A Novel *Xanthomonas* sp. Causes Bacterial Spot of Rose (*Rosa* spp.)

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Abstract

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A bacterial spot of rose (*Rosa* spp.) caused by a xanthomonad was observed in Florida and Texas. Ten representative strains collected from the two states between 2004 and 2010 were used to determine the taxonomic position of this rose pathogen. Fatty acid methyl ester analysis was performed and a nearly 2-kb 16S-23S rRNA intergenic spacer along with flanking portions of the 16S and 23S rRNA genes were sequenced for selected strains, showing that they were members of the genus *Xanthomonas*. Multilocus sequence typing and analysis (MLST/MLSA) and pathogenicity tests were conducted to further characterize the *Xanthomonas* strains. The MLSA, based on six gene fragments—*fusA*, *gapA*, *gltA*, *gyrB*, *lacF*, and *lepA*—showed that the

rose strains fell into *Xanthomonas axonopodis* subgroup 9.2 and shared the highest similarity values (98.8 to 99.7%) with *X. alfalfae* subsp. *citrumelonis* of the subgroup. However, principal coordinate analysis grouped the rose strains into a unique cluster distinct from other members of the subgroup according to virulence phenotypes on 11 plant species belonging to five plant families (Araceae, Euphorbiaceae, Rosaceae, Rutaceae, and Solanaceae). Moreover, the rose strains were aggressive on rose and Indian Hawthorn (*Raphiolepis indica*). On the basis of the MLSA and virulence phenotypes, the pathovar epithet *rosa* is proposed.

Xanthomonas is a large genus of plant-pathogenic bacteria that are gram negative, rod shaped, and yellow pigmented, causing diseases in up to 400 plant hosts (36). A number of *Xanthomonas* spp. pathogenic on members of the family Rosaceae have been reported, such as *Xanthomonas fragariae* on strawberry (33), *X. pruni* on peach (47), and a *Xanthomonas* sp. on Photinia (23). However, pathogenic *Xanthomonas* spp. have not been documented on rose (*Rosa* spp.), a member of the family Rosaceae. Species-level classification of *Xanthomonas* spp. has been based on DNA-DNA hybridization (DDH; 45), repetitive sequence-based polymerase chain reaction (rep-PCR; 32), and multilocus sequence typing and analysis (MLST/MLSA) (51). Some species of *Xanthomonas* are subdivided into pathovars (6). More than 140 pathovars have been defined within the genus *Xanthomonas* (46). The term pathovar was established to name pathogens that are specific for certain diseases but the classification is merely derived from the pathogenic reaction phenotype of a plant rather than the intrinsic characteristics of these pathogens (46,52). This special classification was adopted as a provisional solution until more adequate data would be established. Since 1995, genomic fingerprinting and DDH have been used to delineate many pathovar groups within the genus *Xanthomonas* into separate species, especially within *X. axonopodis* subgroups (7,9,11,15,31,32,45). Although limitations of DDH have been reported, it has played a central role in the delineation of bacterial species. However, the need for a more flexible approach to define bacterial species has been proposed to avoid the restrictions of DDH (8).

MLST/MLSA is an alternative method to classify bacteria based on comparing sequences of various chromosomal loci among

closely related organisms (20). MLSA results have been compared with species delineated by DDH for *Xanthomonas* spp., suggesting that MLSA mimics groupings derived from DDH, amplified fragment length polymorphism, and rep-PCR and rapidly differentiates species (51). In addition, MLSA can provide an equivalent level of the maximum possible resolution among bacterial isolates determined by whole-genome sequencing (19). More importantly, MLSA offers a relatively simple and rapid way to allocate strains to known species or group them as members of new species (1,51). An MLSA database and website was recently developed for plant-associated microbes, known as the PAMDB, for identifying and comparing strains (1). MLSA data of the PAMDB can assign unknown *Xanthomonas* strains to any of the phylogenetic groups but it has only limited power to differentiate the strains belonging to the same pathovar or subspecies (1). Whole-genome sequencing and additional informative MLSA loci may provide the means to further facilitate identification of *Xanthomonas* strains and to investigate their microevolution. However, clustering may not occur or may include different groups of strains because important phenotypes such as pathogenicity determinants may not be conferred by chromosomal loci or sampled strains may reveal a continuous spectrum of genotypic variation or both (8).

A congruence of systematics using different methods reveals real phylogenetic relationships between strains tested (24,50). Due to the limitations of DDH, MLSA has offered a relatively simple and rapid way to classify strains as members of known species of *Xanthomonas* (1,51). The polyphasic classification using DDH and phenotypic information has elevated a number of *Xanthomonas* pathovars to species (15,45). In addition to MLSA data, determining the host range of pathogenic *Xanthomonas* strains is an important part of phenotypic information because the pathogen is usually specific to a number of related plants and, therefore, often codified in pathovar nomenclature (6). Virulence phenotypes and DNA sequence changes may explain host specialization processes, supporting an appropriate reflection of the pathovar classification (27).

