ulès, France).

Hybridization with AAF-labelled rRNA probe was performed according to the manufacturer's instructions (Eurogentec). The procedure was repeated on different blots at least twice for each strain.

Hybridization with the DNA probes was performed at 65°C. Blots were washed once in 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) (pH 7.0) and 0.1% sodium dodecyl sulfate (SDS) for 20 min at room temperature, twice in 1× SSC and 0.1% SDS for 10 min at 65°C, and once in 0.7× SSC for 15 min. The blots were exposed to X-ray films at −80°C with an intensifying screen (Amersham, Les Ulis, France). Strain NCPPB1159 of *X. campestris* pv. manihotis as well as molecular weight marker Raoul I (Appligene) were used as internal standards on each blot.

RFLP data analysis. For the different digest-probe combinations, a unique number was assigned to each band. This allowed conversion to binary data, i.e., the presence or absence of a band at one particular level was coded as 1 or 0, respectively. Band density was not taken into account.

Pairwise distances for all combinations were calculated with the complement to the Jaccard similarity coefficient (14). As the probes used are very different from one another, the analysis was performed for each individual probe. The results of the different digests revealed with probe pBSF2 have been combined.

The distance matrix was subjected to cluster analysis by the unweighted pair group method with averages by using Progiciel R software (A. Vaudor, Laval, Canada).

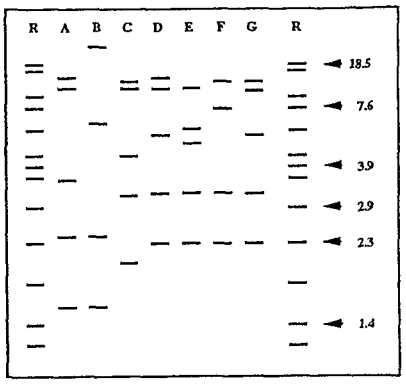

FIG. 1. Schematic representation of the rRNA gene restriction patterns obtained for the *Eco*RI-digested genomic DNA of *X. campestris* pathovars probed with AAF-labelled 16S+23S rRNA genes from *E. coli* and with AAF-labelled pBR322 DNA. All 40 strains of the translucens group exhibited one of these representative patterns. Lanes: A, *X. campestris* pv. manihotis NCPPB1159; B, deviant *X. campestris* pv. undulosa UPB670 (ribotype 6); C, *X. campestris* pv. graminis NCPPB2700 (ribotype 5); D, *X. campestris* pv. hordei NCPPB2389 (ribotype 4); E, *X. campestris* pv. cerealis UPB721 (ribotype 3); F, *X. campestris* pv. undulosa UPB513 (ribotype 2); G, *X. campestris* pv. undulosa NCPPB2821 (ribotype 1); R, molecular mass standard Raoul I. The numbers at the right of the lanes are sizes in kilobases.

RESULTS

Pathogenicity. Seventeen of the strains isolated from bread wheat and all strains (18) isolated from durum wheat (*Triticum durum* Desf.), triticale (×*Triticosecale* Wittm.), rye, bromegrass, and canary grass (*Phalaris canadiensis* L.) produced elongated watersoaked areas evolving into translucent streaks on bread wheat cv. Alondra within 3 days. Then greasy lesions appeared, and they were covered with bacterial exudates after 5 to 7 days. Similar symptoms appeared on barley cv. Corona, except that exudate formation was delayed or restricted. Lesions on barley were often bordered by a yellow margin.

The formation of watersoaked lesions is considered a compatible reaction. Strains isolated from barley could be divided into two pathogenicity groups. Group 1 includes two strains (UPB633 and NCPPB973) that are pathogenic for barley and bread wheat and are similar to the strains described above, and Group 2 includes strains that are pathogenic only for barley, in the manner of reference strain NCPPB2389. The effect of Group 2 strains on bread wheat was limited to the production of light yellow streaks.

Six strains isolated from bread wheat samples collected in Bolivia and Brazil showed atypical symptoms. Lesions were readily formed, but only to a limited extent. The bacteria produced small watersoaked areas on wheat cv. Alondra, sometimes with exudates. Lesions never extended more than 25 mm from the inoculation point and were usually limited to 5 mm. Small watersoaked areas on barley cv. Corona were limited by a necrotic border and no exudation was noticed, but the yellowing extended up to 10 mm from the inoculation point. These strains are temporarily considered to be deviant pv. undulosa strains.

