Outbreaks of Bacterial Spot Caused by *Xanthomonas gardneri* on Processing Tomato in Central-West Brazil

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ABSTRACT

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Severe epidemics of bacterial spot have been observed in central-west Brazil in fields of processing tomato. Several xanthomonads, *Xanthomonas axonopodis* pv. *vesicatoria*, *X. vesicatoria*, or *X. gardneri*, can cause the disease; therefore, attempts were made to identify the pathogen species present in this region. A total of 215 strains were obtained from 10 commercial areas in 1997, 1998, and 2000. The strains were characterized using pulsed-field gel electrophoresis (PFGE) and by their amylolytic and pectolytic activities. Representative strains from each PFGE haplotype then were tested for pathogenicity on tomato and pepper, carbon source utilization, and whole protein sodium dodecyl sulfate–polyacrylamide gel electrophoresis. rRNA sequence comparisons also were performed. All strains recovered from six fields were classified as *X. gardneri*, whereas *X. vesicatoria* and *X. axonopodis* pv. *vesicatoria* also were detected in the remaining four fields. Strains of *X. gardneri*, which could be grouped into two PFGE haplotypes, were unable to hydrolyze starch and pectate and to utilize gentiobiose and maltose. They expressed the β protein of 27 kDa and were pathogenic on tomato but variable on pepper. This is the first report of outbreaks of bacterial spot on tomato caused by *X. gardneri*.

Additional keywords: *Lycopersicon esculentum*

Processing tomato (*Lycopersicon esculentum* Mill.) production areas in Brazil gradually are moving from the southeastern and northeastern regions towards the central-west, in the states of Goiás and Minas Gerais (1,24). In this new agricultural frontier, production increased from 5,000 ha in 1991 to 11,000 ha in 1998 and represents one-half of the total Brazilian acreage of processing tomato. Also, the edaphoclimatic conditions are favorable for the crop, resulting in a superior average yield of 63 t/ha in 1999 compared with the Brazilian average of 46 t/ha (24).

Bacterial spot, a worldwide important disease of tomato and pepper (*Capsicum annuum* L.), has become a major problem in sprinkler-irrigated fields of processing tomato in Brazil. The disease has the potential to cause heavy yield losses due to its seedborne nature, the low efficiency of chemical control, and the lack of resistant

cultivars (10,11,14). Three *Xanthomonas* spp. have been shown to cause bacterial spot on tomato and pepper: *Xanthomonas axonopodis* pv. *vesicatoria*, *X. vesicatoria*, and *X. gardneri* (15,16,27). The latter was first described in Yugoslavia and was initially classified as *Pseudomonas gardneri* (26). Since then, it had been considered a synonym of *X. vesicatoria* (8). However, the taxonomical status of *X. gardneri* is still undefined (30) in spite of being genetically and phenotypically distinct from *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria* (7,12,15,16). Thus, we will refer to this unique population as *X. gardneri* for lack of a universally accepted species name.

The criteria used to phenotypically characterize the *Xanthomonas* spp. associated with bacterial spot on tomato include amylolytic and pectolytic activities, patterns of carbon utilization, serology, fatty acid profiles, silver staining of protein profiles using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and pathogenicity (4,15,25). Also, their genomic structures have been differentiated by analysis of restriction profiles of *hrp* genes, pulsed-field electrophoresis (PFGE) of digested genomic DNA, DNA-DNA reassociation kinetics, repetitive DNA sequence-based polymerase chain reaction (rep-PCR), and rRNA sequence homology

(5,15,18,19). *X. gardneri* belongs to group D of the xanthomonads causing bacterial spot disease on tomato. The bacterium is characterized by its inability to hydrolyze starch and pectate and to utilize gentiobiose as a source of carbon (5), a reaction pattern with a group of monoclonal antibodies, and the presence of a 27-kDa protein typical of this group (16). In addition to the reference strain, originally described in Yugoslavia in 1957 (26), other *X. gardneri* strains recently were identified in Costa Rica (5).

This work reports the identification and characterization of strains of *X. gardneri* associated with severe outbreaks of bacterial spot in commercial fields of processing tomato in the central-west region of Brazil.

MATERIALS AND METHODS

Isolation of strains. Strains were isolated from diseased plants, most of them hybrid cultivars, grown in sprinkler-irrigated fields from the states of Goiás and Minas Gerais (Table 1). Leaves and fruit showing symptoms of bacterial spot were collected randomly throughout the fields.

