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Polyphasic Characterization of *Xanthomonas* Strains from Onion

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ABSTRACT

Gent, D. H., Schwartz, H. F., Ishimaru, C. A., Louws, F. J., Cramer, R. A., and Lawrence, C. B. 2004. Polyphasic characterization of *Xanthomonas* strains from onion. *Phytopathology* 94:184-195.

Xanthomonas leaf blight has become an increasingly important disease of onion, but the diversity among *Xanthomonas* strains isolated from onion is unknown, as is their relationship to other species and pathovars of *Xanthomonas*. Forty-nine *Xanthomonas* strains isolated from onion over 27 years from 10 diverse geographic regions were characterized by pathogenicity to onion and dry bean, fatty acid profiles, substrate utilization patterns (Biolog), bactericide resistance, repetitive sequence-based polymerase chain reaction fingerprinting, rDNA internally transcribed spacer (ITS) region, and *hrp b6* gene sequencing. Multiplication of onion *Xanthomonas* strain R-O177 was not different from *X. axonopodis* pv. *phaseoli* in dry bean, but typical common bacterial blight disease symptoms were absent in dry bean. Populations from each geographical region were uniformly sensitive to 100 µg of CuSO₄, 100 µg of ZnSO₄, and 100 µg of streptomycin sulfate per ml. Biolog substrate

utilization and fatty acid profiles revealed close phenotypic relatedness between onion strains of *Xanthomonas* and *X. axonopodis* pv. *dieffenbachiae* (57% of strains) and *X. arboricola* pv. *poinsetticola* (37% of strains), respectively. A logistic regression model based on fatty acid composition and substrate utilization classified 69% of strains into their geographical region of origin. Sequencing of a portion of the *hrp B6* gene from 24 strains and ITS region from 25 strains revealed greater than 97% sequence similarity among strains. DNA fingerprinting revealed five genotype groups within onion strains of *Xanthomonas* and a high degree of genetic diversity among geographical regions of origin. Based on pathogenicity to onion, carbon substrate utilization, fatty acid profiles, rDNA genetic diversity, and genomic fingerprints, we conclude that the strains examined in this study are pathovar *X. axonopodis* pv. *allii*. Implications of genetic and phenotypic diversity within *X. axonopodis* pv. *allii* are discussed in relation to an integrated pest management program.

Additional keywords: *Allium cepa*, *Allium fistulosum*, *Capsicum annuum*, *Phaseolus vulgaris*.

A leaf blight of onion caused by *Xanthomonas* (Pammel) Dowson strains has appeared as a major disease of onion since it was first described in Hawaii in 1978 (2). Disease symptoms are varied and include leaves with lenticular water-soaked lesions that elongate into chlorotic streaks, necrosis, tip dieback, and stunting of plants, which reduces bulb size. A bulb rot has never been reported and apparently does not occur, but yield losses of 19 to 100% have been reported (26,29,37).

Since its original description by Alvarez et al. (2), the disease has been reported in Texas (14), Colorado (36), California (26), Barbados (29), South Africa (38), Reunion Island, France (34), and Georgia (35). Unreported epidemics of the disease have also been observed in Venezuela and Brazil (R. Gitaitis, *personal communication*). A similar disease caused by strains classified as *X. striaformans* was reported on onion in southern Colorado in 1953 (40), but differed in 8 of 18 physiological characteristics from the *Xanthomonas* sp. described by Alvarez et al. (2). A bacterial blight of Welsh onion (*Allium fistulosum* L.) classified as *X. campestris* pv. *allii* has also been described in Japan (16), but its relationship to other strains causing disease on *A. cepa* L. is unknown.

The host range of *Xanthomonas* isolated from onion remains uncertain. Strains from Barbados are reportedly pathogenic to leguminous hosts such as snap bean (*Phaseolus vulgaris* L.), lima

bean (*Phaseolus lunatus* L.), soybean (*Glycine max* (L.) Merr.), winged bean (*Psophocarpus tetragonolobus* (L.) DC.), moth bean (*Vigna aconitifolia* (Jacq.) Marechal), and field pea (*Pisum sativum* L.) (27), as well as other *Allium* spp. including leek (*A. ampeloprasum* var. *porrum* L.), chives (*A. schoenoprasum* L.), garlic (*A. sativum* L.), and shallot (*A. cepa* var. *aggregatum*) (8). Strains from Hawaii, however, induced a hypersensitive response in snap bean (2). *X. campestris* pv. *allii* is nonpathogenic to chive and Chinese chive (*A. tuberosum* Rottler) (16).

The diversity among strains of *Xanthomonas* strains causing disease on onion is unknown, as is their relationship to other species and pathovars of *Xanthomonas*. Therefore, this study was initiated to characterize a broad collection of onion *Xanthomonas* strains from multiple geographic locations over many years by a polyphasic approach. A preliminary report of this work has been presented (12).

MATERIALS AND METHODS

Bacterial strains, culture, and DNA isolation. Forty-nine strains of *Xanthomonas* originally isolated from *A. cepa* or *A. fistulosum* were obtained from California, Texas, Colorado, Georgia, Hawaii, Brazil, Venezuela, Barbados, South Africa, and Japan, including the type strain of *X. campestris* pv. *allii* (MAFF 311173) (Table 1). Spontaneous rifampicin and streptomycin mutants of onion *Xanthomonas* strain O177 (ATCC 508) and *X. axonopodis* pv. *phaseoli* strain B458, respectively, were generated as described previously (44) and are referred to as R-O177 and AS-B458, respectively. All antibiotics were purchased from Sigma Chemical (St. Louis). Strain B458 was isolated from dry

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TABLE 1. *Xanthomonas* and *Stenotrophomonas* strains used in this study

| Strain | Other strain designations | Origin | | | Source ^a | ITS ^b | RFLP ^b | <i>hrp B6</i> ^b |
|--------------|---------------------------|----------------------------------|--------------|------|---------------------|------------------|-------------------|----------------------------|
| | | Host | Location | Year | | | | |
| A94-1b | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | ... | ... | ... |
| A118-2a | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | ... | ... | ... |
| A206-2a | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | ... | ... | + |
| A206-2b | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | + | ... | + |
| A206-5 | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | ... | ... | ... |
| A225-2 | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | ... | ... | ... |
| A227-2 | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | ... | ... | ... |
| A229-1 | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | + | + | + |
| A255-4 | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | ... | ... | ... |
| A256-3 | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | ... | ... | ... |
| A274-3 | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | ... | ... | ... |
| A274-7a | ... | <i>Allium cepa</i> | Hawaii | 1977 | A. Alvarez | ... | ... | + |
| A551-3 | ... | <i>Allium cepa</i> | Hawaii | 1980 | A. Alvarez | ... | ... | ... |
| A554-3 | ... | <i>Allium cepa</i> | Hawaii | 1980 | A. Alvarez | ... | ... | ... |
| A579-7 | ... | <i>Allium cepa</i> | Hawaii | 1980 | A. Alvarez | ... | ... | ... |
| A1889 | ... | <i>Allium cepa</i> | Hawaii | 1987 | A. Alvarez | ... | ... | ... |
| A1890 | ... | <i>Allium cepa</i> | Hawaii | 1987 | A. Alvarez | ... | ... | ... |
| JV 594 | CFBP 6362 | <i>Allium cepa</i> | Brazil | 1987 | O. Pruvost | + | ... | + |
| JV 595 | CFBP 6363 | <i>Allium cepa</i> | Brazil | 1987 | O. Pruvost | + | ... | + |
| ATCC 504 | O130 | <i>Allium cepa</i> | Colorado | 1996 | H. Schwartz | + | ... | + |
| ATCC 505 | O153 | <i>Allium cepa</i> | Colorado | 1996 | H. Schwartz | ... | ... | + |
| ATCC 506 | O154 | <i>Allium cepa</i> | Colorado | 1996 | H. Schwartz | + | + | + |
| ATCC 507 | O155 | <i>Allium cepa</i> | Colorado | 1996 | H. Schwartz | + | ... | + |
| ATCC 508 | O177 | <i>Allium cepa</i> | Colorado | 1998 | H. Schwartz | + | ... | + |
| B458 | ... | <i>Phaseolus vulgaris</i> | Colorado | 2000 | H. Schwartz | + | + | + |
| F2:22 | ... | <i>Allium cepa</i> | Barbados | 1996 | L. O'Garro | + | ... | + |
| JX 721 | CFBP 6387 | <i>Allium cepa</i> | Venezuela | 1997 | O. Pruvost | + | ... | ... |
| JX 727 | CFBP 6388 | <i>Allium cepa</i> | Venezuela | 2001 | O. Pruvost | + | ... | + |
| TX-1a | ... | <i>Allium cepa</i> | Texas | 1998 | T. Isakeit | ... | ... | ... |
| TX-2b | ... | <i>Allium cepa</i> | Texas | 1998 | T. Isakeit | + | + | ... |
| TX-2c | ... | <i>Allium cepa</i> | Texas | 1998 | T. Isakeit | ... | ... | ... |
| TX-3 | ... | <i>Allium cepa</i> | Texas | 1998 | T. Isakeit | + | ... | + |
| TX-10 | ... | <i>Allium cepa</i> | Texas | 1998 | T. Isakeit | + | ... | + |
| MAFF 311173 | ... | <i>Allium fistulosum</i> | Japan | 1998 | MAFF | + | ... | + |
| MAFF 311174 | ... | <i>Allium fistulosum</i> | Japan | 1998 | MAFF | + | ... | + |
| MAFF 311175 | ... | <i>Allium fistulosum</i> | Japan | 1998 | MAFF | ... | ... | + |
| MAFF 311176 | ... | <i>Allium fistulosum</i> | Japan | 1998 | MAFF | ... | ... | + |
| MAFF 311177 | ... | <i>Allium fistulosum</i> | Japan | 1998 | MAFF | ... | + | ... |
| MAFF 311178 | ... | <i>Allium fistulosum</i> | Japan | 1998 | MAFF | + | ... | ... |
| MAFF 311179 | ... | <i>Allium fistulosum</i> | Japan | 1998 | MAFF | ... | ... | ... |
| ATCC BAA 575 | BD 142 | <i>Allium cepa</i> | South Africa | 1999 | ARC-PPRI | + | ... | + |
| ATCC BAA 576 | BD 143 | <i>Allium cepa</i> | South Africa | 1999 | ARC-PPRI | + | ... | + |
| ATCC BAA 577 | BD 211 | <i>Allium cepa</i> | South Africa | 1999 | ARC-PPRI | ... | ... | ... |
| Xcu 200-2 | ... | <i>Allium cepa</i> | Georgia | 2000 | R. Gitaitis | ... | ... | ... |
| Xcu 01-1 | ... | <i>Allium cepa</i> | Georgia | 2001 | R. Gitaitis | + | ... | + |
| Xcu 01-2 | ... | <i>Allium cepa</i> | Georgia | 2001 | R. Gitaitis | + | ... | + |
| Calandri-1 | ... | <i>Allium cepa</i> | California | 2002 | R. Gilbertson | + | ... | ... |
| Calandri-3 | ... | <i>Allium cepa</i> | California | 2002 | R. Gilbertson | ... | ... | ... |
| Calon-1 | ... | <i>Allium cepa</i> | California | 2002 | R. Gilbertson | + | + | ... |
| Calon-5 | ... | <i>Allium cepa</i> | California | 2002 | R. Gilbertson | + | ... | + |
| ATCC 10547 | ... | <i>Dacus carota</i> | USA | ... | ATCC | ... | ... | ... |
| ATCC 11633 | ... | <i>Capsicum annuum</i> | USA | ... | ATCC | ... | ... | ... |
| ATCC 11672 | ... | <i>Xanthium strumarium</i> | India | 1950 | ATCC | ... | ... | ... |
| ATCC 11765 | ... | <i>Medicago sativa</i> | India | ... | ATCC | ... | ... | ... |
| ATCC 13461 | ... | <i>Holcus sp.</i> | Texas | 1978 | ATCC | ... | ... | ... |
| ATCC 13637 | ... | <i>Homo sapiens</i> | ... | 1960 | ATCC | ... | ... | ... |
| ATCC 19047 | ... | <i>Piper betle</i> | India | 1964 | ATCC | ... | ... | ... |
| ATCC 19312 | ... | <i>Axonopus scoparius</i> | Columbia | ... | ATCC | ... | ... | ... |
| ATCC 19313 | ... | <i>Corylus maxima</i> | Oregon | ... | ATCC | ... | ... | ... |
| ATCC 29088 | ... | <i>Lolium multiflorum</i> | Switzerland | 1975 | ATCC | ... | ... | ... |
| ATCC 29091 | ... | <i>Dactylis glomerata</i> | Switzerland | 1975 | ATCC | ... | ... | ... |
| ATCC 33913 | ... | <i>Brassica oleracea</i> | UK | ... | ATCC | ... | ... | ... |
| ATCC 35937 | ... | <i>Lycopersicon lycopersicum</i> | New Zealand | ... | ATCC | ... | ... | ... |
| ATCC 49079 | ... | <i>Raphanus sativus</i> | USA | ... | ATCC | ... | ... | ... |
| ATCC 49083 | ... | <i>Juglans regia</i> | New Zealand | ... | ATCC | ... | ... | ... |
| ATCC 49119 | ... | <i>Phaseolus vulgaris</i> | USA | 1989 | ATCC | ... | ... | ... |
| ATCC 49120 | ... | <i>Citrus sp.</i> | Florida | 1989 | ATCC | ... | ... | ... |