Strains isolated from orchard grass (*Dactylis glomerata* L.), timothy (*Phleum pratense* L.), rough-stalked meadow grass (*Poa trivialis* L.), rice (*Oryza sativa* L.), and maize (*Zea mais* L.) failed to induce compatible lesions on the tested barley and wheat cultivars.

Serology. All strains inducing a compatible pathogenic reaction either on barley or on barley and bread wheat gave a cells stained with fluorescein isothiocyanate were clearly distinguishable on the dark background. Strains considered to be deviant pv. undulosa were negative. No positive reaction was observed with xanthomonad strains that are nonpathogenic for barley or wheat.

DNA ribotyping. The rRNA probe allowed nine different patterns among all the tested strains to be distinguished (Table 2). Some of these patterns are presented in Fig. 1 as examples. A total of 18 different bands was counted for strains of *X. campestris* pv. cerealis, graminis, hordei, manihotis, secalis, translucens, and undulosa, with three to five bands per pattern. All of the strains of the translucens group shared two common bands of 2.3 kb and 3 kb each. The 16 other fragments allowed the characterization of the nine different patterns.

Cluster analysis resulted in nine ribotypes (Fig. 2). Each ribotype represents strains with an identical pattern.

Most of the strains clustered in ribotypes 1 and 2. These two ribotypes correspond to 35 strains fully pathogenic for bread wheat and barley. They could be closely related, since they are located on the same branch of the cluster. Neopathotype strains of *X. campestris* pv. phleipratensis, *X. campestris* pv. poae, *X. campestris* pv. secalis, and *X. campestris* pv. undulosa belong to ribotype 1, which includes strains isolated from diverse host plants (5) from or collected in Africa, America, and Asia. Ribotype 2 corresponds to strains isolated from bread wheat or triticale samples from Central and South America as well as to strain UPB644 from Zambia.

Strains strictly pathogenic for barley clustered in a distinct group corresponding to ribotype 4, which includes the neopathotype strain of *X. campestris* pv. hordei. Ribotype 3 includes strains isolated from bromegrass and the neopathotype strain of *X. campestris* pv. cerealis, as well as the neopathotype strain of *X. campestris* pv. translucens. The six strains isolated from bread wheat and temporarily considered to be deviant pv. undulosa grouped in ribotype 6. These strains shared no common bands with those of ribotypes 1 to 5.

The reference strains of the other pathovars, i.e., UPB686 (*X. campestris* pv. holcicola), NCPPB2700 (*X. campestris* pv.

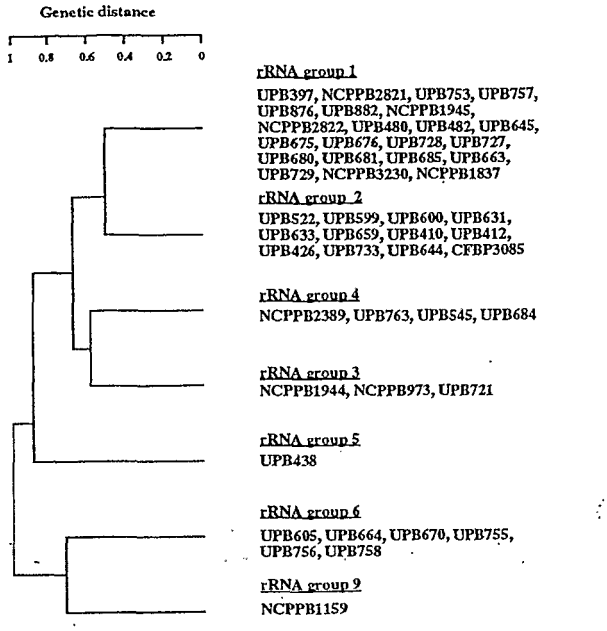

FIG. 2. Dendrogram of genetic distances produced with the computer program Progiciel R (A. Vaudor, Laval, Canada), showing the relationships among strains of *X. campestris* on the basis of an RFLP analysis in which AAF-labelled rRNA 16S+23S was used as a probe. Genetic distances are measured in kilobases.