Plant material was washed in tap water and pieces containing typical spot lesions were excised, surface disinfested with 70% ethanol for 10 s and with 3% sodium hypochloride for 30 s, and finally rinsed with sterile distilled water. Bacteria were isolated by grinding the tissue in sterile distilled water (100 μ l) with a pestle in a sterile mortar and streaking the undiluted extract onto nutrient agar (NA) plates (23). The plates were incubated at 28°C for 3 to 5 days. Single, yellow colonies were selected and transferred to another NA plate to secure purity. The strains were stored in phosphate buffer (pH 7.0) at room temperature over the short term and in nutrient broth amended with 30% glycerol at –80°C for long-term storage (21). Strains 75-3, XV56 (4), and XG101 (26) were included in the study as representatives of *X. axonopodis* pv. *vesicatoria*, *X. vesicatoria*, and *X. gardneri*, respectively. These strains were obtained from the working collection of Instituto Agronômico do Paraná, Londrina, Brazil.

PFGE of digested genomic DNA. PFGE analysis was performed with all 215 field strains as described by Egel et al. (9), with minor modifications. Genomic DNA from lysed agar-embedded cells of bacte-

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rial strains was digested with *Xba*I (Invitrogen Life Technologies, Carlsbad, CA). DNA fragments were resolved in 1.2% agarose gels prepared with 0.5× TBE running buffer (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA, pH 8.0). Runnings were carried out in a CHEF-DR II apparatus (Bio-Rad Laboratories, Hercu-

les, CA) at 12°C and 6V/cm of gel with pulse times of 4 s for 1 h followed by 15 s for 24 h. Gels were stained with ethidium bromide (0.5 µg/ml). Co-migrating DNA fragments larger than 100 kb (9) were compared and distinct banding patterns were regarded as different haplotypes. Dice coefficients of similarity were calcu-

lated from the restriction fragment data for all pairwise strain combinations, including strains 75-3, XV56, and XG101 (PFGE groups A, B, and D, respectively [16]), with NTSYS-pc (version 1.70; Exeter Software, Setaukaat, NY). Parsimony analysis was used to establish genetic relationships among representative strains from each haplotype, including strains 75-3, XV56, and XG101. Analyses of 500 bootstrap replicates were performed using the PARS routine of PHYLIP version 3.6a3 (10) and a consensus tree was generated using the CONSENSE routine.

Starch hydrolysis and pectate utilization. The ability to hydrolyze starch and pectate was determined by placing 10-µl droplets of bacterial suspensions (10^8 CFU/ml, absorbance at 600 nm [A_{600}] = 0.1) prepared in distilled water on NA amended with 1% starch and on crystal violet-pectate medium without crystal violet (6). All 215 field strains were used in these tests. A strain was considered positive for amylolytic activity if the bacterial growth was surrounded by a clear zone after flooding the plates with lugol. A depression in the medium was interpreted as a positive reaction for pectolytic activity. Strain XV56 was used as positive control and strains 75-3 and XG101 as negative controls for both tests.

Pathogenicity tests. The susceptible cultivars Walter (tomato) and Early Calwonder (pepper) were inoculated with 94 strains which included the PFGE haplotypes and representative strains from each field. Bacterial cultures were obtained from colonies transferred from NA to nutrient broth and shaken (12,000 rpm) overnight at 28°C. Bacterial suspensions then were centrifuged and the resulting pellets were resuspended in distilled water to a final concentration of 5×10^7 CFU/ml (1:10 dilution of A_{600} = 0.3). Plants with four to five true leaves (6 and 5 weeks after sowing for pepper and tomato plants, respectively) were spray inoculated with bacterial suspension and then maintained in a moist chamber for 2 days before being transferred to a greenhouse (25 to 35°C). Two plants of each cultivar were used for each strain. Symptom development was observed periodically up to 21 days after inoculation.

Carbon source utilization. The carbon sources tested were gentiobiose, maltose, D(+) melezitose, D(+) raffinose, and D(+) trehalose (5,8). Bacterial suspensions (10 µl each) prepared with distilled sterile water (10^8 CFU/ml, A_{600} = 0.1) were deposited on plates of medium C of Dye (13) amended with 0.5% of one of the carbon sources (24). Bacterial growth was compared with plates devoid of carbon after incubation for up to 5 days at 28°C. Two plates were used per strain. The test was performed with 21 strains representing the PFGE haplotypes and strains 75-3, XV56, and XG101.