^a Source: H. Schwartz, Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins; R. Gilbertson, Department of Plant Pathology, University of California, Davis; T. Isakeit, Department of Plant Pathology and Microbiology, Texas A&M University, College Station; MAFF = Ministry of Agriculture, Forestry, and Fisheries of Japan, Okinawa; ATCC = American Type Culture Collection, Manassas, VA; L. O'Garro, Department of Biology, University of the West Indies, Bridgetown, Barbados; A. Alvarez, Department of Plant Pathology, University of Hawaii, Honolulu; R. Gitaitis, Department of Plant Pathology, University of Georgia, Tifton; ARC-PPRI = Agricultural Research Council-Plant Protection Research Institute, Pretoria, South Africa; O. Pruvost, Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Reunion Island, France.

^b Internal transcribed spacer (ITS); restriction fragment length polymorphism (RFLP). + Indicates strain was used for this analysis; ... Indicates strain was not used for this analysis.

bean pinto cv. Bill Z in southwestern Colorado. Strain R-O177 and AS-B458 are resistant to greater than 200 µg of rifampicin or streptomycin per ml, respectively, but selection routinely was performed on nutrient agar amended with 50 µg of the appropriate antibiotic per ml. Other strains were routinely cultured on nutrient agar or broth lacking antibiotic during incubation at 26°C, and bacterial strains were preserved in 15% nutrient glycerol broth at -80°C for long-term storage.

Strains were cultured in 1.5 ml of nutrient broth for 24 h for DNA isolation procedures. The culture was adjusted to an optical density of 0.1 at 600 nm in sterile 0.02 M potassium phosphate buffer (PB), pH 7.2, and DNA was isolated with the CTAB (hexadecyltrimethylammonium bromide) method (4). DNA was stored in Tris-EDTA (TE) buffer (10 mM Tris and 1 mM EDTA, pH 8.0) at -20°C.

Pathogenicity and host range. All strains were tested for pathogenicity on *A. cepa* (cv. Blanco Duro) in growth chamber assays. Pathogenicity on *A. fistulosum* (cv. White Welsh) was confirmed with representative strains from nine geographical regions, including O177, Calon-1, TX-3, ATCC BAA 576, Xcu 01-1, MAFF 311173, A229-1, JV 594, and JX 727. Colonies of the strain to be tested were inoculated into 3 ml of nutrient broth in 15-ml culture tubes and were incubated at 26°C with vigorous shaking (250 oscillations per min) for 24 h. The cultures were adjusted to approximately 10⁷ CFU/ml in sterile PB before spraying (Crown SpraTool, Aerovoe Industries, Inc., Gardnerville, NV) the foliage of 6- to 8-week-old plants to runoff with the bacterial suspension. Control plants were inoculated with sterile PB. The plants were placed in a growth chamber and incubated for 7 days with a 28°C/24°C day/night temperature regime, light intensity of 350 µMs⁻¹m⁻², 100% relative humidity, and daily misting with tap water to runoff. At least four plants were inoculated with each strain. Plants were observed daily for symptom development, and the pathogen was isolated from characteristic lesions by grinding leaf sections in 1 ml of PB with a sterile mortar and pestle and streaking loopfuls of the homogenate onto nutrient agar. The pathogen was confirmed as *Xanthomonas* by carbon and nitrogen substrate utilization patterns on Biolog GN Microplates (Biolog, Inc., Hayward, CA) as described previously (43).

Pathogenicity on dry bean (cv. Sacramento Light Red Kidney) was also evaluated by the spray inoculation method described for onion, except plants were kept in the growth chamber for 14 days. All plants were 4 to 5 weeks old when inoculated.

Multiplication of the rifampicin resistant onion strain R-O177 and streptomycin resistant bean strain AS-B458 in inoculated dry bean, pepper (*Capsicum annuum* L., cv. Sweet California Wonder), and onion were quantified as described previously (27). Briefly, a single colony of each pathogen was picked from a 72-h nutrient agar plate and grown overnight in nutrient broth (26°C and 250 oscillations per min) to the mid-logarithmic phase. The culture was adjusted to approximately 10⁸ CFU/ml spectrophotometrically ($A_{600} = 0.12$) with sterile PB, diluted to 10⁴ CFU/ml in sterile PB, and pressure infiltrated into the youngest, fully expanded bean and pepper leaflets of 4- to 5-week-old plants with a sterile 1-ml syringe. Trace levels of nutrient broth (1 µl or less per 10 ml of sterile PB) from the cultivation media were also introduced into the plant during inoculation but were assumed not to contribute to bacterial replication in planta. Five 20-mm² leaf disks were immediately removed (each from a different leaflet) with an ethanol-sterilized cork borer and surface-disinfested in 95% ethanol followed by several rinses in sterile PB before grinding individually in 100 µl of PB with a sterile mortar and pestle. The homogenate was serially diluted in sterile PB, and 100-µl droplets were plated in duplicate onto nutrient agar with the appropriate selection antibiotic. Plants serving as controls were infiltrated with sterile PB, and the homogenate was plated onto nutrient agar. Leaf disks were removed daily for 7 days to generate population growth curves.

Populations in onion were determined as described by O'Garro and Paulraj (27). Briefly, the youngest, fully extended leaves of 8-week-old onion plants (cv. Blanco Duro) were pinpricked seven times at 1-cm intervals with a 22-gauge needle bearing strain R-O177 or AS-B458 removed from 72-h-old nutrient agar culture plates. Each pinpricked leaf area was inoculated with a bacterial matrix approximately equal in size to the needle tip. Leaf sections (5 by 1 cm in length), each having an inoculated area, were removed every 2 days, surface-disinfested in 95% ethanol, rinsed in sterile PB, and ground aseptically in 1 ml of sterile PB with a mortar and pestle. Recoveries were done up to 14 days after inoculation to generate population growth curves. The homogenate was serially diluted and plated onto nutrient agar amended with 50 µg of the appropriate selection antibiotic per ml. Colonies were enumerated after 72 h of incubation at 26°C, and characteristic colonies were confirmed as strain R-O177 or AS-B458 by genomic fingerprinting with BOX primers as described previously (20). Pathogenicity of recovered onion *Xanthomonas* strain R-O177 and *X. axonopodis* pv. *phaseoli* strain AS-B458 on onion cv. Blanco Duro and dry bean cv. Sacramento Light Red Kidney, respectively, was conducted as described previously.

Copper, zinc, and streptomycin resistance screening. The minimum inhibitory concentrations (MIC) of CuSO₄, ZnSO₄, and streptomycin sulfate were determined using a modification of the broth microdilution test guidelines from the National Committee for Clinical Laboratory Standards (25). Serial dilutions (1:2) of each bactericide were made in nutrient broth in round-bottom 96-well plates to a final volume of 100 µl per well. Each well was inoculated with 5 µl of a 10⁷ CFU/ml suspension of *Xanthomonas* sp. from a 3-ml nutrient broth overnight culture. The MIC was considered the lowest concentration of each bactericide that completely inhibited visual growth after 24 h. *Xanthomonads* are generally considered resistant to copper, zinc, or streptomycin if they are able to grow on artificial media amended with 100 to 200 µg of a given bactericide per ml (6,7,17,24,39). In this study, strains were considered resistant to CuSO₄, ZnSO₄, or streptomycin sulfate if the MIC was greater than 100 µg/ml. *X. axonopodis* pv. *vesicatoria* strains 81-23 and E3 (provided by J. Jones, University of Florida) were included as copper and streptomycin resistant positive controls, respectively.

