Polyphasic Characterization of *Xanthomonas axonopodis* pv. *allii* Associated with Outbreaks of Bacterial Blight on Three *Allium* Species in the Mascarene Archipelago

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Accepted for publication 15 April 2008.

ABSTRACT

Picard, Y., Roumagnac, P., Legrand, D., Humeau, L., Robène-Soustrade, I., Chiroleu, F., Gagnevin, L., and Pruvost, O. 2008. Polyphasic characterization of *Xanthomonas axonopodis* pv. *allii* associated with outbreaks of bacterial blight on three *Allium* species in the Mascarene archipelago. *Phytopathology* 98:919-925.

Based on the number of new reports during the last two decades, bacterial blight of onion (*Allium cepa*) is considered an emerging disease. The causal agent, *Xanthomonas axonopodis* pv. *allii*, is pathogenic to several *Allium* species after inoculation, but outbreaks worldwide have been primarily reported on onion. We describe a unique epidemiological situation in Réunion Island, France, with concomitant outbreaks on three *Allium* species, onion, leek (*A. porrum*), and garlic (*A. sativum*). There was no host specialization within *Allium* spp. among strains associated with the three host species. Based on amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism, strains

associated with these outbreaks in Réunion Island were highly related genetically to strains isolated from diseased plant samples and contaminated seed lots in the neighboring island of Mauritius, where the disease has occurred since 1984. All AFLP haplotypes were identified as *X. axonopodis* pv. *allii* based on polymerase chain reaction analysis using specific primers, biochemical tests, and/or pathogenicity tests. Two genetically related groups of strains (A and B) that can be distinguished by AFLP, differential utilization of three carbon sources, and xanthomonadin pigment production were detected initially after establishment of the pathogen. In less than 10 years after the establishment of the pathogen there was nearly an extinction of group A strains in Réunion Island, suggesting differences in fitness between strains in the two groups.

Additional keywords: emerging pathogen, in planta growth rates, seed transmission.

Since the original description of the disease and its causal agent in Hawaii (2), bacterial blight of onion (*Allium cepa* L.) (BBO) has been reported from many countries worldwide, with an increasing number of disease reports since then (26). Consequently, the causal agent, *Xanthomonas axonopodis* pv. *allii* (26) (synonym *X. campestris* pv. *allii* [19]), is an emerging pathogen and was recently included on the alert list of the European and Mediterranean Plant Protection Organization (EPPO). In the Mascarene archipelago (South West Indian Ocean), BBO was first recorded in Mauritius in 1984 (S. Benimadhu, *personal communication*), and subsequently in the neighboring Réunion Island, where the disease was first observed in onion fields in 1993. After 2000, major outbreaks were also reported in Réunion on leek and garlic.

X. axonopodis pv. *allii* is pathogenic after inoculation on several *Allium* species including onion, garlic (*A. sativum* L.), leek (*A. porrum* L.), and Welsh onion (*A. fistulosum* L.) (26). On onion, lesions consist of lenticular water-soaked leaf spots which turn into dry chlorotic lesions that eventually coalesce. When

disease is severe, leaf dieback can occur, resulting in a reduction of bulb size. The morphology of leaf lesions on other *Allium* species is similar to those on onion (26). *X. axonopodis* pv. *allii* was also pathogenic to citrus after bacterial infiltration, and produced lesions that were similar to citrus bacterial spot (12). There is no experimental evidence of strains of this bacterium being pathogenic to citrus under field conditions.

Amplified fragment length polymorphism (AFLP), a technique widely used for studying the epidemiology of human bacterial pathogens (8,9,15,21,25,31), successfully distinguished local inoculum from inoculum resulting from migration events in epidemiological studies of the BBO pathosystem (17). In this study, we describe a polyphasic characterization (including AFLP and restriction fragment length polymorphism [RFLP] analyses, polymerase chain reaction [PCR] amplification with specific primers, biochemical and physiological assays, and pathogenicity tests) of *X. axonopodis* pv. *allii* strains originating from the Mascarene archipelago (Réunion and Mauritius Islands). The objectives of this study were to evaluate the relatedness between strains of *X. axonopodis* pv. *allii* from Réunion and Mauritius Islands, and to examine genetic and pathological variations among strains from BBO outbreaks recorded in Réunion Island on onion, garlic, and leek.

