A Leaf Spot and Blight of Greenhouse Tomato Seedlings Incited by a *Herbaspirillum* sp.

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ABSTRACT

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A leaf spot and blighting were observed on leaves of tomato transplants from a producer in Florida in 2001 and 2002. A nonfluorescent bacterium was isolated consistently from affected tissue. The typical bacterium was a gram negative, strictly aerobic, slightly curved rod with one or two flagella. Sequence analysis of the 16S rRNA indicated that two representative strains, F1 and SE1, had greater than 99% nucleotide sequence identity with *Herbaspirillum huttiense* and *H. rubrisubalbicans*. The cellular fatty acid composition of the total of 16 tomato strains was very similar to *H. huttiense* and *H. rubrisubalbicans*. Based on carbon utilization, six of nine strains tested with the Biolog system were identified as *Herbaspirillum* spp. The tomato strains were oxidase positive and grew at 40°C, but were negative for levan production, pectate hydrolysis, and arginine dihydrolase activity. Based upon this polyphasic analysis, we concluded that the strains were most closely related to *H. huttiense*, although placement in this species would require further analyses. However, the tomato strains and *H. rubrisubalbicans*, but not *H. huttiense*, caused confluent necrosis when infiltrated at high concentrations into tomato leaves and were able to produce leaf spot symptoms on inoculated tomato seedlings in the greenhouse. Using pulsed-field gel electrophoresis, we determined that there was considerable variability between the strains collected in 2001 and 2002.

Additional key words: Bacterial spot, *Pseudomonas huttiensis*

In Florida, bacterial spot of tomato (*Solanum lycopersicum* L.), caused by *Xanthomonas euvesicatoria* and *X. perforans* (15) (previously designated *X. campestris* pv. *vesicatoria*), is a major disease causing significant problems on tomato transplants as well as field-grown tomato. Tomato transplant production supplies virtually all plants to the $400 million fresh-market tomato industry in Florida. As such, control measures are used to limit losses associated with this disease on transplants. Integrated with cultural practices and other foliar treatments, bacteriophages have been shown to effectively control bacterial spot, resulting in increased yields (5,10,17). Recently, bacteriophages received Environmental Protection Agency registration (EPA registration no. 67986-1) for use on tomato and, therefore, are currently being used in greenhouses and production fields in Florida to control bacterial spot.

In October 2001 and July 2002, a leaf spot and blight was observed on 3- to 4-week-old tomato seedlings of a commercial cultivar grown for transplants in a greenhouse in southern Florida. The greenhouse operation had used bacteriophages as part of a strategy to control bacterial spot. The seedlings displayed various symptoms. Some plants showed discrete lesions along the leaf veins and isolated leaf spots (Fig. 1A). The spots were irregular and varied in size from 2 to 4 mm. Affected tissue was discolored and necrotic (Fig. 1B). Most of the seedlings had apical or marginal leaf necrosis. The diseased tissue collapsed, turned brown, and necrotized. Blighting of younger plants resulted in those plants being discarded. The disease remained localized on individual plants or on plants within one tray. Symptom development stopped within a week of the initial appearance. The symptoms did not occur on older plants. Less than 5% of the plants grown in the greenhouse were affected. Due to concern of possible spread of pathogens on tomato transplants in the greenhouse and the unusual symptoms of the disease, we investigated the causal agent of the seedling leaf spot and marginal leaf necrosis. In this article, we describe the isolation and identification of the bacterium associated with this new disease of tomato seedlings.