Substrate utilization and fatty acid methyl ester profiles. Substrate utilization and fatty acid methyl ester profiles were generated for all 49 onion *Xanthomonas* strains presented in Table 1 by Microbe Inotech Laboratories, Inc. (St. Louis) by standard procedures (42,43). Cultures were streaked onto trypticase soy broth agar at 26°C for 24 h before inoculating onto Biolog GN microplates according to Biolog protocols for gram-negative aerobic bacteria. The resulting metabolic fingerprints were read by an automated plate reader and compared with version 4.1 of the Biolog database. Fatty acid compositions were determined by gas chromatographic analysis by standard methods and compared with the Microbial Identification Aerobe (version 4.1) and Clinical Aerobe (version 4.0) databases (MIDI, Newark, DE).

***hrp B6* gene amplification and sequencing.** A portion of the highly conserved putative ATPase gene *hrp B6* was amplified from extracted genomic DNA with oligonucleotide primers RST2 (5'-AGGCCCTGGAAGGTGCCCTGGA-3') and RST3 (5'-ATC-GCACTGCGTACCGCGCGCA-3'), which direct the amplification of an approximately 840-bp fragment (11,18). DNA was extracted from 24 strains (Table 1) as described previously; one to five representative strains from each geographic location were selected for the experiment. A polymerase chain reaction (PCR) product for this gene is expected from most phytopathogenic xanthomonads, but is not expected from nonpathogenic xanthomonads that lack the *hrp* gene cluster. DNA was amplified in reaction mixtures containing 12.5 µl of PCR Master Mix (Promega, Madison, WI), 1 µM each primer, 100 ng of CTAB extracted DNA, and PCR-grade water to a total volume of 25 µl. PCR

amplification cycle conditions were as described by Leite et al. (18). Amplified DNAs were separated by electrophoresis in 1.0% agarose (Bio-Rad Laboratories, Hercules, CA) gels in Tris-acetate buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.2). Ethidium bromide (0.5 µg/ml) was added to each gel, and the DNA fragments were visualized over a UV transilluminator (Fotodyne, Hartland, WI). The approximate 840-bp amplicon was gel purified (Qiagen MinElute Gel Extraction Kit, Qiagen, Inc., Valencia, CA) and sequenced (Davis Sequencing, Davis, CA). Sequence alignment, calculation of similarity values, and cluster analysis were performed with MegAlign software (DNASTAR, Inc., Madison, WI). The National Center for Biotechnology Information GenBank nonredundant database was searched for sequence similarity using the BLASTn algorithm (1).

16S and intergenic spacer region restriction fragment length polymorphism and sequencing. PCR experiments were performed as described previously (22) to amplify the entire 16S and 16S-23S rDNA internally transcribed spacer (ITS) region or only the 16S-23S rDNA ITS region using universal primer pairs 27F (5'-AGAGTTTGTATCCTGGCTCAG-3')/FGPS-132 (5'-CCGGGTTTCCCCATTCCGG-3') or G1 (5'-GAAGTCGTAACAAAGG-3')/L1 (5'-CAAGGCATCCACCGT-3'), respectively. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA). One to five representative strains from each geographic location were used in each experiment. A 10-µl aliquot of the resulting DNA fragment from the 16S and ITS region amplified by primers 27F and FGPS-132 was restricted with *EcoRI* or *HaeIII* as recommended by the manufacturer (Promega) and separated by electrophoresis in 1.0% agarose (Bio-Rad Laboratories) gels, visualized, and photographed as described previously. The ITS amplicon was gel purified (Qiagen) and sequenced (Davis Sequencing). Sequence alignment, calculation of similarity values, and cluster analysis were performed with MegAlign software. The National Center for Biotechnology Information GenBank nonredundant database was searched for sequence similarity using the BLASTn algorithm (1).

Repetitive sequence based-PCR genomic fingerprinting. Genomic fingerprints were determined for each strain as described by Louws et al. (19–21) using primers corresponding to prokaryotic enterobacterial repetitive intergenic consensus (ERIC)

sequences, repetitive extragenic palindromic (REP) sequences, and the BOXA subunit of the BOX element. *Stenotrophomonas maltophilia* was included as a positive control. Additionally, 45 other strains of phytopathogenic xanthomonads representative of the DNA:DNA homology groups described by Vauterin et al. (41) were included to determine the relationship of onion strains of *Xanthomonas* to other species and pathovars of *Xanthomonas*. Seventeen of these strains are included in this report (Table 1).

Images were imported into GelCompar software (version 4.1, Applied Maths, Kortrijk, Belgium) and linearly combined, and similarity was calculated using Pearson's correlation coefficient applied to the entire densitometric curves of the gel tracks as described previously (31). Gels were standardized with a 1-kb DNA molecular weight ladder (Invitrogen Corp., Carlsbad, CA). Cluster analysis was performed by the unweighted pair group method with arithmetic averages clustering. All PCR reactions were repeated at least twice.

Statistical analysis. Analysis of in planta population growth was conducted with SAS version 8.0 (PROC MIXED, SAS Institute, Cary, NC) to generate means and standard errors of log-transformed data. Factor analysis was conducted on merged Biolog and fatty acid profile data sets to generate 10 unique and uncorrelated factors (SAS PROC FACTOR) with orthogonal varimax rotation. These factors were subsequently used in logistic regression (PROC LOGISTIC) with stepwise selection to predict the region of origin of each strain. Cluster and principal component analysis of fatty acid composition content and substrate utilization profiles were conducted with MINITAB 13 (Minitab, Inc., State College, PA) using the unweighted pair group method with arithmetic averages clustering. The percent color change in each Biolog GN2 MicroPlate well, compared with the water control, was used in cluster and principal component analysis. Percent compositions of each fatty acid were used in the analysis of fatty acid profiles.

RESULTS

Pathogenicity and host range. All onion strains of *Xanthomonas* used in this study were pathogenic on onion except strains

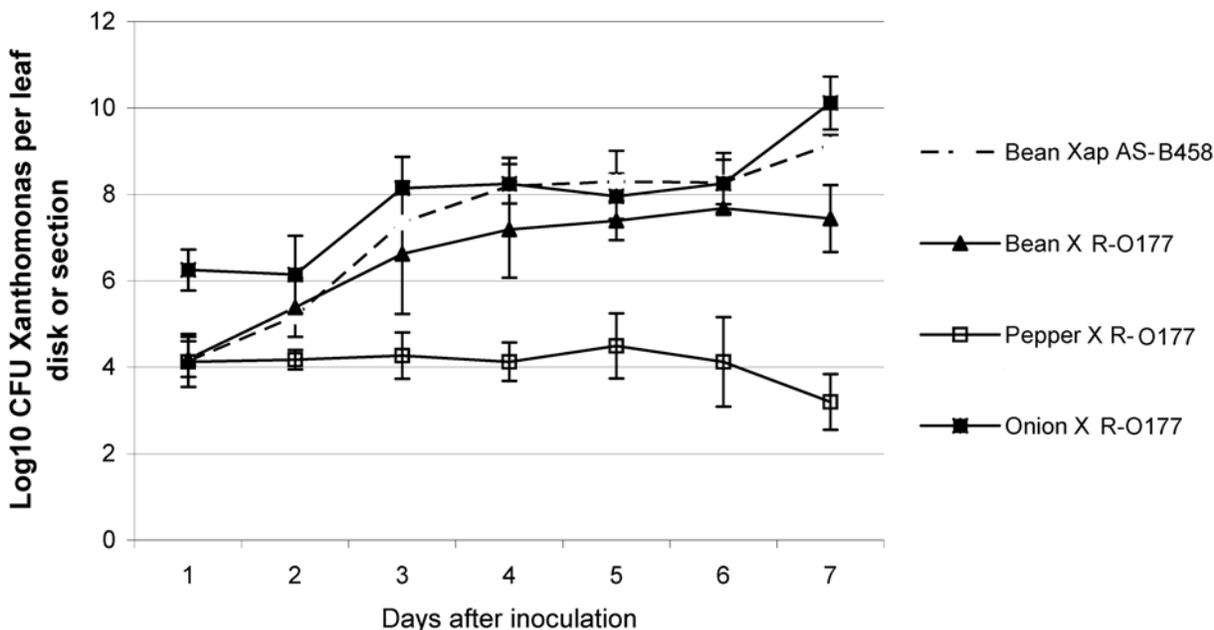


Fig. 1. Population dynamics of onion *Xanthomonas* strain R-O177 and *X. axonopodis* pv. *phaseoli* strain AS-B458 in dry bean, onion, and pepper. Populations of *Xanthomonas* strain R-O177 in onion leaf sections were enumerated every 2 days for 14 days, but are scaled to 7 days for comparison. AS-B458 populations decreased to less than 10^3 CFU per leaf disk or section 7 days after inoculation in pepper and onion, respectively (data not shown). Data are means of five replications repeated twice ($n = 10$) \pm standard error of the mean. X = *Xanthomonas*; Xap = *Xanthomonas axonopodis* pv. *phaseoli*.

A1889, A1890, and TX-2b. Strain TX-2b was identified as *X. campestris* pv. *carotae* by repetitive sequence-based (rep)-PCR, Biolog, and ITS sequence and produced typical bacterial blight symptoms on carrot (*Daucus carota* L.). Characteristic lenticular-shaped, water-soaked lesions also developed on *A. fistulosum* within 7 days following inoculation with representative strains from nine geographical regions. Disease symptoms were not observed with any onion strain of *Xanthomonas* on dry bean

cv. Sacramento Light Red Kidney, but typical common bacterial blight symptoms were observed on dry bean 5 days after inoculating with *X. axonopodis* pv. *phaseoli* strain AS-B458.