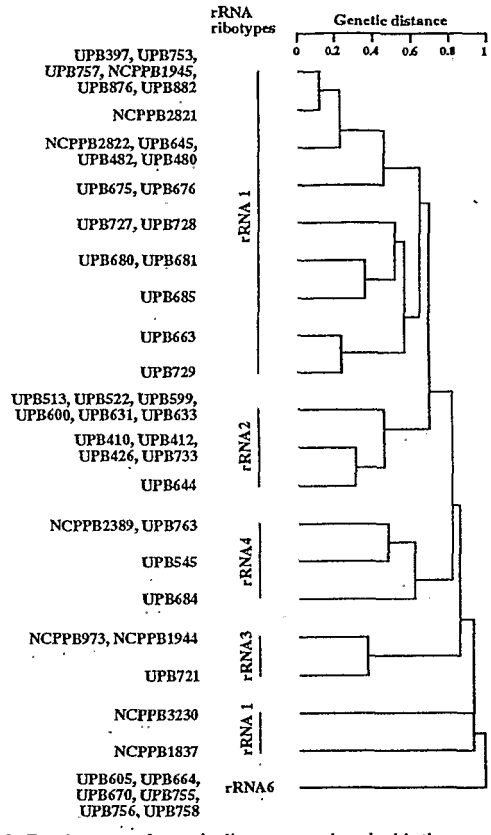

FIG. 3. Dendrogram of genetic distances produced with the computer program Progiciel R (A. Vaudor, Laval, Canada), showing the relationships among strains of *X. campestris* on the basis of an RFLP analysis in which ^{32}P-labelled pBSF2 was used as a probe. Genetic distances are measured in kilobases.

NCPPB1585 (*X. campestris* pv. oryzicola), showed patterns different from those of the strains of the translucens group.

DNA RFLP analysis. With the pBS8 DNA probe, no hybridization was observed with any of the tested strains, except with *X. campestris* pv. manihotis NCPPB1159.

Forty-eight strains of eight *X. campestris* pathovars were examined for DNA polymorphism with the plasmid DNA probe pBSF2. With the four restriction endonucleases used, *Xho*I, *Eco*RI, *Bam*HI, and *Bgl*II, 7, 13, 14, and 11 different patterns, respectively, were found. The different digests totaled 67 different bands. The dendrogram calculated from the Jaccard similarity coefficient and unweighted pair group method with averages cluster analysis is presented in Fig. 3. A total of 20 possible different combinations was obtained.

Strains from Bolivia and Brazil considered to be deviant *X. campestris* pv. undulosa (UPB605, UPB664, UPB670, UPB755, UPB756, and UPB758) fell into a separate cluster, as was the case with ribotyping.

Four other major clusters that correspond to ribotypes for strains of the translucens group could be delineated.

Strains NCPPB1837 of *X. campestris* pv. phleipratensis and NCPPB3230 of *X. campestris* pv. poae fell into a separate cluster.

DNA digestions with *Xho*I revealed that groups with the same pattern corresponded exactly to groups delineated by ribotyping, except that strains of *X. campestris* pv. phleipratensis and pv. poae showed distinct patterns. The different patterns produced by hybridization with *Eco*RI-digested genomic DNA are shown in Fig. 4.

The plasmid probe pBSF2 produced different hybridization patterns. A high level of polymorphism was observed for ribotype 1 with the four endonucleases tested. In contrast, no or poor hybridization was observed for ribotype 6.

DISCUSSION

On the basis of the present data, strains of *X. campestris* pv. cerealis, pv. hordei, pv. secalis, pv. translucens, and pv. undulosa, designated the translucens group (15, 20), all induce the same compatible reaction in pathogenicity tests on barley and are similar in serological tests. However, they revealed heterogeneity by RFLP analysis. They can be distinguished from

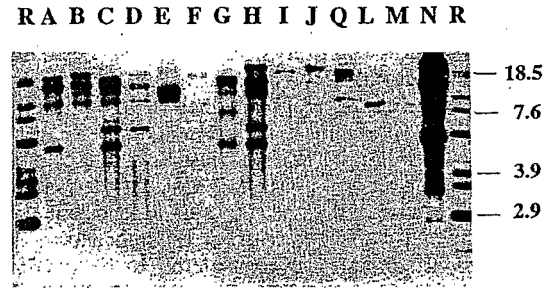

FIG. 4. Southern hybridization of *Eco*RI-digested genomic DNA of 13 *X. campestris* strains of the translucens group. Lanes: R, molecular mass standard Raoul I; A, strain UPB882 (Yemen, group 1); B, strain UPB727 (Ethiopia, group 2); C, strain UPB729 (United States, group 3); D, strain UPB681 (South Africa, group 4); E, strain UPB454 (Switzerland, group 5); F, strain UPB670 (Bolivia, group 13); G, strain UPB600 (Mexico, group 7); H, strain UPB426 (Argentina, group 6); I, strain UPB763 (United States, group 7); J, strain UPB684 (Iran, group 9); K, strain UPB545 (Mexico, group 8); L, strain UPB448 (United States, group 10); M, strain UPB721 (Japan, group 11); N, *X. campestris* pv. manihotis NCPPB1159 (group 14). The numbers at the right are sizes in kilobases.

strains of pathovars of *X. campestris*, such as pv. manihotis, pv. graminis, or pv. oryzicola, by ribotyping.