MATERIALS AND METHODS

Bacterial strains and generic media. The *X. axonopodis* pv. *allii* strains used in this study included 19 strains from diseased

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains two tables showing the geographic origins and other characteristics of the 279 strains of *X. axonopodis* pv. *allii* used in this study.

doi:10.1094/PHYTO-98-8-0919

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onion collected from 1996 to 2000 in Mauritius, 15 strains from onion seeds harvested in 1996 in Mauritius, 158 strains from onion collected from 1993 to 2005 in Réunion Island, two strains from a single diseased garlic plant collected in 1994 in Réunion Island, 30 strains from diseased garlic collected from 2001 to 2004 in Réunion Island, 46 strains from diseased leek collected from 2001 to 2005 in Réunion Island, and nine *X. axonopodis* pv. *allii* strains from other regions: Brazil (CFBP 6362, 6363, 6377, 6378, and JVJ93), Barbados (CFBP 6367 and 6368), and Japan (CFBP 6107^{PT} and 6108) (Collection Française de Bactéries Phytopathogènes, INRA Angers, France). Strains were stored at -80°C on beads in cryovials (Microbank Prolab Diagnostics, Austin, TX) and/or as lyophiles. Bacteria were cultured on yeast peptone glucose agar (YPGA; yeast extract 7 g liter⁻¹, peptone 7 g liter⁻¹, glucose 7 g liter⁻¹, agar 18 g liter⁻¹, pH 7.2).

AFLP analysis. A total of 225 strains were analyzed by AFLP. For each strain, at least two independent DNA extractions obtained from a single bacterial colony were used for AFLP analyses. AFLP was performed as described previously (1). Briefly, DNA was digested with *SacI* and *MspI* (New England Biolabs Ozyme, Saint Quentin Yvelines, France), as recommended by the manufacturer. Then, 2.5 µl of the digested products was added to 22.5 µl of a ligation mix containing 2 µM *MspI* adaptor, 0.2 µM *SacI* adaptor (Applied Biosystems, Courtaboeuf, France), and 2 U of T4 DNA ligase (New England Biolabs Ozyme) in 1× T4 DNA ligation buffer. Ligations were performed for 3 h at 37°C before enzyme inactivation at 65°C for 10 min. Ligation products were diluted 10-fold with high-performance liquid chromatography (HPLC)-grade water before preselective PCR. The reactions were done in 15 µl and contained 5 µl of diluted ligation product, 2.5 mM MgCl₂, 0.23 µM of each *MspI* and *SacI* primer, 0.45 mM of each dNTP (New England Biolabs Ozyme), 0.5 U of Taq DNA polymerase (Goldstar Red, Eurogentec, Seraing, Belgium), in 1× Goldstar buffer. The following PCR conditions were used: initial extension to ligate the second strand of the adaptors at 72°C for 2 min, a denaturation step at 94°C for 2 min, 25 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, and a final extension step at 72°C for 10 min. PCR products were diluted 10-fold with HPLC-grade water before selective amplification. The selective-amplifications using the unlabeled *MspI*+ A, C, T, or G primer and the labeled *SacI*+C primer (four different fluorochromes) were performed in the same conditions as the preselective PCR except that the *SacI*+C primer concentration was 0.12 µM. The following PCR conditions were used during selective amplifications: initial denaturation at 94°C for 2 min followed by 37 cycles at 94°C for 30 s, annealing for 30 s at 65°C for the first cycle, decreased by 0.7°C per cycle for the next 12 cycles, and then 56°C for the last 24 cycles, and extension at 72°C for 2 min with a final extension step at 72°C for 10 min. Samples were then prepared for capillary electrophoresis by adding 1 µl of the final PCR product to 18.7 µl of formamide and 0.3 µl of LIZ500 DNA ladder (Applied Biosystems) as an internal standard. The mixture was then denatured for 5 min at 95°C and placed on ice for at least 5 min. Electrophoresis was performed in an ABI PRISM-3100 Genetic Analyzer (Applied Biosystems) using performance optimized polymer POP-4 at 15,000 V for about 20 min at 60°C, with an initial injection of 66 s. The AFLP fingerprints were analyzed visually using Genescan software 3.7 (Applied Biosystems). Strain CFBP 6369 was used as a control in each experiment.