Table 1. Number of strains of *Xanthomonas* species isolated from processing tomato plants with bacterial spot in the central-west region of Brazil

State, county and year, field number	Tomato cultivars ^a	Number of strains
Goiás		
Itapaci, 1997		
1	CNPH 401-8	9
2	Andino	10
3	Topmech	4
Rio Verde, 1997		
1	Mixture of hybrids of APTX and Heinz series, Agrocres 7022 and Andino	44
2	Santa Adelia	3
Morrinhos, 1998		
1	Hypeel 108, Hypeel 45	39
2 ^b	Hypeel 45	6
2	Hypeel 45, Rio Grande	20
3	Petomech	4
Morrinhos, 2000		
1	RPT 1570, Hypeel 108, Petoseed 6914	65
Minas Gerais		
Patos de Minas, 1998		
1	Viradoro, Ragu 9414, Heinz 9498	11

^a Rio Grande, Topmech, Petomech, CNPH 401-8, and Viradoro are open-pollinated cultivars, whereas the others are hybrids.

^b Samples collected at seedling stage.

Fig. 1. *Xba*I-pulsed-field gel electrophoresis (PFGE) patterns of strains of *Xanthomonas* spp. Lane M, molecular weight marker λDNA-PFGE (New England Biolabs, Beverly, MA). Lanes 1 to 9, strain XG101, CNPH467, CNPH496, 75-3, CNPH411, CNPH468, CNPH485, XV56, and CNPH345. Molecular sizes are given in kilobases.

Electrophoresis of soluble proteins.

Twenty-one strains and strains 75-3, XV56, and XG101 were characterized by their protein profiles by one-dimensional electrophoretic separation (3,25). Extracts of whole cell proteins were obtained from 2 ml of bacterial suspension (5×10^8 CFU/ml, $A_{600} = 0.3$). The pellet was suspended in 200 μ l of lysis buffer (51.4 mM Tris-HCl, pH 6.8, 1.67% SDS, 10% 2-mercaptoethanol, 16.7% glycerol, 0.01% bromophenol blue) and the suspension heated at 100°C for 10 min and centrifuged at $16,000 \times g$ for 10 min. Protein samples of 12.5 μ l were loaded onto a discontinuous gel consisting of a stacking gel (5% total acrylamide, 62 mM Tris-HCl, pH 8.9, 0.05% SDS) and a separation gel (10% total acrylamide, 378 mM Tris-HCl, pH 6.8, 0.05% SDS). Solutions were deaerated for 15 min and polymerized by adding 0.03% of ammonium persulfate and 0.001% of tetramethylethylenediamine (Temed). Electrophoresis was performed at 4°C by using a Protean II double slab vertical electrophoresis apparatus (Bio-Rad Laboratories) filled with electrophoresis buffer (52 mM Tris, 53 mM glycine, pH 8.9, 0.1% SDS). The voltage was 100 V until the samples reached the separation gel and increased to 180 V until the end of running. The gel was silver stained at room temperature (3).

Sequencing of the 16S rRNA gene and the intergenic spacer. The 16S rRNA gene and the intergenic spacer (IGS) between the 16S and the 23S rRNA genes of strains CNPH345, CNPH411, CNPH467, and CNPH496 were amplified using primers FGPS6-63 and FGPL132'-38 and sequenced using the HAB1-6 primers (16). Amplification was performed in a final volume reaction of 25 μ l using 50 ng of DNA, 0.4 mM each primer, 200 μ M dNTPs, 1 U of ThermalAce DNA polymerase (Invitrogen Life Technologies), and 1 $\times$ ThermalAce buffer. Amplification conditions consisted of one cycle of 95°C for 2 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min; followed by a final cycle of 72°C for 4 min. PCR products were gel purified with a GFX PCR purification kit (Amersham Biosciences, Piscataway, NJ) and sequenced in an ABI 377 sequencing machine (Applied Biosystems, Foster, CA) using the DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences) according to the manufacturer's protocols. PCR products were sequenced to a minimum of a 2 $\times$ coverage.

Sequences were assembled using Sequencher V 3.1 (GeneCodes, Ann Arbor, MI) and edited when necessary based on a visual inspection of the chromatograms. Consensus sequences from each strain were compared with sequences of strains Xv1111 (*X. vesicatoria*), XV153 (*X. axonopodis* pv. *vesicatoria*), and XCGA2 (*X. gardneri*) deposited in GenBank (accession numbers AF123088, AF123089, and AF 123093) (15) using Clustal W (28).

Strains XV153 and XCGA2 are other designations for the original strains 75-3 and XG101, respectively (16). Sequence divergences were estimated with the Kimura two-parameter model with a 3:1 transition:transversion ratio (16) using the DNADIST routine of PHYLIP, version 3.6a3 (10). Parsimony analyses were done as described above except that the DNAPARS routine was used.