Populations of onion *Xanthomonas* strain R-O177 increased in planta in dry bean and onion, but not in pepper (Fig. 1). Populations of *X. axonopodis* pv. *phaseoli* strain AS-B458 and onion *Xanthomonas* strain R-O177 increased in dry bean to greater than 10^8 and 10^7 CFU per 20-mm² leaf disk, respectively, 7 days after

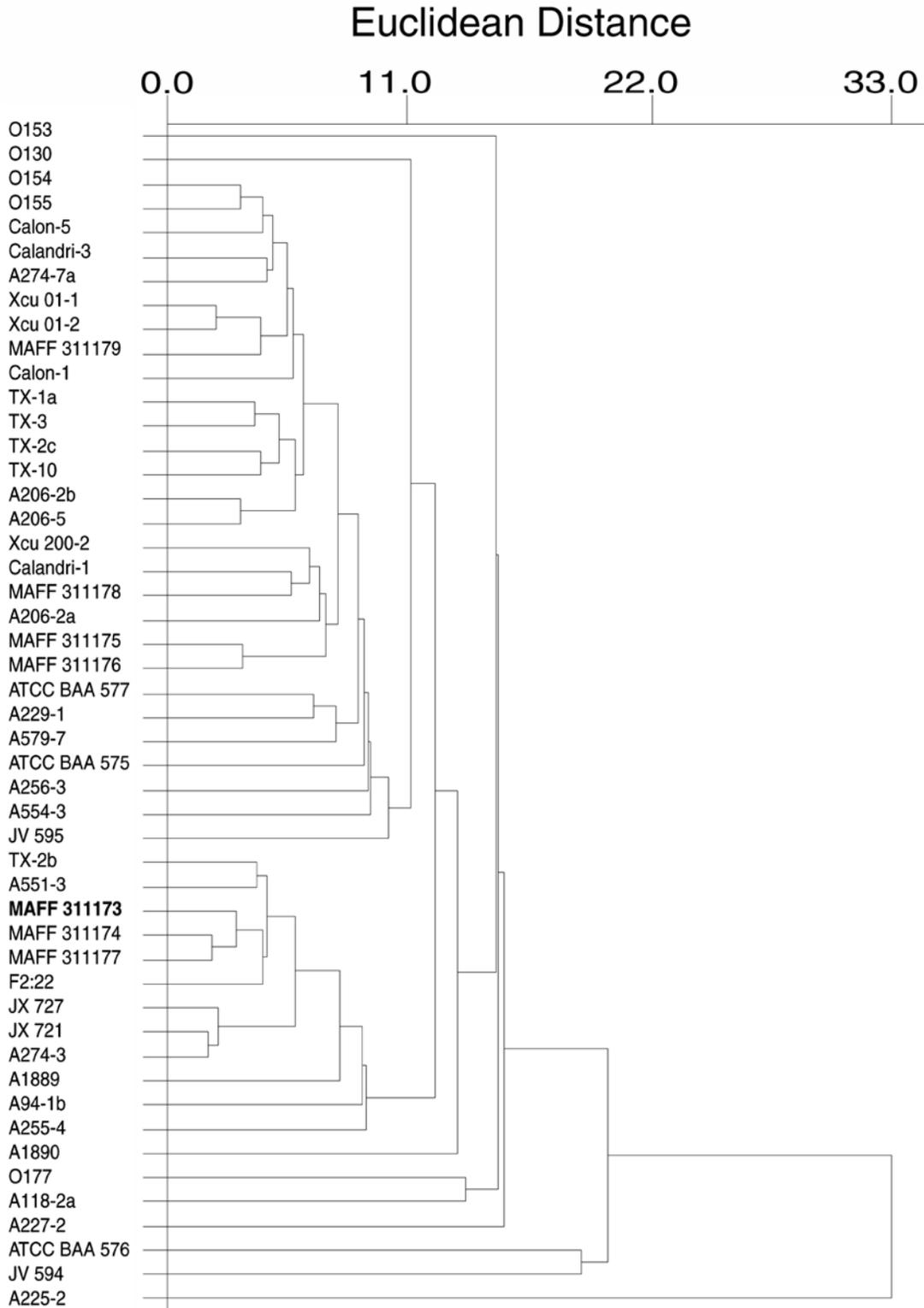


Fig. 2. Relationships among 49 onion *Xanthomonas* strains isolated from various geographical regions based on Biolog GN Microplate (Biolog, Inc., Hayward, CA) substrate utilization patterns. The type strain of *X. campestris* pv. *allii* MAFF 311173 is noted in bold.

inoculation. Strain R-O177 increased to greater than 10⁸ CFU per 1-cm leaf section in onion during the study time course. Populations of *X. axonopodis* pv. *phaseoli* decreased to less than 10³ CFU per sample area in onion and pepper 7 days after inoculation (data not shown).

Copper, zinc, and streptomycin resistance screening. Resistance to copper, zinc, or streptomycin was not observed among onion strains of *Xanthomonas*; all were inhibited by 100 µg/ml or less of copper sulfate, zinc sulfate, or streptomycin. All strains were highly sensitive to streptomycin and were inhibited by 1.6 µg/ml or less.

Substrate utilization and fatty acid methyl ester profiles. A diversity of substrate utilization and fatty acid methyl ester profiles was observed in onion strains of *Xanthomonas*. Mean Biolog and fatty acid profile similarity indices of the 49 onion *Xanthomonas* strains tested were 0.62 and 0.78, respectively, with the *X. axonopodis* profile. Corresponding distance coefficients for Biolog and fatty acid profiles were 4.428 and 2.581, respectively, compared with the *X. axonopodis* profile. The closest pathovar matches within the Biolog database were *X. axonopodis* pv. *dieffenbachiae* (57%), *X. axonopodis* pv. *begonia* (16%), *X. axonopodis* pv. *phaseoli* (4%), and *X. axonopodis* pv. *malvacearum* (4%). Nearly 12% of the strains did not have a closest match and were simply identified as *X. axonopodis*. The MIDI Aerobe and Clinical Aerobe fatty acid databases indicated that onion strains of *Xanthomonas* were most closely related to *X. arboricola* pv. *poinsetticola* (37%), *X. axonopodis* pv. *dieffenbachiae* (18%), and *X. axonopodis* pv. *citrumelo* (16%).

Biolog substrate utilization profiles revealed diversity in carbon and nitrogen utilization patterns by onion strains of *Xanthomonas*, but this diversity was not fully explained by geographic origin (Fig. 2). Euclidean distances among strains were less than 13 with the exception of strains JV 594 (Brazil), ATCC BAA 576 (South Africa), and A225-2 (Hawaii). Principal component analysis did not reveal distinct clustering of strains (data not shown).

Onion strains of *Xanthomonas* contained high proportions of 15:0 ISO (26.91%) and 15:0 ISO 2OH/16:1ω7c (18.87%) fatty acids. The 10:0, 10:0 2OH, 11:0 3OH, 12:0, 13:0 ISO, 13:0 2OH, 17:1 ω6c, 17:1 10methyl, and 17:0 ISO 3OH fatty acids comprised less than 1.22% of the total fatty acid profile or were absent in all strains. Table 2 presents fatty acid composition profiles for five representative strains that characterize the diversity of fatty acid profiles in the 49 onion strains of *Xanthomonas*

included in this study. Cluster analysis of fatty acid profiles revealed phenotypic diversity in onion strains of *Xanthomonas* that was partially explained by geographic origin (Fig. 3). Euclidean distances among strains were generally less than 5, except for six strains from Hawaii and strains O177, TX-2b, Xcu 01-1, and F2:22, suggesting little diversity in fatty acid composition. Strains from California formed a distinct group that also included Georgia strain Xcu 200-2. Japanese strains formed two distinct but highly related subgroups (Euclidean distance less than 4), but other strains were not grouped solely by geographic origin. Strains A1889 and A1890, which were not pathogenic to onion, had a nearly identical fatty acid profile but were dissimilar from all other evaluated strains. Additionally, principal component analysis did not reveal distinct clustering of strains (data not shown). Several fatty acids were only occasionally recovered from strains or were present at low levels and may have obscured the variability among geographical regions, reducing the power of principal component analysis to separate strains by geography. Fatty acid methyl ester profiles alone were not useful for determining geographic origin of all strains.

Factor analysis of combined Biolog substrate utilization and fatty acid profile data and subsequent logistic regression yielded a model with two factors (Factors 1 and 9) that correctly placed 69.3% of strains into their geographical region; 23.5% were misclassified and 7.2% were tied. The likelihood equations for each equation were computed as $Z = \beta_0 + 0.782(\text{Factor 1}) + 0.865(\text{Factor 2})$, where β_0 is the cumulative logit intercept for each region given in Table 3. The probability of group membership of a strain in a region (Z_i) is described by $p(R_i) = e^{Z_i}/(1 + e^{Z_i})$, where e is the natural logarithm. The probability of group membership in Venezuela is given by $p(R_{\text{Venezuela}}) = 1 - p(R_{\text{Texas}})$.

hrp B6 gene amplification and sequencing. A single DNA fragment was amplified with all onion *Xanthomonas* strains examined in this study. Sequence similarity of 97 to 98% was observed with onion *Xanthomonas* strains compared with the partial *hrp* gene sequence of *X. axonopodis* pv. *vesicatoria* (Accession No. U33548), which was previously deposited in GenBank (10). Cluster analysis revealed five distinct groups that generally grouped by geographical region of origin (data not shown). Strains from Texas, South Africa, and Colorado did not group solely by geographical region of origin.