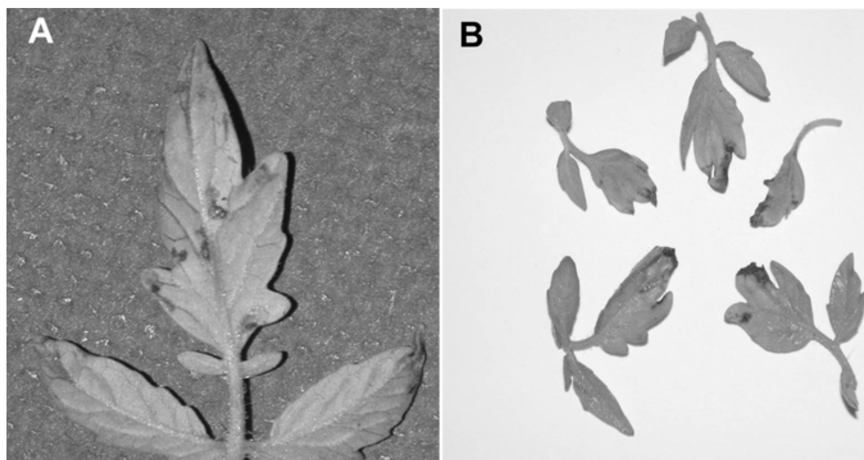

Fig. 1. A, Leaf spot and **B,** apical leaf necrosis of naturally infected tomato seedlings grown in the greenhouse.

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MATERIALS AND METHODS

Isolation. Diseased transplants were received in October 2001 and in July 2002. Lesions from leaves of the diseased transplants were triturated in sterile tap water and the resulting suspensions were streaked onto nutrient agar (NA) plates. The plates were incubated at 28°C for 48 h. Representative colonies were transferred to new NA plates and used for further testing. Twelve strains were isolated from two samples received in 2001 and four strains from one sample obtained in 2002.

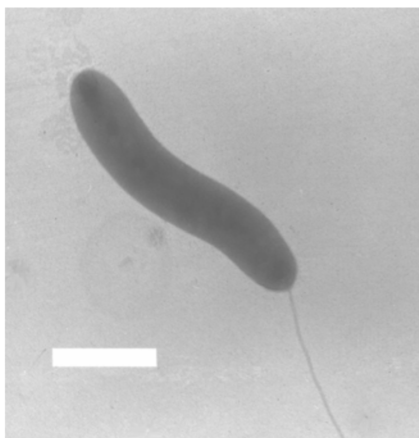

Fig. 2. A cell of representative strain SE1 with one polar flagella observed by transmission electron microscopy. The white bar represents 1 µm.

Physiological and biochemical tests. Characteristics of 16 strains isolated from diseased seedlings were determined using standard bacteriological tests, including gram reaction, production of a fluorescent pigment on King's medium B, oxidase reaction, levan production, pectate hydrolysis, arginine dihydrolase, and oxidative-fermentative (O/F) metabolism of glucose, as described by Schaad et al. (20). Carbon substrate utilization using Biolog GN MicroPlates (Biolog, Inc., Hayward, CA) was performed on nine strains representing isolations made in 2001 and 2002.

Cellular fatty acid composition of 16 tomato strains was determined and used to identify the pathogen. Bacteria were grown, extracted, and analyzed according to the standard Microbial Identification System (MIDI) protocol (MIDI, Newark, DE; 19). Extracts were analyzed using the Sherlock System. Results then were used to compare unknown strains with each other, with control strains, and with data from the MIDI database (MIDI Library Generation System version 4.5) in order to identify the pathogen.

Restriction endonuclease analysis. Genomic DNA of the bacterial strains was prepared and digested with *Spe*I as described by Egel et al. (9). The resulting large DNA fragments were separated by pulsed-field gel electrophoresis (PFGE). The gels were stained with ethidium bromide. All strains were run on the same gel so that accurate comparison could be made. SE1 and F1 were selected as representative strains based on their PFGE patterns.

Electron microscopy. Cells were grown in nutrient broth and incubated on a shaker

Fig. 3. Dendrogram representing cluster analysis based on fatty acid methyl ester profiles of tomato strains (F1, F2, F3, F4, F5, F6, SE1, SE3, SE4, U4, U5, U6, KB1, KB2, KB5, and KB6), control strains (*Herbaspirillum rubrisubalbicans*: LMG1278, LMG2286, and LMG6418; and *H. huttiense*: LMG2199) and data from the Microbial Identification System (MIDI) software database (MIDI Library Generation System version 4.5).

at 28°C for 24 h. A representative strain (SE1) was used and cell morphology was observed by transmission electron microscopy (TEM; 6).