RESULTS

PFGE analysis. Six haplotypes represented by strains CNPH345, CNPH411,

CNPH467, CNPH468, CNPH485, and CNPH496 were distinguished among the 215 strains based on their *Xba*I restriction patterns (Fig. 1). These haplotypes and those of reference strains 75-3, XV56, and XG101 were further clustered into three PFGE groups by the parsimony analysis (Fig. 2). The first group was represented by strains 75-3, CNPH411, CNPH468, and CNPH485, with coefficients of similarity ranging from 86 to 93%. The second group was represented by strains XV56 and CNPH345, which shared only 46% similarity. The third group comprised strains

Fig. 2. Genetic relationships among strains representative of pulsed-field gel electrophoresis (PFGE) haplotypes of *Xanthomonas* spp. associated with tomato bacterial spot based on parsimony analysis of **A**, PFGE data, and **B**, sequence of 16S rRNA and intergenic sequence. **A**, Numbers of strains of each haplotype are in parenthesis. **B**, Strains XV153 and XCGA2 are other designations of the original strains 75-3 and XG101, respectively (16). Bootstrap values above 50% are depicted at the corresponding nodes.

XG101, CNPH467, and CNPH496, with coefficients of similarity ranging from 64 to 91%.

Phenotypic characterization. The phenotypic features were rather uniform among strains belonging to the same PFGE group (Table 2). None of the strains from PFGE group 1, for instance, was able to hydrolyze starch and pectate, but all were pathogenic on tomato and pepper, causing brown spots with raised borders and sunken centers. Furthermore, in addition to strain 75-3, three strains from this group were characterized by protein profiles and four by carbon source utilization. All of these strains contained the characteristic α protein (3) of 32 kDa and were able to use gentiobiose, maltose, and D(+) trehalose. However, they were unable to use D(+) melezitose differently from strain 75-3 and differed in their ability to use D(+) melezitose and D(+) raffinose. Strains of PFGE group 2, on the other hand, showed amylolytic and pectolytic activity and were characterized by the presence of the β protein (3) of 27 kDa. They were pathogenic only on tomato, causing dark brown

and flat spots surrounded by water-soaked areas. Strains XV56 and CNPH345 of this group utilized gentiobiose, maltose, and D(+) trehalose, as in PFGE group 1. Both strains also utilized D(+) raffinose, but only strain CNPH345 utilized D(+) melezitose. PFGE group 3 strains, like the PFGE group 1 strains, were unable to hydrolyze starch or pectate. Most of them were pathogenic on both tomato and pepper, whereas 14% were pathogenic only on tomato. The symptoms on this host were the same as those caused by strains of PFGE group 2. When 22 group 3 strains were characterized by protein profile and carbon source utilization, 18 reacted similarly to the reference strain XG101. They were characterized by the presence of the β protein as well as the inability to use gentiobiose, maltose, and D(+) melezitose and the ability to use D(+) trehalose. The utilization of D(+) raffinose was variable.

rRNA sequence analysis. The sequences of the 16S rRNA gene and the IGS region were determined for strains CNPH345, CNPH411, CNPH467, and CNPH496 (GenBank accession numbers

AY288080, AY288081, AY288082, and AY288083, respectively) and compared with those of reference strains. The genetic distances (d) between strains CNPH411 and XV153 (*X. axonopodis* pv. *vesicatoria*) and among strains CNPH496, CNPH467, and XCGA2 (*X. gardneri*) were 0.0000, whereas strain CNPH345 was most closely related to Xv1111 (*X. vesicatoria*; $d = 0.0010$). The parsimony clustering agreed with the genetic distance data; strains CNPH496 and CNPH467 clustered with XG101, CNPH411 clustered with 75-3, and CNPH345 with Xv1111 (Fig. 2).

Classification and haplotypic frequency. Based on the phenotypic and genotypic traits presented above, strains CNPH496 and CNPH467 were classified as *X. gardneri*, strain CNPH411 as *X. axonopodis* pv. *vesicatoria*, and CNPH345 as *X. vesicatoria*. Based on these species assignments, *X. gardneri* was the most prevalent species recovered from all 10 fields; in 6 fields, it was the only species identified (Table 1). Of the two *X. gardneri* haplotypes, the one represented by strain CNPH467 was by far the most prevalent (206 of 207 cases). This strain was deposited in the culture collection of the Instituto Biológico (Campinas, Brazil) under IBSBF No. 1782.