The presence or absence of reproducible DNA fragments obtained with the four combinations was scored in a binary matrix. A neighbor-joining tree (11) with bootstrap values (1,000 repeated samplings) was constructed based on the Dice dissimilarity coefficient using DARwin 5.0 software (CIRAD, Montpellier, France).

Physiological, biochemical tests, and carbon source utilization assay. A subset of 106 strains representative of the genetic diversity based on AFLP analysis were used for physiological,

biochemical, and carbon source utilization tests. Physiological and biochemical tests used included Gram test, O/F glucose metabolism, oxidase, urease, indole production, production of fluorescent pigments, starch hydrolysis, gelatin hydrolysis, esculin hydrolysis, milk proteolysis, cellulose hydrolysis, Tween esterase, H₂S production from cysteine, and pectolytic activity. Physiological and biochemical tests were conducted as described by Vernière et al. (32) except for pectolytic activity (14) and gelatin hydrolysis (20). Carbon source utilization patterns were determined as described previously (26) using the Biolog GN system (Biolog Inc., Hayward, CA) (4).

Absorption spectrum of xanthomonadin pigment. Because two colony color types (bright yellow and pale yellow) were observed visually within the strain collection, the xanthomonadin pigments from a subset of nine strains were analyzed. Methanol extraction of xanthomonadin was slightly modified from Schaad (30). Bacterial cells obtained from 48-h-old cultures on nutrient agar (3 g of meat extract, 5 g of bactopectone, 15 g of agar, and 1,000 ml of distilled water, pH 7.3) were centrifuged (4,500 rpm for 5 min) in 0.01 M sterile Sigma 7 to 9 buffer (Sigma Aldrich, Saint-Quentin Fallavier, France) pH 7.2. This washing step was repeated eight times until a clear supernatant was obtained. Bacterial suspensions were adjusted spectrophotometrically (OD₆₀₀ of 100-fold-diluted suspensions = 0.04) to contain approximately 8 × 10⁹ CFU ml⁻¹. Bacterial pellets were allowed to dry overnight at room temperature and were then suspended in 5 ml of methanol. Tubes were then placed in boiling water for 1 min. After centrifugation of the suspensions, supernatants were saved in new tubes. The absorption spectra of the xanthomonadin extracts were measured spectrophotometrically (Hewlett Packard spectrophotometer HP8453, Les Ulis, France) between 250 and 600 nm.

PCR amplification with PXaa1 and NXaa1 primers. Genomic DNAs from 129 strains were used for PCR. Template consisted of 1 µl of bacterial suspension boiled for 1 min and chilled on ice. Suspensions were prepared from 24-h-old cultures grown on YPGA in HPLC grade water and contained approximately 1 × 10⁸ CFU ml⁻¹. PCR reactions were performed as described previously (17). The first round of PCR was performed with primers PXaa1U (5'-GGCTCTAATACGACGTTGACGAT-3') and PXaa1L (5'-AAATTCATGCGCGTTTTCAATAG-3'), which complement bases 197 to 219 and bases 871 to 893, respectively, of a sequence-characterized amplified region (SCAR) specific to *X. axonopodis* pv. *allii* strains. The amplification program included initial denaturation at 94°C for 5 min, 40 cycles consisting of denaturation at 95°C for 1 min, annealing at 68°C for 1 min and extension at 72°C for 2 min, and a final extension step at 72°C for 5 min. Strains negative with PXaa1 primers were further evaluated using internal primers. The primers used in the second PCR round were NxaaU1 (5'-TTACGTCGCAACAATCCAGATA-3') and NxaaL1 (5'-GGGCACCATTGACATTATCAGTT-3'), which complement bases 424 to 446 and 848 to 870 of the SCAR sequence, respectively. For this second round of PCR, 1 µl from the first reaction was used as template and the amplification program consisted of denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 40 s, and a final extension at 72°C for 5 min. PCRs were performed in 25 µl reaction mixtures containing 3 mM MgCl₂, 100 µM of each dNTP, 0.2 µM of each primer (PXaa1U/PXaa1L or NxaaU1/NxaaL1), 1 µl template DNA and 1.25 U Taq polymerase Goldstar Red (Eurogentec) in 75 mM Tris-HCl, 20 mM (NH₄)₂SO₄, 0.01% Tween 20 buffer (pH 8.8). Negative controls in which water was used as template were included in all experiments. PCR reaction products were separated by electrophoresis in 1% Seakem LE agarose (FMC Bioproducts, Philadelphia, PA), stained with ethidium bromide, and visualized with UV light. Because nested PCR is known to sometimes produce false positives, only samples from which the expected DNA fragment was detected at least twice were scored as positive.