16S and ITS region restriction fragment length polymorphism and sequencing. All onion strains of *Xanthomonas*

TABLE 2. Cellular fatty acid composition of five representative onion strains of *Xanthomonas* sp. from different geographical regions and belonging to five repetitive sequence based-polymerase chain reaction (rep-PCR) genotype groups

| Strain ^a | rep-PCR genotype | % Fatty acid composition | | | | | | | | | | | | | | |
|---------------------|------------------|--------------------------|----------|----------|--------------|----------|----------|--------------|----------|----------|--------------|----------|------------|----------|--------------|-------|
| | | 10:0 | 11:0 iso | 10:0 3OH | 11:0 ISO 3OH | 11:0 3OH | 13:0 ISO | 12:0 ISO 3OH | 12:0 3OH | 14:0 ISO | 13:0 ISO 3OH | 13:0 2OH | 15:1 ISO F | 15:0 ISO | 15:0 ANTEISO | |
| TX-10 | 1 | 1.02 | 3.57 | 0.32 | 1.68 | ... | ... | ... | 3.26 | 0.35 | 1.88 | 3.39 | ... | ... | 26.63 | 6.82 |
| O177 | 2 | 0.92 | 3.68 | 0.38 | 1.50 | ... | ... | ... | 2.27 | ... | 1.80 | 2.50 | ... | ... | 29.37 | 7.48 |
| Calon-5 | 3 | 0.79 | 3.63 | 0.33 | 1.66 | 0.16 | 0.31 | 0.21 | 2.71 | 0.32 | 1.57 | 3.61 | 0.28 | 0.20 | 28.22 | 8.61 |
| MAFF 311173 | 4 | 0.78 | 4.13 | 0.34 | 1.80 | ... | ... | ... | 2.91 | 0.34 | 1.42 | 3.96 | 0.29 | ... | 29.10 | 8.16 |
| JV 594 | 5 | 1.22 | 4.07 | 0.31 | 1.46 | ... | ... | 0.27 | 2.50 | 0.71 | 1.66 | 2.43 | ... | ... | 25.43 | 11.57 |

| Strain | rep-PCR genotype | % Fatty acid composition | | | | | | | | | | | | | |
|-------------|------------------|--------------------------|----------|----------|----------|------|----------|----------|--------------|----------|----------|----------|--------------|---------------------|---------|
| | | 15:1 ω6c | 15:0 ISO | 16:0 ISO | 16:1 ω9c | 16:0 | 17:1 ω9c | 17:0 ISO | 17:0 ANTEISO | 17:1 ω8c | 18:1 ω9c | 18:1 ω7c | 17:0 ISO 3OH | Sum F3 ^b | Unknown |
| TX-10 | 1 | ... | 1.52 | 1.86 | 2.16 | 7.19 | 4.86 | 8.36 | 0.52 | 1.02 | 0.77 | 0.91 | ... | 20.34 | 1.60 |
| O177 | 2 | ... | 1.74 | 1.56 | 1.76 | 6.99 | 4.94 | 8.02 | 0.51 | 0.96 | 0.69 | ... | ... | 19.98 | 2.96 |
| Calon-5 | 3 | ... | 1.49 | 1.93 | 2.02 | 6.91 | 4.81 | 8.24 | 0.64 | 0.94 | 0.66 | 0.66 | 0.30 | 17.56 | 1.22 |
| MAFF 311173 | 4 | 0.28 | 1.33 | 2.04 | 1.55 | 6.17 | 5.28 | 8.07 | 0.62 | 0.95 | 0.55 | 0.59 | ... | 18.18 | 1.17 |
| JV 594 | 5 | 0.47 | 1.66 | 3.69 | 1.90 | 5.47 | 4.54 | 6.06 | 0.69 | 1.09 | 0.65 | 0.67 | ... | 18.97 | 2.50 |

^a Fatty acid composition data for 5 of the 49 onion *Xanthomonas* strains analyzed that represent the diversity of fatty acid profiles observed among strains pathogenic to onion.

^b Sum F3 = summed feature 3 representing 16:1ω7c and 15 ISO 2OH fatty acids. Fatty acids 10:0 2OH, 12:0, 17:1 ω6c, and 17:1 10methyl were occasionally detected in some strains but comprised less than 1% of the total fatty acid profile. The representative strains presented lacked these four fatty acids and are not shown.

analyzed contained a single 16S-23S ITS region. Sequencing of the PCR amplified ITS region yielded an approximately 600-bp amplicon with greater than 99% sequence similarity to the ITS region of *X. axonopodis* pv. *citrumelo* strain F1 (Accession No. AF442741), which was previously deposited in GenBank (9). All strains differed from *X. axonopodis* pv. *citrumelo* strain F1 by 5 bp or less except for strains A299-1 (9 bp) and TX-2b (18 bp). The ITS sequence of strain TX-2b was 100% similar to Accession No. AF279428 from *X. campestris* pv. *carotae* (5). Onion strains of *Xanthomonas* also appear to contain the transfer RNA (tRNA) genes tRNA^{Ala} and tRNA^{Ile}.

Variability within the ITS region was limited among onion strains of *Xanthomonas*. Greater than 98% sequence similarity was observed among strains evaluated, but strains from Japan, California, Brazil, Venezuela, Texas, Hawaii, and South Africa generally grouped by geographical region of origin in cluster analysis (data not shown). Strains from Colorado and Georgia did not group by region.

No polymorphisms were detected among onion strains of *Xanthomonas* evaluated when the entire 16S + ITS region was amplified and subsequently restricted with *EcoRI* or *HaeIII*. However, *X. axonopodis* pv. *phaseoli* strain B458 did exhibit a polymorphic

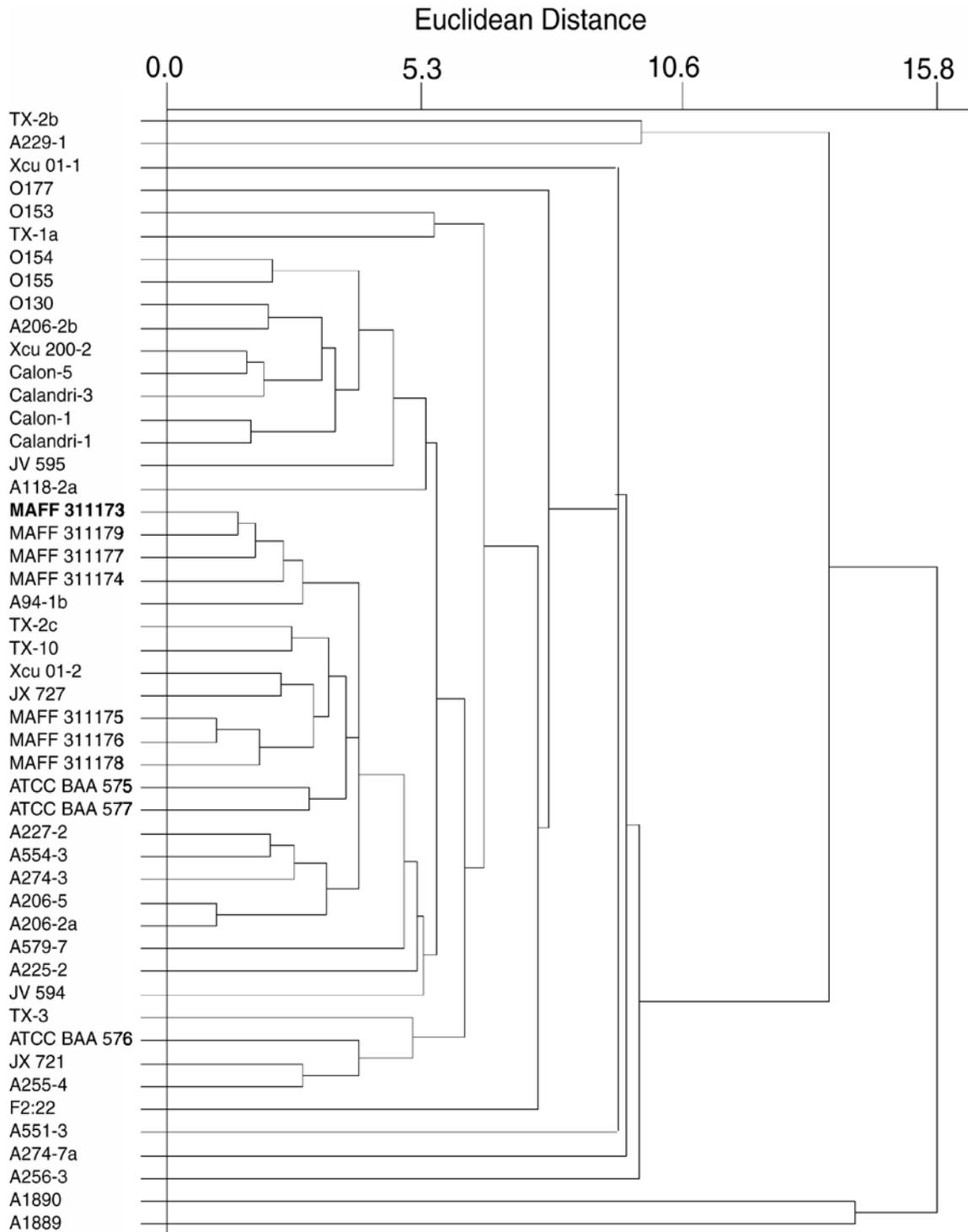


Fig. 3. Relationships among 49 onion *Xanthomonas* strains isolated from various geographical regions based on fatty acid composition. The type strain of *X. campestris* pv. *allii* MAFF 311173 is noted in bold.

band, approximately 150 bp, compared with onion strains of *Xanthomonas* when restricted with *Hae*III (data not shown), suggesting onion strains of *Xanthomonas* are genetically distinct from *X. axonopodis* pv. *phaseoli*.

rep-PCR genomic fingerprinting. Complex DNA fingerprints were generated from genomic DNA extracted from 49 onion *Xanthomonas* strains, 45 other *Xanthomonas* species and pathovars, and the *Stenotrophomonas maltophilia* positive control (Fig. 4). Amplified DNA fragments ranged in size from approximately 200 bp to greater than 4 kb and revealed a high degree of genetic diversity among onion strains of *Xanthomonas*. DNA fingerprint similarity among onion *Xanthomonas* strains ranged from greater than 98% to less than 50%. Onion *Xanthomonas* strains formed five distinct genotypes that were largely but not entirely explained by geographical origin. The majority of strains (30%) belonged to genotype 2, which all originated from Hawaii except for Colorado strain O177. Genotype 4 strains comprised 25% of the strains analyzed and represented strains originating from California, Georgia, Colorado, and Venezuela. Seven strains originally isolated from *A. fistulosum* in Japan, including the type strain of *X. campestris* pv. *allii*, comprised genotype 4. Thirteen percent of the strains comprised the heterogeneous genotype 5 and included strains from Brazil, South Africa, Barbados, and Texas strain TX-2c. Genotype 1 comprised three strains (6%) from Texas. TX-2b had a nearly identical DNA fingerprint as the *X. campestris* pv. *carotae* type strain, and produced typical bacterial leaf blight symptoms on carrot, but was not pathogenic to onion. Strains A1890, A1889, and A91-1a did not cluster with the five other onion *Xanthomonas* genotypes.