Sequence analysis of 16S rRNA. Amplification of the *rrs* (i.e., the 16S rRNA gene) and the intergenic spacer (IGS) located between *rrs* and *rrl* (i.e., the 23S rRNA gene) was performed under standard polymerase chain reaction (PCR) conditions using primers FGPS6-63 and FGPL132′ as described by Ponsonnet and Nesme (18). We sequenced the 16S rRNA of two representative strains (SE1 and F1) and the sequences were compared in the GenBank, EMBL, and DJB databases using the gapped BLASTN 2.0.5 program through the National Center for Biotechnology Information server (1).

Pathogenicity and hypersensitive reaction tests. Hypersensitivity of the strains was tested by infiltration of bacterial suspensions (10^8 CFU/ml) in tomato leaves. Plants were incubated at approximately 24°C and monitored for confluent necrosis for 48 h.

In order to determine whether these strains induce a compatible or incompatible interaction in tomato leaflets, population dynamics and electrolyte leakage assays were performed. Six-week-old tomato plants (cv. Bonny Best) were infiltrated with bacterial suspensions (10^6 and 10^5 CFU/ml, respectively) of two representative strains (SE1 and F1), a strain of *Herbaspirillum huttiense* (LMG2199), and a tomato race 3 strain of *X. perforans* (91-118) and monitored for electrolyte leakage and population dynamics. Plants were incubated in a growth room at 28°C on a 12-h light cycle. Immediately after inoculation and subsequently for 3 days, samples of the infiltrated tissue were taken with a cork borer and the pathogen populations and electrolyte leakage were measured as previously described (7,11).

In order to reproduce the symptoms of natural infection, 3-week-old tomato cv. Bonny Best seedlings were inoculated using two different methods. The first method consisted of spraying the plants with bacterial suspensions (10^8 CFU/ml) followed by incubation in high humidity conditions for 24 h in a growth chamber. The second method consisted of preincubating the tomato seedlings in high humidity provided by covering plants with plastic bags 24 h prior and after inoculation. The seedlings were inoculated by dipping the plants in bacterial suspension of the investigated strains (10^6 CFU/ml + Silwet L77, 0.025%) for 15 s. Both sets of inoculated plants were removed from the high-humidity conditions and transferred to the greenhouse for symptom development. Diseased leaves of inoculated plants were used for reisolation of bacterial strains, as described previously. In both experiments, the same procedure was applied for control plants with no bacteria added to the treatment.

In order to evaluate disease severity, tomato cv. Florida 47 transplants were grown in 10-cm pots containing commercial substrate (Floradur Anzuchterde, Typ-fein; Floragard Product Gartnererde, Germany). Prior to inoculation, 4-week-old plants were incubated in high humidity for 24 h. Five plants per strain were inoculated by spraying with a hand-held plastic sprayer the bacterial suspensions (approximately 10^8 CFU/ml) of representative strains isolated from tomato (F1 and SE1) and control strains of *H. rubrisubalbicans* (LMG2286^T = ATCC 19308), *H. huttiense* (LMG2199^T = ATCC 14670), and *X. perforans* (91-118) onto the foliage, followed by incubating under high humidity conditions for an additional 24 h. Control plants were sprayed with sterile water. Both, inoculated and control plants were transferred to the greenhouse bench and spread out in a completely randomized design, followed by daily observation for symptom

Table 1. Composition of major fatty acids of selected strains isolated from tomato transplants and *Herbaspirillum rubrisubalbicans* and *H. hutiense*

Fatty acid	SE1	F1	*H. rubrisubalbicans*	*H. huttiense*
10:0 3OH[a]	1.9	1.8	1.7	1.7
12:0	3.8	4.0	3.5	3.7
12:0 3OH	3.5	3.5	3.3	3.0
14:0 2OH	2.7	2.9	2.3	2.6
Sum feature 3[b]	37.5	37.9	33.1	38.4
16:0	23.7	23.0	25.8	26.5
17:0 cyclo	2.4	1.9	5.4	1.7
18:1 ω7c	21.4	21.3	20.9	19.3

[a] Only fatty acids with greater than 1% of total composition were included in this table.
[b] Sum feature 3 (15:0 ISO 2OH/16:1w7c).