Restriction fragment length polymorphism (RFLP) analysis. Genomic DNAs from 115 strains representative of the genetic diversity based on AFLP were extracted by the CTAB method (3). DNAs were digested to completion with *Ngo*MIV (New England Biolabs) or *Eco*RI (New England Biolabs). Digested-DNA fragments were separated in a 1% agarose gel (25-cm long) by electrophoresis at 50 V for 20 h (*Eco*RI) or 26 h (*Ngo*MIV) in TAE buffer, and gel blotting was performed as described previously (10). Southern blots with *Ngo*MIV-digested DNA were hybridized with the labeled 16S-ITS1-23S rDNA fragment. The rDNA fragment used as probe was prepared by PCR amplification from strain CFBP 6369 (Collection Française de Bactéries Phytopathogènes, Beaucouzé, France). Primer FGPS6 (5'-GGAGAGTTAGATCTTGGCTCAG-3') and 23S4675 (5'-CACTCCGGTCCTCTCGTA-3') were used, and are located at the beginning of the 16S rRNA gene (22) and at the end of the 23S rRNA gene, respectively. Primer 23S4675 was designed using OLIGO 5.0 (National BioSciences Inc., Plymouth MN) from rDNA operon sequences of *X. citri* pv. *citri* (6). Primers were synthesized by Sigma-Genosys (Cambridge, UK). PCR reactions were performed in a 100- μ l volume containing 10 ng of template DNA, 10 U of a mixture of *Taq*, and *Tgo* DNA polymerases (Expand Long Template PCR System, Roche Diagnostics, Meylan, France), 1 $\times$ reaction buffer 3 (supplied by the manufacturer with the polymerase), 0.4 μ M of each primer, 40 μ M of each dNTP (Roche Diagnostics, Meylan, France), and HPLC grade water (Sigma-Aldrich, Saint-Quentin Fallavier, France). The amplification consisted of a 3 min denaturation step at 95°C, followed by 30 cycles of DNA denaturation step at 95°C for 1 min, 1 min annealing at 60°C, and 5 min extension at 72°C. A final extension step was performed at 72°C for 15 min. PCRs were performed using a PE-9600 thermocycler (Applied Biosystems, Courtaboeuf, France). Blots with *Eco*RI-digested DNA were hybridized with the labeled plasmid pXV9 (extracted and purified using the plasmid midi kit (Qiagen, Courtaboeuf, France) containing an approximately 25-kb *hrp* cluster from *X. axonopodis* pv. *vesicatoria* (5). Probe labeling, hybridization, and detection were performed with the kit ECL as described by the manufacturer (GE Healthcare, Saclay, France) under high stringency conditions.

Pathogenicity tests. At least five strains per AFLP haplotype (when available) were inoculated on the host species from which they originated. Six strains (three from each AFLP group) originating from the intercepted seed lot also were checked for pathogenicity on onion cv. Red Creole. In addition, a subset of 28 strains (5 strains identified as AFLP group A, 18 strains identified as AFLP group B, strain LMG 16528, and 4 strains from other countries) were checked for pathogenicity on three host species (onion cv. Red Creole, garlic cv. Vacoa, and leek cv. Gros Long d'Été). Spray-inoculations of bacterial suspensions containing approximately 1×10^7 and 1×10^4 CFU ml⁻¹ and subsequent analyses were performed as described previously (27). Briefly, 200 ml of bacterial suspension prepared in 0.01 M sterile Sigma 7 to 9 buffer (pH 7.2) was sprayed on 10 cv. Red Creole onion plants at the 4 to 5 leaf stage. In each experiment, a negative control (buffer inoculation) and a positive control (CFBP 6366 and/or JQ632-1) were used. Inoculated plants were incubated in growth chambers at 30 $\pm$ 1°C day, 26 $\pm$ 1°C night, 95% relative humidity (RH) and a 12-h photoperiod with a light intensity of approximately 390,000 lm. Disease ratings were recorded up to 12 days after inoculation (dai). Disease scores were the sum of BBO leaf lesions observed on the 10 inoculated plants 8 dai. The pathogen was re-isolated from up to 15 leaf lesions per strain–host combination using NCTM1 semi-selective medium as previously reported (27). All experiments were replicated at least once.