Eight rep-PCR DNA fragments were common to the five genotype groups of onion *Xanthomonas* strains (Fig. 5) and appear to be signature bands of these strains. Strains pathogenic on onion share these DNA fragments, including *X. campestris* pv. *allii* strains, independent of geographical region of origin. All eight of these bands are present in the *X. axonopodis* pv. *citrumelo* type strain, but seven bands are also present in *X. axonopodis* pv. *alfalfae* and *X. axonopodis* pv. *betlicola*. The DNA fingerprint of *X. axonopodis* pv. *citrumelo* was nearly identical to Colorado strain O177 and grouped within genotype 2 (Figs. 4 and 6). The *X. axonopodis* pv. *betlicola* type strain DNA fingerprint was also highly similar to genotype 2 strains. *X. axonopodis* pv. *alfalfae* grouped with genotype 4 strains from Japan, and *X. axonopodis* pv. *vesicatoria* grouped with the heterogeneous genotype 5 strains. The other pathovars of *Xanthomonas* that grouped with onion *Xanthomonas* strains all belong to DNA homology group 9-2 (41), providing strong evidence that onion *Xanthomonas* strains also belong within DNA homology group 9-2.

DISCUSSION

In this paper, we describe the phenotypic and genetic diversity among strains of *Xanthomonas* causing *Xanthomonas* leaf blight of onion and the relationship of these strains to other described *Xanthomonas* pathovars and species. Of particular importance is the relationship of onion *Xanthomonas* strains to the described pathovar *X. campestris* pv. *allii*. We conclude that the onion *Xanthomonas* strains examined in this study are pathovar *X. campestris* pv. *allii* based upon pathogenicity to onion, as well as phenotypic (carbon substrate utilization, fatty acid profiles) and genetic (*hrp b6* gene sequence, 16S rDNA restriction fragment length polymorphism [RFLP] profile, ITS sequence, and rep-PCR-mediated DNA fingerprints) similarities. A pathovar is designated solely based on distinctive pathogenicity to one or more plant hosts (10). The *X. campestris* pv. *allii* and onion *Xanthomonas* strains included in this study were indistinguishable based on pathogenicity to *A. cepa* and *A. fistulosum* and, therefore, satisfy the basic requirement for pathovar designation. DNA fingerprinting by rep-PCR demonstrated that although con-

siderable genetic diversity is present within onion *Xanthomonas* strains, the strains included in this study clearly group with and belong to the described pathovar *X. campestris* pv. *allii*. No onion strains of *Xanthomonas*, including the type strain of *X. campestris* pv. *allii*, grouped with the *X. campestris* DNA homology group 15 strains. However, they all grouped within the *X. axonopodis* DNA homology group 9-2, indicating the correct species epithet should be *X. axonopodis* and not *X. campestris*. Therefore, we propose onion strains of *Xanthomonas* be classified as *X. axonopodis* pv. *allii* to correctly represent their genotypic and phylogenetic relationship to other *X. axonopodis* species within DNA homology group 9-2. Onion strains of *Xanthomonas* are hereafter referred to as *X. axonopodis* pv. *allii*. The results of this polyphasic characterization and transfer of *X. campestris* pv. *allii* strains to *X. axonopodis* pv. *allii* are in agreement with similar research independently conducted by Rougmanac et al. (33).

Additionally, the high ITS sequence similarity and 16S RFLP profiles among strains suggest that a close phylogenetic relationship exists within *X. axonopodis* pv. *allii*. The limited genetic diversity within the 16S rDNA region is consistent with other findings (13) and may limit utility of the ITS region for differentiating *X. axonopodis* pv. *allii* from other xanthomonads. However, the rDNA spacer region is useful for identifying other bacteria (15) and could provide presumptive identification of *X. axonopodis* pv. *allii*. Describing this relationship among these strains and between other *Xanthomonas* species and pathovars should clarify the taxonomic position of *X. axonopodis* pv. *allii*, and elucidation of this relationship should aid in identification and future epidemiological studies of *X. axonopodis* pv. *allii*.

Pathogenicity on onion and multiplication in dry bean were clearly demonstrated, but the absence of disease symptoms on dry bean suggests that most *X. axonopodis* pv. *allii* strains may be only weakly virulent on dry bean and perhaps other pulse crops. Alternatively, dry bean may be a nonsymptomatic host of some *X. axonopodis* pv. *allii* strains. Pathogenicity and/or virulence on alternate hosts (i.e., pulse crops) appears to be highly variable among populations of *X. axonopodis* pv. *allii*. In this study, bean common bacterial blight disease symptoms were absent in all *X. axonopodis* pv. *allii* strains evaluated, but the Barbados population is reportedly highly virulent on dry bean and other pulse crops (27). Although most *X. axonopodis* pv. *allii* strains appear weakly virulent, dry beans may serve as a reservoir of *X. axono-*

TABLE 3. Maximum likelihood estimates of fatty acid methyl ester and substrate utilization profile multiple logistic regression used to predict geographic region of origin of onion strains of *Xanthomonas*

| Parameter ^a | df | Maximum likelihood estimates | | | |
|------------------------|----|------------------------------|----------------|---------------|---------------------------|
| | | Estimate ^b | Standard error | Wald χ^2 | $P > \chi^2$ ^c |
| Intercept Barbados | 1 | -4.662 | 1.08 | 18.48 | <0.0001 |
| Intercept Brazil | 1 | -3.529 | 0.68 | 26.56 | <0.0001 |
| Intercept California | 1 | -2.495 | 0.49 | 26.44 | <0.0001 |
| Intercept Colorado | 1 | -1.670 | 0.39 | 18.56 | <0.0001 |
| Intercept Georgia | 1 | -1.260 | 0.35 | 12.68 | 0.0004 |
| Intercept Hawaii | 1 | 0.916 | 0.33 | 7.81 | 0.0052 |
| Intercept Japan | 1 | 1.704 | 0.38 | 19.71 | <0.0001 |
| Intercept South Africa | 1 | 2.166 | 0.43 | 24.97 | <0.0001 |
| Intercept Texas | 1 | 3.641 | 0.72 | 25.77 | <0.0001 |
| Factor 1 | 1 | 0.782 | 0.27 | 8.38 | 0.0038 |
| Factor 9 | 1 | 0.865 | 0.27 | 9.91 | 0.0016 |

^a Each equation is given by $Z = \beta_0 + 0.782(\text{Factor 1}) + 0.865(\text{Factor 2})$, where β_0 is the cumulative logit intercept for each region; where Intercept Barbados = -4.662, Intercept Brazil (Barbados + Brazil) = -3.529, and Intercept Texas = (Barbados + Brazil... + Texas) = 3.641. The probability of group membership of a strain in a region (Z_i) is described by $p(R_i) = e^{Z_i} / (1 + e^{Z_i})$, where e is the natural logarithm and $p(R_{\text{Venezuela}}) = 1 - p(R_{\text{Texas}})$.

^b Intercept estimate = β_0 .

^c Probability of obtaining a greater χ^2 statistic than that observed if the null hypothesis is true.

podis pv. *allii* surviving as weak pathogens or epiphytically. Greenhouse and field studies are in progress to quantitate the survival of *X. axonopodis* pv. *allii* between onion crops on dry bean and other hosts.

Resistance to commonly applied bactericides did not appear within the collection of strains included in this study. This is

consistent with the findings of Paulraj and O'Garro (28) in Barbados.

Resistance to copper, zinc, and streptomycin has been widely reported in phytopathogenic bacteria (6,7,17,23,24,39), and insensitivity is often conferred by resistance genes located on self-transmissible plasmids (6,7,24,39). Xanthomonads are generally

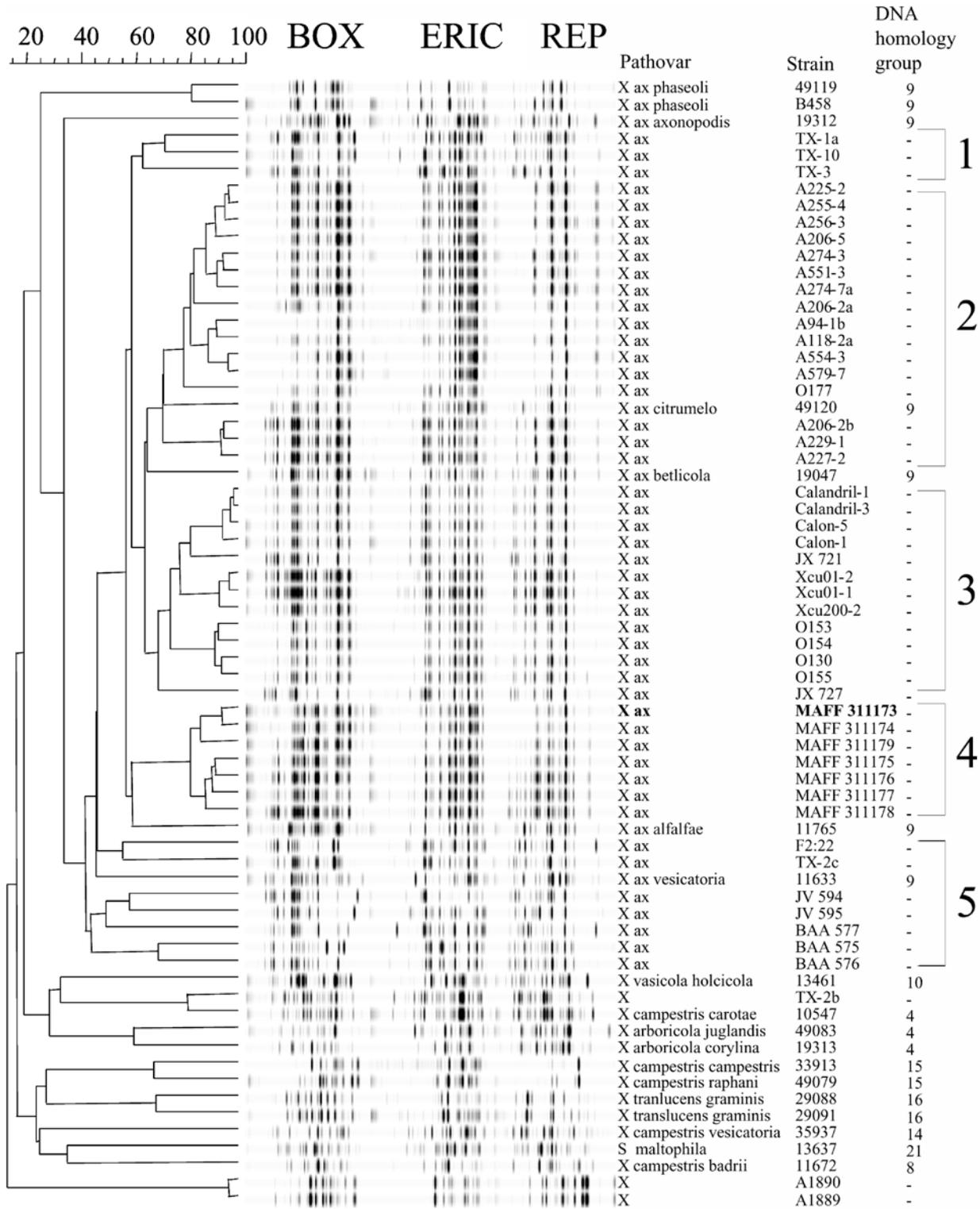


Fig. 4. Similarity among repetitive sequence based-polymerase chain reaction DNA fingerprints of onion *Xanthomonas* strains from various geographical areas and between other species and pathovars of *Xanthomonas* sp. DNA fingerprints were generated using primers corresponding to prokaryotic enterobacterial repetitive intergenic consensus (ERIC) sequences, repetitive extragenic palindromic (REP) sequences, and the BOXA subunit of the BOX element (BOX) and analyzed using the product-moment correlation coefficient (r). DNA homology groups correspond to those reported by Rademaker et al. (30). The type strain of *X. campestris* pv. *allii* MAFF 311173 is noted in bold. Genotype groups 1 to 5 are noted with brackets. X = *Xanthomonas*, X ax = *X. axonopodis*, S maltophila = *Stenotrophomonas maltophilia*, and - = not known.