X. campestris strains such as *X. campestris* pv. phleipratensis and *X. campestris* pv. poae pathogenic for grasses and other cereals can also be identified with the pBSF2 DNA probe. This confirms previous serological (6) and fatty acid methyl ester test results (24).

Neopathotype strains of *X. campestris* pv. cerealis, hordei, and undulosa fell into distinct clusters with the rRNA probe as well as with the pBSF2 DNA probe. Ribotype 4 strains isolated from barley and pathogenic for barley only correspond to the description of *X. campestris* pv. translucens, a synonym of *X. campestris* pv. hordei. This pathovar is distinguishable from the others on the basis of pathogenicity tests and looks somewhat different in its fatty acids by comparison with those of other pathovars of the translucens group (23). Our results confirm the differences detected earlier at the pathovar and infrapathovar levels among *X. campestris* pathovars pathogenic for small grains (5).

Strains NCPPB973 and UPB633 have also been isolated from barley but proved to be pathogenic for wheat. Hence, they correspond to the description of *X. campestris* pv. undulosa. The fact that strains isolated from one host might be pathogenic for another underlines the need to perform pathogenicity tests before the strains are named.

Strains that come from diverse hosts but have similar RFLP patterns should be considered nearly identical strains. The study indicates that neopathotype strain NCPPB973 of *X. campestris* pv. translucens is very similar to strains isolated from bromegrass and identified as *X. campestris* pv. cerealis. Strain ribotyping might be an easy way to distinguish strains of *X. campestris* pv. cerealis from those of *X. campestris* pv. undulosa, but the pathogenicities of strains of both pathovars on a wider host range should be compared so that the pertinence of the distinction between the pathovars can be evaluated.

The cloned plasmid DNA fragment (pBSF2) revealed heterogeneity among strains isolated in one location on the same host (e.g., in Mexico). Also, it allowed the distinction of groups according to geographical origin, pathogenicity, and even host plant among *X. campestris* pathogens of small grains. This DNA fragment harbors pathogenicity genes of *X. campestris* pv. manihotis (21a) and could therefore account for a general mechanism of pathogenesis.

The utility of RFLP analysis for epidemiological studies of *X. campestris* pv. undulosa is underlined by the relationships that have been found among isolates from different geographical areas. In this study, strains from Mexico formed a homogeneous subgroup in ribotype 2, which also contains one strain from Zambia. The strain from Madagascar isolated from wheat grown from seed imported from Brazil is similar to other Brazilian strains. This emphasizes the potential risk of seedborne transmission, even if a zero level is not needed (19). Strains isolated from subsamples from the same seed lot, i.e., strains UPB727 and UPB728, Ethiopia, and strains UPB410, UPB412, and UPB426, Argentina, showed the same profiles. Nevertheless, the high diversity found among several locations reflects the intensive exchange of wheat germplasm in the world. This contrasts with other bacterial pathosystems, such as banana-*Pseudomonas solanacearum* or cassava-*X. campestris* pv. manihotis (22).

X. campestris pv. undulosa strains that are characterized as deviant by pathogenicity tests form a homogeneous cluster of strains that originated in South America. These strains did not react with monoclonal antibody AB3-B6, which is directed against the lipopolysaccharides (LPSs) of *X. campestris* pv. undulosa (4a). Moreover, they did not hybridize with the plasmidic probe pBSF2, which is related to the pathogenicity of *X. campestris* pv. manihotis. Also, by hybridization with an rRNA probe, they showed a pattern totally different from those of the other strains analyzed. They might be related to the *X. campestris* pv. translucens strain described by Ojanen et al. (18) as different from other reference strains on the basis of antigenicity and LPS profiles.

Further studies of more strains by means of different probes related to host specificity are needed to assess the genetic distances separating the different groups and to correlate groups with similar host ranges and mechanisms of pathogenicity.

ACKNOWLEDGMENTS

We thank E. Duveiller (International Center for Wheat and Maize Improvement [CIMMYT]) for reviewing the manuscript.

This research was funded by the Belgian Administration for Development Cooperation (BADC) and was a collaborative research effort of CIMMYT, ORSTOM (Unité de Phytopathologie, J.-P. Geiger), and UCL (Unité de Phytopathologie, Faculté des Sciences Agronomiques).

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