In planta multiplication in detached onion leaves. Nine strains originating from Réunion Island were used for this study. They included two strains from onion identified as AFLP group A, the unique AFLP haplotype LMG 16528, and six strains

identified as AFLP group B (two from onion, two from leek, and two from garlic). As BBO lesions induce a quick lodging and decay of onion leaves, we developed a detached leaf assay for measuring in planta multiplication over time of *X. axonopodis* pv. *allii*. Onion leaves (cv. Véronique) were surface disinfested with 1% NaOCl and cut into approximately 5-cm-long fragments. Leaf fragments were placed on the surface of water agar (10 g liter⁻¹) supplemented with benlate at 50 mg ml⁻¹. Bacterial suspensions prepared in 0.01 M sterile Sigma 7 to 9 Tris buffer (pH 7.2) and containing approximately 1×10^4 cells ml⁻¹ were obtained from serial dilutions of suspensions adjusted spectrophotometrically (OD₆₀₀ = 0.05) to contain approximately 1×10^8 CFU ml⁻¹. For inoculation, a 10 μ l droplet of inoculum was deposited on a single wound made in the center of each fragment using a sterile needle. Plates were sealed with Parafilm, and were incubated for 6 days at 26 $\pm$ 1°C and a 12-h photoperiod with a light intensity of approximately 40,000 lm. Each day, eight fragments per strain were used for population size determination. For this, fragments were individually processed using a stomacher device (Seward Medical, London, UK) at high speed for 30 s in 20 ml of 0.01 M sterile Sigma 7 to 9 buffer (pH 7.2). Appropriate dilutions were plated on semi-selective NCTM1 medium as described previously (27). Experiments were replicated once.

The fit of the log-transformed bacterial population data to a normal distribution was examined and variances homogeneity was checked with the Bartlett test. Nonlinear exponential, Gompertz, complementary log-log, logistic, and probit models were fitted to the data, and parameters were estimated for each bacterial strain using R software (version 2.6.0; R Development Core Team, Vienna, Austria). The appropriateness of each model was assessed by visual examination of the residual plots, correlation analysis of observed versus predicted values, and the use of the Akaike information criterion (7). For the model which was most suitable overall, a comparison of two of the three parameters (the daily multiplication rate r^* and the maximum population size K) was performed using a maximum likelihood ratio test ($P = 0.05$). Confidence intervals for the model parameters were estimated by the bootstrap method (16).

RESULTS

AFLP analysis. The overall AFLP data set was composed of 183 markers, 16 (8.7%) of which were monomorphic. All strains originating from the Mascarene archipelago clearly differed from *X. axonopodis* pv. *allii* strains from other countries (Fig. 1). The tree representation separated strains originating from the Mascarene archipelago into two major groups supported by bootstrap values $\geq 97\%$. Group A strains were characterized by four unique markers (i.e., markers that are present in all group A genomes and absent in all group B genomes), whereas group B strains were characterized by eight unique markers. When compared pairwise, any group A and B pair of strains differed by 20 to 30 markers. Both groups contained strains from both countries that were isolated from diseased onion leaves, scapes, and contaminated seed. Group A contained six haplotypes that differed by one to four markers. This group contained only strains producing pale yellow colonies on agar media ($n = 35$) that were isolated from onion diseased plants and seed in 1998 or earlier. Group B contained nine haplotypes that differed by one to six markers. This group contained only strains producing bright yellow colonies on agar media ($n = 179$) collected from diseased onion, garlic, and leek and onion seed from 1993 to 2005. Only a single strain isolated from diseased onion in Réunion Island (i.e., LMG 16528) did not cluster within A and B groups.

Physiological, biochemical tests, and carbon source utilization assay. Biochemical and physiological tests did not reveal variability among strains originating from diseased plant material. The biochemical and physiological properties of the strains were

characteristic of the genus *Xanthomonas*. These strains hydrolyzed starch, gelatin, casein, and had pectolytic activity at pH 5.0, 7.0, and 8.5.