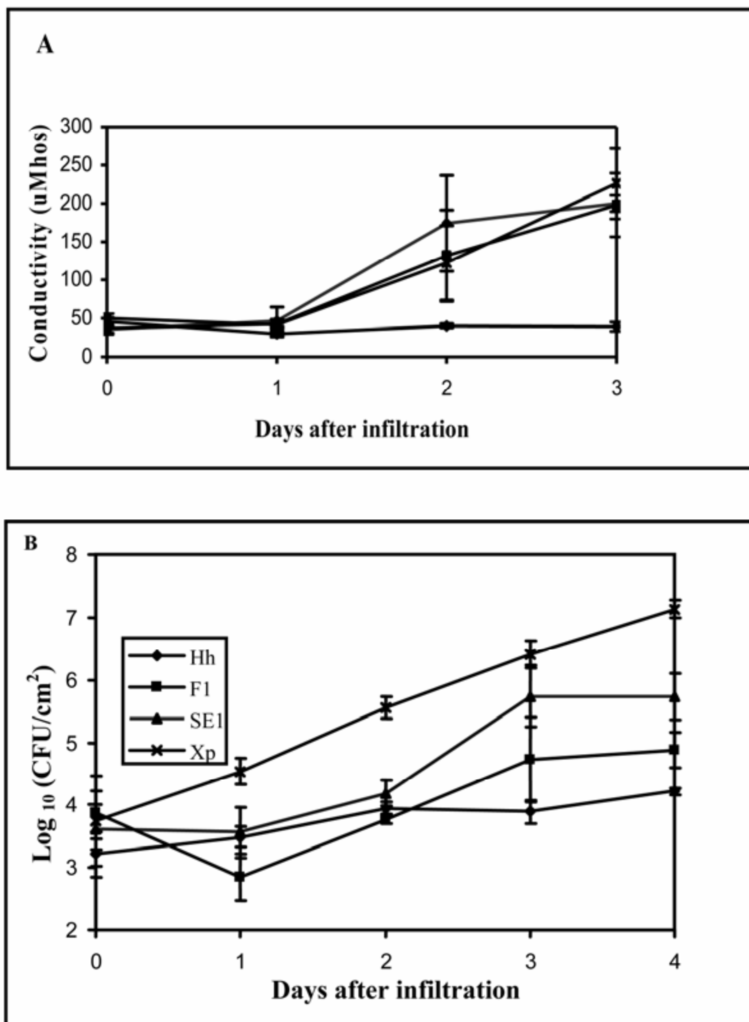

Fig. 4. A, Electrolyte leakage and **B,** internal populations from leaf tissue of tomato cv. Bonny Best plants, infiltrated with suspension of two tomato strains (SE1 and F1), *Herbaspirillum huttiense* (*Hh*), and *Xanthomonas perforans* (*Xp*).

development. Disease severity was evaluated by estimating the leaf area affected with necrotic lesions using the Horsfall-Barratt (HB) rating scale (12) 7 and 14 days after inoculation. The HB scale values were transformed to percent values and the data then were analyzed by using SAS software using analysis of variance (Proc ANOVA; SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Characterization of bacterial strains. The consistent isolation of nonfluorescent bacterial strains forming creamy white colonies indicated that the bacterium was different from known pathogens causing symptoms on tomato foliage (*X. perforans*, *Pseudomonas syringae* pv. *tomato*, *P. syringae* pv. *syringae,* and *P. viridiflava*; 13, 14). When observed by TEM, cells of the representative strain (SE1) were slightly curved, with one or two flagella on one or both poles (Fig. 2). The strains were gram negative, strict aerobes, and oxidase positive, and grew at 40°C, but were negative for levan, pectate hydrolysis, and arginine dihydrolase. According to the fatty acid profiles, the strains displayed greatest similarity with the bacterium *P. huttiensis* (16), indicated as a first choice by MIDI system, with the similarity index ranging from 0.538 to 0.849. Because this organism was proposed to be transferred to the genus *Herbaspirillum* (2,3,8), we also included for comparison one *H. huttiense* (LMG2199^{T}) and three *H. rubrisubalbicans* (LMG2286^{T}, LMG6418, and LMG1278) strains. When the data generated by fatty acid analysis were processed by MIDI software, the strains isolated from tomato and the control strains clustered into one group (Euclidean distance ≤6), indicating a high degree of similarity in fatty acid composition (Fig. 3). However, within the group, the strains of *H. rubrisubalbicans* (LMG2286, LMG1278, and LMG6418) and two tomato strains formed a cluster or subgroup separate from *H. huttiense* (LMG2199) and 14 of the tomato strains which included SE1 and F1. The fatty acid composition of two representative tomato strains (SE1 and F1) was very close to *H. rubrisubalbicans* and *H. huttiense* (Table 1). The two tomato strains and *H. huttiense* had relatively low amounts of 17:0 cyclo and relatively high amounts of sum feature 3 compared with *H. rubrisubalbicans*. Results of PFGE showed that there was considerable variability in banding pattern between the strains collected in 2001 and 2002 (*data not shown*).