considered resistant to copper, zinc, or streptomycin if they are able to grow on artificial media amended with 100 to 200 µg of the bactericide per ml, but resistance to streptomycin concentrations of 800 µg/ml or greater have been reported (24). Growers in Colorado and other onion-producing regions rely heavily on copper-based bactericides for disease suppression, and resistance may emerge with continued use (37), underscoring the need for an integrated pest management program to manage *Xanthomonas* leaf blight. The MIC of zinc was equal to or less than copper for all *X. axonopodis* pv. *allii* included in this study (data not shown), and zinc may be an effective bactericide to manage *X. axonopodis* pv. *allii* and delay copper resistance development. However, the relationship between the MICs determined in this study and sensitivity of bacterial cells under field conditions is unknown. Petri dish assays of bacterial sensitivity to copper, zinc, and streptomycin have been extensively used to detect bactericide resistance (6,7,17,24,39), and in vitro sensitivity appears to be a suitable proxy for sensitivity under field conditions (22). The efficacy of zinc sprays is currently under investigation in field trials.

Fatty acid and substrate utilization profiling revealed significant intraspecific diversity. Cluster analysis revealed limited grouping of strains by geographical region of origin based on cellular fatty acid composition and, to a lesser extent, Biolog Microplate substrate utilization. However, the fatty acid and substrate utilization profiles were sufficiently variable among regions to allow for factor analysis and subsequent multiple logistic regression to generate a predictive model. The multiple logistic regression model developed in this study could correctly classify 69% of the strains included in this analysis into their geographical region of origin. Combining this model with rep-PCR-mediated DNA fingerprinting would provide a powerful tool for investi-

gating dissemination of *X. axonopodis* pv. *allii* by seed transmission among onion production regions.

Identification of strains using the MIDI Aerobe database and Biolog version 4.1 database inconsistently identified strains. Fatty acid profiling identified the 49 strains of *X. axonopodis* pv. *allii* used in this study as 11 different *Xanthomonas* pathovars. Biolog identified the strains closest match as 10 different pathovars. Users of these systems are encouraged to create a new profile for *X. axonopodis* pv. *allii* if fatty acids or Biolog substrate utilization are to be used for identification.

The conserved rep-PCR genomic fingerprints within geographic locations suggest that the population structure is largely clonal within production areas. The tight phylogenetic cluster formed within a given production region suggests *X. axonopodis* pv. *allii* was introduced into these production regions in one or a few events, perhaps on contaminated seed (3,34). The onion production regions considered in this study are sufficiently isolated that gene flow from other populations would be low and distinct clonal populations may have formed following genetic drift and selection of fit genotypes. The climates, cultivars, and production practices within the different onion production regions considered may have selected for fitness and adapted genotypes unique for each region. However, the signature bands of *X. axonopodis* pv. *allii* pathogenic on onion are conserved among strains, and the recent evolution of *X. axonopodis* pv. *allii* was revealed by the identical rDNA RFLP profiles and high sequence similarity of the nonconserved ITS region.

The relationship between *X. axonopodis* pv. *allii* and *X. axonopodis* pv. *alfalfae*, *X. axonopodis* pv. *betlicola*, *X. axonopodis* pv. *citrumelo*, and *X. axonopodis* pv. *vesicatoria* and other DNA homology 9-2 strains remains unclear. Our study included a small

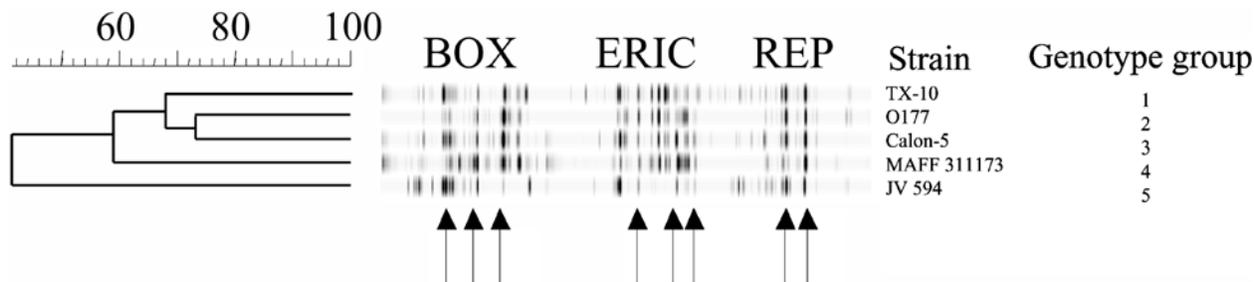


Fig. 5. Diversity of repetitive sequence based-polymerase chain reaction DNA fingerprints among onion *Xanthomonas* strains isolated from various geographical regions and representing different genotype groups. Arrows indicate DNA bands that are conserved across all strains pathogenic on onion. The scale indicates the product-moment correlation coefficient (r). Strain MAFF 311173 is the type strain of *X. campestris* pv. *allii*. MAFF = Ministry of Agriculture, Forestry, and Fisheries GeneBank accession.

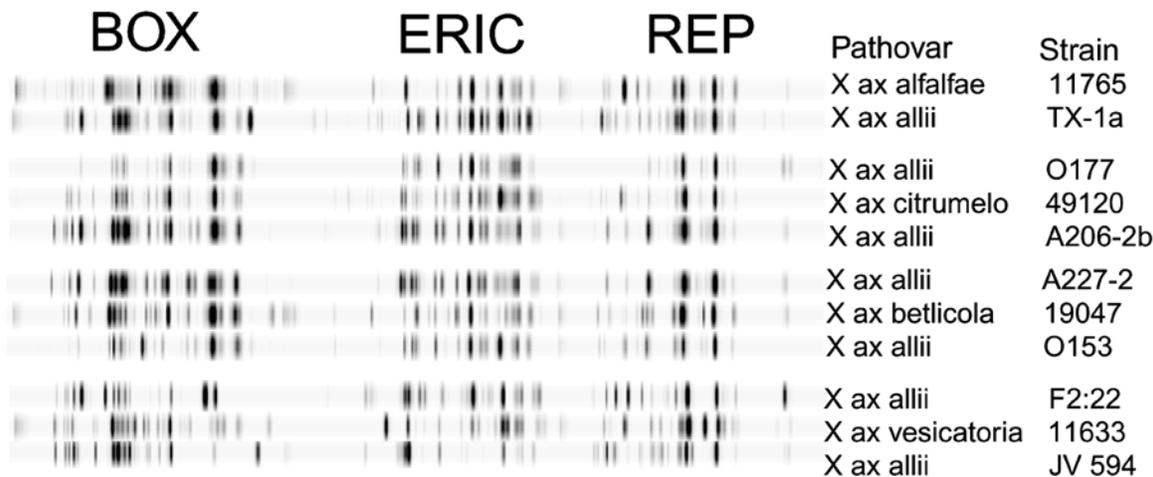


Fig. 6. Repetitive sequence based-polymerase chain reaction DNA fingerprint profile matches of onion *Xanthomonas* strains and other *Xanthomonas* pathovars within DNA homology group 9-2. X ax = *Xanthomonas axonopodis*.

subset of strains from each geographical region and few representative strains from other *Xanthomonas* species and pathovars. We are currently studying a larger collection of DNA homology group 9-2 strains to more fully describe the genetic and pathogenic relationships between these pathovars and *X. axonopodis* pv. *allii*.

The high level of genetic diversity within *X. axonopodis* pv. *allii* is in stark contrast to many other DNA fingerprinting studies with *Xanthomonas* and other plant-pathogenic bacteria (5,21,32). Louws et al. (20) found nearly identical rep-PCR DNA fingerprints within the subspecies of *Clavibacter michiganensis*. Genetic similarity within subspecies was greater than 80%, but less than 40% between subspecies. The level of diversity within *X. axonopodis* pv. *allii* approaches that between *Clavibacter* subspecies. However, signature rep-PCR bands conserved within *X. axonopodis* pv. *allii*, identical rDNA RFLP profiles, and high ITS region sequence similarity do not suggest a polyphyletic population structure exists. Rademaker et al. (30) demonstrated that rep-PCR fingerprinting explains 81% of the variability in DNA:DNA homology and can be used to reflect true genotypic and phylogenetic relationships. *X. axonopodis* pv. *allii* rep-PCR fingerprints among strains were at least 40% similar, corresponding approximately to at least 70% DNA:DNA homology (30). Therefore, despite high levels of genetic diversity within the population, *X. axonopodis* pv. *allii* appears to constitute a single species. Genetic drift of geographically isolated populations could account for the observed diversity among rep-PCR DNA fingerprints. Knowledge of the population structure should aid in future epidemiological and ecological studies of *X. axonopodis* pv. *allii*.