Carbon source utilization varied among strains originating from the Mascarene archipelago. All strains producing pale yellow colonies and identified as AFLP group A utilized L-fucose, D-melibiose, and citric acid, in contrast to strains identified as AFLP group B. Strain LMG 16528, which was identified as a unique haplotype by AFLP and produced bright yellow colonies on agar media, also utilized L-fucose, D-melibiose, and citric acid. Among strains from other genetic groups, strains from Barbados and two strains from Brazil (CFBP 6362 and 6378) utilized all three carbon sources. Strains originating from Japan utilized D-melibiose and citric acid. The three remaining strains from Brazil utilized L-fucose and D-melibiose.

Absorption spectrum of xanthomonadin pigment. The maximal absorption of the pigment extracts ranged from 440 to 445 nm. The absorption values observed for pale yellow cultures (AFLP group A) was approximately half of those observed for bright yellow cultures (AFLP group B), therefore confirming the visual observations made on YPGA plates.

RFLP analysis. Five and four different hybridization profiles were identified based on RFLP using the *hrp* and rDNA probes, respectively. All strains from Réunion and Mauritius Islands were of a single *hrp* (*hrp*-1) and rDNA profile (rDNA-1). Strains from other countries were distinguishable from those using either probe (Fig. 2A and B), except for one Brazilian strain (CFBP 6378). Strain CFBP 6378 did not differ from strains originating from the Mascarene archipelago based on the ribosomal probe (rDNA-1), but was distinguishable by the *hrp* probe (*hrp*-5). RFLP haplotypes were consistent with AFLP data (Fig. 1).

PCR amplification with PXaa1 and NXaa1 primers. A PCR product of the expected size (697 bp) was observed for most, but not all, strains using PXaa1 primers. For all PXaa1-negative strains, no amplicon was obtained (expected size 447 bp) using internal NXaa1 primers. PCR-positive strains included all strains from the Mascarene archipelago, as well as strains of *X. axonopodis* pv. *allii* isolated in Japan. No amplicon was obtained for strains originating from Barbados. Four out of five Brazilian strains produced a positive reaction.

Pathogenicity tests. The pathogenicity of all tested strains, including strains isolated from onion seed, was confirmed by Koch postulates on the host species from which the strain originated. Moreover, all 28 strains, including AFLP group A and B strains originally isolated from diseased onion, leek, and garlic, were pathogenic to these three *Allium* species. In addition, large *Xanthomonas* population sizes ($\geq 10^7$ CFU lesion⁻¹) were recovered from at least 10 lesions per inoculated strain. On onion, typical lesions developed irrespective of the bacterial concentration used for inoculation. The length of the latent infection period ranged from 3 to 4 (with inocula of 10^7 CFU/ml⁻¹) to 7 to 11 days (with inocula of 10^4 CFU/ml⁻¹). On leek and garlic, lesions were observed primarily on plants inoculated with suspensions containing 10^7 CFU/ml⁻¹, suggesting a lower susceptibility of these two species. Because *X. axonopodis* pv. *allii*, together with secondary pathogens, induced a rapid decay of leaves on inoculated plants, it was not possible to reliably assess the aggressiveness of inoculated strains.

In planta multiplication in detached onion leaves. Based on nonlinear regression analysis of bacterial multiplication in planta over time, the Gompertz model was the most appropriate model. Daily multiplication rates (*r**) differed significantly among strains, and no obvious relationship with the host of origin was noted (Table 1). Similarly, the maximum population sizes derived from the Gompertz model (i.e., the asymptote, *K*) differed significantly among strains, by up to 0.7 log unit (Table 1). However, *K* may lack pertinence, as strains characterized by high multiplication rates tended to produce bacterial ooze on the agar media, which was not taken into account in the population size measurement.