Based on differential oxidation of the 95 carbon substrates using the Biolog GN MicroPlate, five of the nine tested strains, including F1, were identified as *H. huttiense* and one as *H. rubrisubalbicans*, while the remaining three, including SE1, were most closely related to the carbon source utilization profile of genus *Burkholderia* saved in the database. The type strains of *H. huttiense* (LMG2199) and *H. rubrisubalbicans* (LMG2286) were identified correctly using the Biolog GN database.

Sequence analysis of the 16S rRNA indicated that the two strains F1 (GenBank accession no. EF216331) and SE1 (GenBank accession no. EF216332) had 99.6% homology between each other, 99.5 and 99.1% nucleotide sequence identity with *H. huttiense* (LMG2199 = ATCC 14670), and 99.8 and 99.3% nucleotide sequence identity with *H. rubrisubalbicans* (LMG 2286 = ATCC 19308), respectively.

Based on results of polyphasic analysis, the strains isolated from tomato transplants had greatest similarity with *H. huttiense*. The strongest evidence for the close relatedness of the tomato strains with this species is based on the 16S rRNA sequence analysis. Both strains had greater than 99% sequence identity with *H. huttiense*. Fatty acid analysis and Biolog also were useful for showing a closer relationship between the tomato strains and *H. huttiense* than with *H. rubrisubalbicans*.

Pathogenicity and hypersensitive tests. The tomato strains (SE1 and F1) and the *H. rubrisubalbicans* strain, when infiltrated at 10^{8} CFU/ml into tomato leaves, caused a hypersensitive reaction within 24 h, whereas *X. perforans* caused tissue collapse after 48 h and the *H. huttiense* strain caused no symptoms. In the experiments where leaflets were infiltrated with bacterial suspensions adjusted to approximately 10^{6} CFU/ml, the curves for electrolyte leakage for strains SE1 and F1 were similar to that of the bacterial spot pathogen, *X. perforans*. Electrolyte leakage basically remained unchanged in leaflets infiltrated with the *H. huttiense* strain (Fig. 4A). In population determinations where the bacterial suspensions were adjusted to 10^{5} CFU/ml and infiltrated into tomato leaflets, the two tomato strains did not grow to the same level as the *X. perforans* strain, but reached levels at least 10-fold lower (Fig. 4B). This may indicate that these strains are less virulent in tomato leaves. Again, the *H. huttiense* strain showed basically no multiplication in the infiltrated areas. Thus, the tomato strains acted like bacterial plant pathogens, whereas the known *H. huttiense* strain was typical of a nonpathogen.

In inoculation tests, only the strains isolated from tomato seedlings were able to produce symptoms similar to those observed in natural infection. Marginal tissue of leaves of inoculated plants collapsed and became necrotic 3 days after inoculation. Necrotic lesions occurred along the leaf margins. Small, pinpoint spots were