DISCUSSION

Genotypic and phenotypic approaches were used to assign strains of xanthomonads causing bacterial spot disease on processing tomato in central-west Brazil to established *Xanthomonas* spp. Based on PFGE analysis and ribosomal RNA sequence analysis, two haplotypes were identified as *X. gardneri*. This classification is strengthened by the fact that, like reference strain XG101, all strains were not able to use D(+) melezitose but used D(+) trehalose (8). Finally, the finding that the Brazilian strains were unable to use gentiobiose agrees with an earlier report on *X. gardneri* strains in Costa Rica (5). Among the carbon sources utilized, gentiobiose and maltose were the most discriminatory, with negative results only for strains of *X. gardneri*. The substrate maltose might have diagnostic value, but more strains representing all phenotypic groups of *X. axonopodis* pv. *vesicatoria* (A, A1, and C; 16), and *X. vesicatoria* should be evaluated.

Members of *X. gardneri* were pathogenic on either tomato and pepper or only on tomato. It is still unknown if the host range observed for *X. gardneri* also is determined by the avirulence genes *avrBsT*, which induces hypersensitive response in all pepper lines (20), as for both *X. axonopodis* pv. *vesicatoria* and *X. vesicatoria*. On artificially inoculated tomato leaves, *X. gardneri* caused symptoms similar to those described for *X. vesicatoria* (3).

Table 2. Phenotypic features of strains from the central-west Brazil within pulsed-field gel electrophoresis (PFGE) groups^a

Features	PFGE group 1	PFGE group 2	PFGE group 3
Amilolytic activity	0	100	0
Pectolytic activity	0	100	0
Specific protein ^b	α	β	β
Carbon source			
Gentiobiose	100	100	0
Maltose	100	100	0
D(+) melezitose	0	100	0
D(+) raffinose	75	100	21
D(+) trehalose	100	100	100
Pathogenicity			
Tomato cv. Walter	100	100	100
Pepper cv. Early Calwonder	100	0	86

^a Percent of strains in the group that was characterized by positive results in the respective test.

^b Specific protein band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 3. Frequency of *Xanthomonas* spp. isolated from processing tomato fields in central-west Brazil

State, county and year, field number	Frequency of <i>Xanthomonas</i> spp. (%) ^a		
	XG	XAV	XV
Goiás			
Itapaci, 1997			
1	100.0	0.0	0.0
2	100.0	0.0	0.0
3	50.0	0.0	50.0
Rio Verde, 1997			
1	100.0	0.0	0.0
2	100.0	0.0	0.0
Morrinhos, 1998			
1	94.9	5.1	0.0
2 ^b	66.7	33.3	0.0
2	95.0	5.0	0.0
3	75.0	25.0	0.0
Morrinhos, 2000			
1	100.0	0.0	0.0
Minas Gerais			
Patos de Minas, 1998			
1	100.0	0.0	0.0

^a XG = *Xanthomonas gardneri*, XAV = *X. axonopodis* pv. *vesicatoria*, XV = *X. vesicatoria*.

^b Samples collected at seedling stage.

The predominant recovery of *X. gardneri* strains from tomato fields reported here may be indicative that this species is becoming an important component of the bacterial spot complex and is no longer as rare in nature as reported (15). Epidemics caused by *X. gardneri* never have been reported before. The occurrence of new species and types of xanthomonads causing bacterial spot on processing tomato in Brazil may be a consequence of the gradual substitution of open-pollinated cultivars, either locally bred or imported from California, by hybrids marketed by international seed companies (1). Contaminated seed seem to be a crucial factor for introducing the pathogen into noninfested areas (2,11,17). Symptoms on seedlings commercially grown in greenhouses, as observed in Morrinhos in 1998 in field 2, indicated that infected seed might have been used. It was the first year of tomato cropping on that farm and, despite the introduction of intensive copper spraying, the disease became widespread. It is intriguing that the frequency of *X. axonopodis* pv. *vesicatoria* strains declined over time while the frequency of *X. gardneri* increased (Table 3). It could be speculated that differences in fitness, as has been demonstrated in *X. axonopodis* pv. *vesicatoria* with tomato race 3 in relation to race 1 (16), also could exist between *X. axonopodis* pv. *vesicatoria* and *X. gardneri*.

This is the first survey of xanthomonads on processing tomato in Brazil; therefore, it cannot be stated whether or not outbreaks caused by *X. gardneri* are recent. The low polymorphism found among strains could be an indication of a recent introduction event. However, this hypothesis is still tentative, because the level of genetic variation within *X. gardneri* stills needs to be assessed. Thus, understanding bacterial spot complex in Brazil is the first step for directing future research on detection, survival, chemical control efficiency, and breeding for attaining acceptable levels of control in the field.

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