DISCUSSION

AFLP fingerprints indicated that *X. axonopodis* pv. *allii* strains from the Mascarene archipelago were genetically different from those isolated in Barbados, Brazil, and Japan. Additional data (not shown) using strains of *X. axonopodis* pv. *allii* from Hawaii, mainland United States, South Africa, and Venezuela confirmed this result (Dice dissimilarity index >0.3). The marked difference between strains originating from the Mascarene archipelago and

Fig. 1. Neighbor-joining tree derived from Dice dissimilarity indexes based on 183 amplified fragment length polymorphism (AFLP) markers showing the relationships between strains of *Xanthomonas axonopodis* pv. *allii* isolated in the Mascarene archipelago, Barbados, Brazil, and Japan (outgroups). Bootstrap values are shown. *n* is the number of haplotypes found within each group. Two main groups (designated A and B) were identified among strains isolated in the Mascarene archipelago. Group A contains strains producing pale yellow colonies isolated solely from onion. Group B contains strains producing bright yellow colonies originating from onion, leek, and garlic. A single strain from Réunion Island (LMG 16528) did not cluster within group A or B. The uniqueness of this strain allowed its efficient tracing in molecular epidemiological studies (17).

strains from the other regions was confirmed by the RFLP (using a ribosomal and an *hrp* probe) and Biolog data (26). Our results are consistent with those by Gent et al. (13), which showed, using a logistic regression model, that differences in fatty acid and carbohydrate utilization profiles correlated fairly well with the geographic origin of the strains. Furthermore, our results confirm the large genetic diversity observed within a worldwide strain collection of *X. axonopodis* pv. *allii* using rep-PCR (13). Such a large intra-pathovar diversity also may explain why some strains of *X. axonopodis* pv. *allii* did not react with the PXaa1 and NXaa1 PCR primer pairs (17).

In contrast, on a regional scale, strains from Réunion Island share a high genetic similarity with those from Mauritius. Two groups of strains, referred herein as groups A and B, were isolated from outbreaks and contaminated seed. In addition, we report a presently unique epidemiological situation in Réunion Island, with concomitant major outbreaks on three *Allium* species (onion, leek, and garlic), which are associated with group B strains of *X. axonopodis* pv. *allii*. However, strains isolated from onion, leek, and garlic could not be distinguished based on AFLP data. Similarly, cross-inoculations indicated that all strains assayed

were pathogenic to all three *Allium* species and that on onion multiplication rates of strains originating from leek and garlic were similar to that of strains originating from onion. Collectively, our data and a previous report (26) suggest a lack of pathological specialization of strains of *X. axonopodis* pv. *allii* from Réunion Island within the genus *Allium*. Spatial and climatic parameters probably play a key role in the concomitant outbreaks on onion, leek, and garlic. First, the three *Allium* crops share the same agricultural areas in both islands and secondly, the inoculum is likely to be shortly dispersed in these areas after the occurrence of wind-driven rains (28). The delayed epidemic situation observed on leek and garlic may be related to their lower susceptibility to *X. axonopodis* pv. *allii*, as determined by pathogenicity tests under controlled environmental conditions. However, AFLP data are not appropriate for deciphering micro-evolutionary events related to a very recent adaptation of strains on leek or garlic (33).

Groups A and B, which were genetically more similar to one another than to other groups, could not be clearly distinguished based on virulence and rates of multiplication in planta on onion. In contrast, these two groups could be clearly distinguished based

Fig. 2. Southern blots of digested DNAs from strains of *Xanthomonas axonopodis* pv. *allii* originating from the Mascarene archipelago, Barbados, Brazil, and Japan obtained with **A**, *hrp* and **B**, rDNA probes. All strains from the Mascarene archipelago, Barbados, and Japan were identified as hrp-1 rDNA-1, hrp-4 rDNA-3, and hrp-3 rDNA-4, respectively. Strains from Brazil were of two haplotypes, hrp-5 rDNA-1 (CFBP 6378) and hrp-2 rDNA-2 (other strains). L: 1-kb ladder.