Genetic diversity within *X. axonopodis* pv. *allii* may have important implications for resistance gene deployment and breeding. Host resistance to *Xanthomonas* leaf blight has only been reported in short day onion cultivars (27) and resistance appears to be associated with one or few genes. A race structure may exist within *X. axonopodis* pv. *allii* that could account for the variability in pathogenicity and virulence on pulse crops and other *Allium*. Studies are currently underway to further characterize genes involved in *X. axonopodis* pv. *allii* pathogenicity on onion and the existence of a population race structure.

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LITERATURE CITED

- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Alvarez, A. M., Buddenhagen, I. W., Buddenhagen, E. S., and Domen, H. Y. 1978. Bacterial blight of onion, a new disease caused by *Xanthomonas* sp. *Phytopathology* 68:1132-1136.
- Audy, P., Laroche, A., Saindon, G., Huang, H. C., and Gilbertson, R. L. 1994. Detection of the bean common blight bacteria *Xanthomonas campestris* pv. *phaseoli* and *X. c. phaseoli* var. *fuscans*, using the polymerase chain reaction. *Phytopathology* 84:1185-1192.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1998. *Current Protocols in Molecular Biology*. John Wiley & Sons, Hoboken, NJ.
- Barak, J. D., and Gilbertson, R. L. 2003. Genetic diversity of *Xanthomonas campestris* pv. *vitiensis*, the casual agent of bacterial leafspot of lettuce. *Phytopathology* 93:596-603.
- Bender, C. L., and Cooksey, D. A. 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: Conjugative transfer and role in copper resistance. *J. Bacteriol.* 165:534-541.
- Bender, C. L., Malvick, D. K., Conway, K. E., George, S., and Pratt, P. 1990. Characterization of pXV10A, a copper resistance plasmid in *Xanthomonas campestris* pv. *vesicatoria*. *Appl. Environ. Microbiol.* 56:170-175.
- Bowen, P., Gibbs, H. A., and O'Garro, L. W. 1996. Garlic, chives, shallot, and leek are alternative hosts to *Xanthomonas campestris*, the pathogen of leaf blight of onion. *Proc. Int. Congr. Plant Pathol.* 7:696.
- Cubero, J., and Graham, J. H. 2002. Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. *Appl. Environ. Microbiol.* 86:1257-1264.
- Dye, D. W., Bradbury, J. F., Goto, M., Hayward, A. C., Lelliott, R. A., and Schroth, M. N. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Rev. Plant Pathol.* 59:153-160.
- Fenselau, S., Balbo, I., and Bonas, U. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. *Mol. Plant-Microbe Interact.* 5:390-396.
- Gent, D. H., Schwartz, H. F., Ishimaru, C. A., Louws, F. J., Cramer, R. A., and Lawrence, C. B. 2002. Polyphasic characterization of *Xanthomonas campestris* from onion. Page 48 in: *Proc. 2002 Natl. Allium Res. Conf., Natl. Allium Res. Conf.*, Pasco, WA.
- Hauben, L., Vauterin, L., Swings, J., and Moore, E. R. B. 1997. Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *Int. J. Syst. Bacteriol.* 47:328-335.
- Isakeit, T., Miller, M. E., Barnes, L. W., Dickstein, E. R., and Jones, J. B. 2000. First report of leaf blight of onion caused by *Xanthomonas campestris* in the continental United States. *Plant Dis.* 84:201.
- Jensen, M. A., Webster, J. A., and Straus, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.* 59:945-952.
- Kadota, I., Uehara, K., Shinohara, H., and Nishiyama, K. 2000. Bacterial blight of Welsh onion: A new disease caused by *Xanthomonas campestris* pv. *allii* pv. *nov.* *J. Gen. Plant Pathol.* 66:310-315.
- Lee, Y., Hendson, M., Panopoulos, N. J., and Schroth, M. N. 1994. Molecular cloning, chromosomal mapping, and sequence analysis of copper resistance genes from *Xanthomonas campestris* pv. *juglandis*: Homology with small blue copper proteins and multicopper oxidase. *J. Bacteriol.* 176:173-188.
- Leite, R. P., Jr., Minsavage, G. V., Bonas, U., and Stall, R. E. 1994. Detection and identification of phytopathogenic *Xanthomonas* strains by amplification of DNA sequences related to the *hrp* genes of *Xanthomonas campestris* pv. *vesicatoria*. *Appl. Environ. Microbiol.* 60:1068-1077.
- Louws, F. J., Bell, J., Medina-Mora, C. M., Smart, C. D., Opgenorth, D., Ishimaru, C. A., Hausbeck, M. K., de Bruijn, F. J., and Fulbright, D. W. 1998. rep-PCR-mediated genomic fingerprinting: A rapid and effective method to identify *Clavibacter michiganensis*. *Phytopathology* 88:862-868.
- Louws, F. J., Fulbright, D. W., Stephens, C. T., and de Bruijn, F. J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl. Environ. Microbiol.* 60:2286-2295.
- Louws, F. J., Fulbright, D. W., Stephens, C. T., and de Bruijn, F. J. 1995. Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* 85:528-536.
- Mahuku, G., Carlos, J., Henriquez, M. A., and Cuasquer, J. 2002. Diversity among *Xanthomonas axonopodis* pv. *phaseoli* strains from different geographical areas and pathogenic on common bean. (Abstr.) *Phytopathology* 92(suppl.):S50.
- Marco, G. M., and Stall, R. E. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that in differ in sensitivity to copper. *Plant Dis.* 67:779-781.
- Minsavage, G. V., Canteros, B. I., and Stall, R. E. 1990. Plasmid-mediated resistance to streptomycin in *Xanthomonas campestris* pv. *vesicatoria*. *Phytopathology* 80:719-723.
- National Committee for Clinical Laboratory Standards. 2000. *Methods for Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*. 5th ed. Approved Standard M7-A5. NCCLS, Wayne, PA.
- Nunez, J. J., Gilbertson, R. L., Meng, X., and Davis, R. M. 2002. First report of *Xanthomonas* leaf blight of onion in California. *Plant Dis.* 86:330.
- O'Garro, L. W., and Paulraj, L. P. 1997. Onion leaf blight caused by *Xanthomonas campestris*: Alternative hosts and resistant onion genotypes. *Plant Dis.* 81:978-982.
- Paulraj, L., and O'Garro, L. W. 1992. Aspects of the epidemiology of a leaf blight of onion in Barbados. Pages 89-96 in: *Proc. Annu. Conf. Barbados Soc. Technol. Agric.*, 10th. Barbados, West Indies.
- Paulraj, L., and O'Garro, L. W. 1993. Leaf blight of onions in Barbados caused by *Xanthomonas campestris*. *Plant Dis.* 77:198-201.

30. Rademaker, J. L. W., Hoste, B., Louws, F. J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P., and de Bruijn, F. J. 2000. Comparison of AFLP and rep-PCR genomic fingerprinting with DNA-DNA homology studies: *Xanthomonas* as a model system. *Int. J. Syst. Bacteriol.* 50:665-677.
31. Rademaker, J. L. W., Louws, F. J., and de Bruijn, F. J. 1999. Computer assisted pattern analysis of electrophoretic fingerprints and database construction. Pages 1-33 in: *Molecular Microbial Ecology Manual*. A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn, eds. Kluwer Academic Publishers, Dordrecht, the Netherlands.
32. Roberts, P. D., Hodge, N. C., Bouzar, H., Jones, J. B., Stall, R. E., Berger, R. D., and Chase, A. R. 1998. Relatedness of strains of *Xanthomonas fragariae* by restriction fragment length polymorphism, DNA-DNA reassociation, and fatty acid analyses. *Appl. Environ. Microbiol.* 64:3961-3965.
33. Roumagnac, P., Gagnevin, L., Gardan, L., Sutra, L., Manceau, C., Dickstein, E. R., Jones, J. B., Rott, P., and Pruvost, O. Polyphasic characterization of xanthomonads isolated from onion, garlic and Welsh onion (*Allium* spp.) and their relatedness to different *Xanthomonas* species. *Int. J. Syst. Evol. Microbiol.* (In Press.)
34. Roumagnac, P., Gagnevin, L., and Pruvost, O. 2000. Detection of *Xanthomonas* sp., the causal agent of onion bacterial blight, in onion seeds using a newly developed semi-selective isolation medium. *Eur. J. Plant Pathol.* 106:867-877.
35. Sanders, F. H., Langston, D. B., Brock, J. H., Gitaitis, R. D., Curry, D. E., and Torrance, R. L. 2003. First report of a leaf blight of onion caused by *Xanthomonas* spp. in Georgia. *Plant Dis.* 87:749.
36. Schwartz, H. F., and Otto, K. 2000. First report of a leaf blight of onion by *Xanthomonas campestris* in Colorado. *Plant Dis.* 84:922.
37. Schwartz, H. F., and Otto, K. J. 1998. Onion bacterial disease management in Colorado. Pages 214-218 in: *Proc. 1998 Natl. Onion (and other Allium) Res. Conf.*
38. Serfontein, J. J. 2001. *Xanthomonas* blight of onion in South Africa. *Plant Dis.* 85:442.
39. Sundin, G. W., Jones, A. L., and Fulbright, D. W. 1989. Copper resistance in *Pseudomonas syringae* from cherry orchards and its associated transfer in vitro and in planta with a plasmid. *Phytopathology* 79:861-865.
40. Thomas, W. D., Jr., and Weinhold, A. R. 1953. *Xanthomonas striiformans*, a new bacterial pathogen of onion. *J. Colo.-Wyo. Acad. Sci.* 4:22.
41. Vauterin, L., Hoste, B., Kersters, K., and Swings, K. 1995. Reclassification of *Xanthomonas*. *Int. J. Syst. Bacteriol.* 45:472-489.
42. Vauterin, L., Yang, P., and Swings, J. 1996. Utilization of fatty acid methyl esters for the differentiation of new *Xanthomonas* species. *Int. J. Syst. Bacteriol.* 46:298-304.
43. Verniere, C., Pruvost, O., Civerolo, E. L., Gambin, O., Jacquemoud-Collet, J. P., and Luisetti, J. 1993. Evaluation of the Biolog substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonas campestris* pv. *citri*. *Appl. Environ. Microbiol.* 59:243-249.
44. Weller, D. M., and Saettler, A. W. 1978. Rifampin-resistant *Xanthomonas phaseoli* var. *fuscans* and *Xanthomonas phaseoli*: Tools for field study of bean blight bacteria. *Phytopathology* 68:778-781.