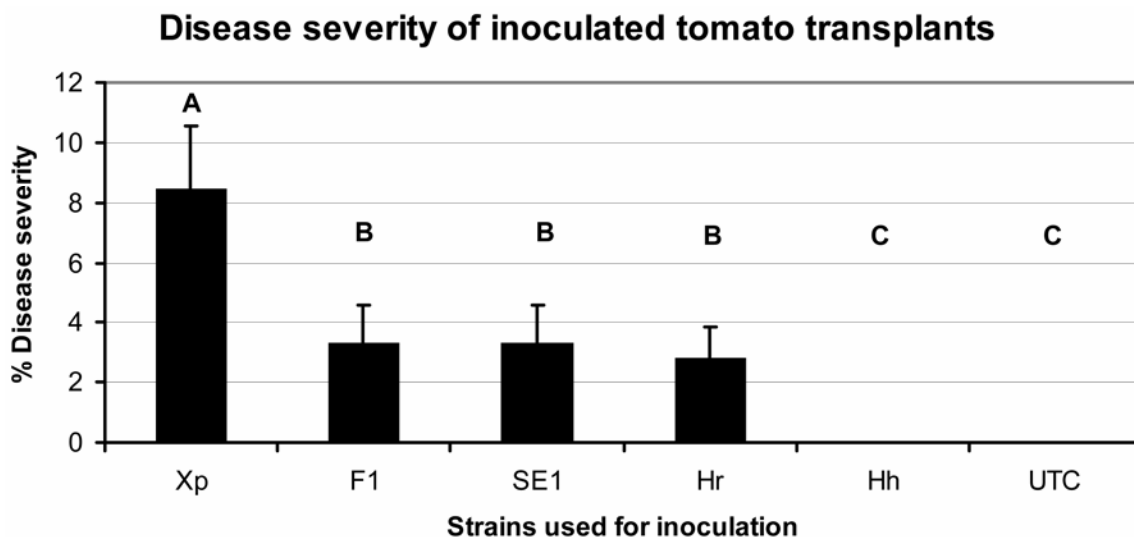

Fig. 5. Disease reaction of tomato transplants inoculated with *Xanthomonas perforans* (Xp; 91-118), tomato strains F1 and SE1, *Herbaspirillum rubrisubalbicans* (Hr; LMG2286), *H. huttiense* (Hh; LMG2199), and untreated control (UTC). The letter above the bars represents statistical difference between treatments according to Waller-Duncan's test.

observed on the abaxial leaf surface 5 days after inoculation. The symptoms were more severe when a 24-h high-humidity incubation period preceded inoculation. Younger plants were more susceptible than older plants (*data not shown*). No secondary spread of symptoms was observed after the plants were transferred to the greenhouse. Based on population and electrolyte experiments, this organism was determined to be a weak pathogen. However, pathogenicity tests demonstrated that, under high-humidity conditions which exist in tomato transplant production, this weak pathogen was able to cause disease.

Statistical analysis of the disease severity data grouped the strains used for inoculation into three groups differing significantly in the severity of symptoms observed on tomato seedlings (Fig. 5). The known tomato pathogen *X. perforans* caused the strongest plant reaction observed 1 and 2 weeks after inoculation. The two tomato strains (F1 and SE1) and *H. rubrisubalbicans* strain also caused a leaf spot on inoculated transplants, although the severity was not as strong as on plants inoculated with *X. perforans*. *H. huttiense* was unable to produce any symptoms on the foliage (Fig. 5).

There are no records of *H. huttiense* being associated with plants. The type strain used for classification was isolated from distilled water (8). However, the endophytic occurrence of *H. seropedicae* and *H. rubrisubalbicans* was reported earlier (4). The habitat of *H. seropedicae* is roots, stems, and leaves of plants from the Gramineae family, whereas *H. rubrisubalbicans* seems to be limited to sugarcane (*Saccharrum officinales*), causing mottled stripe disease (3).

The origin of the strains associated with diseased tomato transplants is unknown. In the location where the transplants were produced, sugarcane was used as a windbreak. Given that *S. officinales* is a natural host for *H. rubrisubalbicans* and that the organism isolated from tomato was closely related to the sugarcane pathogen, it is plausible that sugarcane served as an inoculum source of the pathogen. Furthermore, it may be conceivable that *H. rubrisubalbicans* is heterogeneous and, in fact, may consist of at least two species of bacteria, with one including *H. rubrisubalbicans* and the other representing the strains more closely related to *H. huttiense*. There is definite precedence for more than one bacterial species within a bacterial genus causing disease on the same host, as has been shown with xanthomonads in which four species cause bacterial spot on tomato plants (15), and with several fluorescent pseudomonads being associated with leaf spots or blighting of tomato foliage (13,14).

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