TABLE 1. Nonlinear regression analysis using the Gompertz model and associated statistics for in planta multiplication of strains of *Xanthomonas axonopodis* pv. *allii* from Réunion Island in detached leaves of onion cv. Véronique

Strain	Host of isolation	AFLP group	Rate (r^*) ^x	95% confidence interval of r^* ^y	Maximum population size (K)	95% confidence interval of K ^y	R^{2z}
JQ632-1	Onion	A	0.632 b	0.585 – 0.679	8.901	8.724 – 9.084	0.937
JR511-1	Onion	A	0.533 de	0.483 – 0.587	9.500	9.206 – 9.833	0.923
LMG 16528	Onion	Unique	0.587 d	0.552 – 0.623	9.389	9.233 – 9.572	0.950
JX718-3	Onion	B	0.564 d	0.520 – 0.605	9.523	9.302 – 9.773	0.944
LA187-47	Onion	B	0.605 abc	0.558 – 0.658	9.226	8.999 – 9.469	0.935
JX373-2	Leek	B	0.545 d	0.510 – 0.583	9.633	9.424 – 9.859	0.947
LB239-18	Leek	B	0.568 cd	0.528 – 0.611	9.350	9.140 – 9.586	0.940
JX366-2	Garlic	B	0.630 ab	0.582 – 0.679	9.229	9.028 – 9.451	0.937
LA261-18	Garlic	B	0.653 a	0.597 – 0.712	8.951	8.744 – 9.192	0.924

^x r^* is the daily rate of bacterial multiplication and is expressed as log units leaf⁻¹ day⁻¹. Different letters indicate significant differences based on a likelihood ratio test ($P = 0.05$).

^y Bootstrap confidence intervals for the model parameters (1,000 iterations), as described in Huet et al. (16).

^z Adjusted coefficients of determination.

on several characteristics including AFLP, differential utilization of three carbon sources (L-fucose, D-melibiose, and citric acid), and quantitative variations in xanthomonadin pigment. Xanthomonadins protect against photooxidative damage and play a role in epiphytic survival under natural, high light intensity (18,23,24), which is the case in the tropical environment of the Mascarene archipelago. For instance, a reduction in xanthomonadin pigment production impaired the epiphytic survival of a *X. campestris* pv. *campestris* mutant but did not reduce its in planta growth (23). Interestingly, group A (producing pale yellow colonies) and B (producing bright yellow colonies) strains were isolated from onion field outbreaks in Réunion Island at ratios close to 1:1 in the very first years following pathogen establishment. Less than a decade later, group A strains could no longer be isolated from field samples from the same areas (more than 500 isolation attempts in the main *Allium* production area from 2001 to 2005 yielded only group B strains), suggesting differences in fitness between group A and B strains. We can speculate that the low level of the group A strains xanthomonadin production may have reduced epiphytic fitness and led to the extinction of this group and the predominance of group B strains. However, several factors other than xanthomonadin pigments likely can influence epiphytic and epidemic fitness (29).

Early work on the BBO pathosystem suggested that onion seeds were the most likely sources of inocula for outbreaks recorded in Hawaii, although the causative bacterium was not detected from seed at that time due to the lack of efficient diagnostics tools (2). In Réunion Island BBO outbreaks were first observed on onion in 1993, 9 years after the first BBO observation from the neighboring island of Mauritius (S. Benimadhu, *personal communication*). Based on several molecular methods, strains associated with BBO in Réunion Island are highly related genetically to strains isolated from diseased onion from Mauritius Island. Although long distance movement of *X. axonopodis* pv. *allii* through diseased onion plants (the pathogen has never been detected from onion bulbs) or other pathways cannot be excluded, seed is the most probable pathway for introduction of *X. axonopodis* pv. *allii* in Réunion Island. This hypothesis was experimentally strengthened through the interception of *X. axonopodis* pv. *allii*-contaminated onion seed lots produced in Mauritius Island and imported into Réunion Island. Hence, the present study confirms previous suspicions that a seedborne inoculum of *X. axonopodis* pv. *allii* can become established in a virgin or already contaminated area (2). The importance of international movement of onion seed as a pathway for the worldwide emergence of BBO remains unknown, but may account for the global distribution of *X. axonopodis* pv. *allii*, introduction of *X. axonopodis* pv. *allii* into new onion-growing areas, and worldwide emergence of BBO. Our results suggest the need to develop certification schemes for onion seed against *X. axonopodis* pv. *allii*.

ACKNOWLEDGMENTS

We thank E. L. Civerolo for proofreading in English and manuscript review before submission, J. R. Neto, S. Benimadhu, S. Saumtally, and D. Stead for providing us with bacterial strains, and C. Boyer, K. Vital, and W. Grondin for skilled technical assistance. Odéadom, the European Union (FEOGA), Conseil Général de La Réunion, and CIRAD provided financial support.

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