The objectives of this study were to identify bacterial strains isolated from rose, to evaluate their host range relative to other *Xanthomonas* spp., and to determine the taxonomic placement of the rose strains relative to other xanthomonads based on MLSA.

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Materials and Methods

Strain isolation, storage, and DNA extraction. Bacterial strains were isolated from rose samples collected from production nurseries, local retail stores, or samples submitted to the plant diag-

nostic clinics at University of Florida Research and Education Centers in Apopka and Wimauma, FL (Table 1). Strains were recovered by streaking homogenized tissue sections from the lesion margins of symptomatic plants onto nutrient agar (NA) (Difco Laboratories) and recovering the predominant, yellow-pigmented

Fig. 1. Phylogeny for rose strains of *Xanthomonas* based on the concatenated data set of fragments of the genes *fusA*, *gap-1*, *gltA*, *gyrB*, *lacF*, and *lepA*. Nonredundant sequences of the genes of *Xanthomonas* were downloaded from PAMDB (1) for alignment with sequences of rose strains in this study. Numbers on nodes represent bootstrap support values for maximum parsimony (front), maximum likelihood (middle), and Bayesian posterior probabilities presented as percentage (back). Values represented by an asterisk (*) were less than 50% for bootstrap or 90% for Bayesian posterior probabilities. Numbers 9.1 through 9.6 are the groups described by Rademaker and colleagues within *Xanthomonas axonopodis* (34).

colonies typical of *Xanthomonas* spp. after 3 to 5 days of incubation at 28°C. Representative colonies were subsequently streaked and incubated on NA for further purification before being stored at –80°C in 30% glycerol. Other reference *Xanthomonas* strains (Table 1) were similarly purified prior to storage at –80°C. DNA was extracted using the hexadecyltrimethylammonium bromide method, as previously described (2). DNA was stored in Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8.0) at –20°C.

Pathogenicity tests. The pathogenicity of recovered *Xanthomonas* strains (R07, R09, HDRA, and HDRB) was tested using a bacterial suspension (0.01 M MgSO₄ at 2×10^6 CFU/ml) by either infiltrating the abaxial sides of expanding rose leaves with a needleless syringe or by dipping leaves into the bacterial suspension and enclosing them in plastic bags for 48 h to maintain humidity (34,37). Similar inoculations of shrub rose with *X. perforans* strain T4 (XcT4) and 0.01 M MgSO₄ were included as controls, in addition to inoculating tomato ‘FL 47’ with the recovered *Xanthomonas* strains and XcT4. All plants were maintained in a greenhouse. Strains were reisolated from symptomatic tissues as described above, thus completing Koch’s postulates.

Cross-pathogenicity tests. Four rose strains (R07, R09, HDRA, and HDRB) representing the two haplotypes found in this study (Fig. 1) were prepared from NA with glucose cultures grown for 48 h at 28°C, suspended, and diluted to 2×10^4 , 2×10^6 , and 2×10^8

CFU/ml in 0.01 M MgSO₄. Various horticultural plants common to local nursery and agriculture production and representatives of several plant families were chosen to test the host range of the rose strains (Table 2). Plants were inoculated by infiltrating expanding leaves as described above; a pathogen-free 0.01 M MgSO₄ control and a *Xanthomonas* strain pathogenic to the host was included as a standard (Table 2). The inoculated plants were monitored for disease development, comparing symptoms to the mock-inoculated control and *Xanthomonas* standard. Disease was rated using a 0-to-3 scale where 0 showed no response and 3 represented moderate to severe chlorosis and necrosis. The experiment was repeated two to three times. A matrix based on the virulence differences between each pair of strains was generated using GenAlEx version 6.4 (29). This matrix was used in a principal coordinate analysis (PCA) employed in GenAlEx to visualize any clustering based on virulence difference.

Fatty acid methyl ester analysis. Selected rose strains (R07, HDRA, and HDRB) representing the two haplotypes detected in this study (Fig. 1) were subjected to fatty acid methyl ester (FAME) analysis. Whole-cell FAMEs were extracted and analyzed as described previously (5). FAME profiles of the rose strains were compared with those of known strains stored in the Sherlock Microbial Identification system (version 4.5; Microbial ID, Inc.), which were presented as similarity values.

Table 1. *Xanthomonas* strains used in this study

Strain	Species	Host	Location	Year	Source
R07	<i>Xanthomonas alfalfae</i> pv. <i>rosa</i>	<i>Rosa</i> sp.	Florida	2008	G. Vallad
R09	<i>X. alfalfae</i> pv. <i>rosa</i>	<i>Rosa</i> sp.	Florida	2008	G. Vallad
HDRA	<i>X. alfalfae</i> pv. <i>rosa</i>	<i>Rosa</i> sp.	Florida	2009	G. Vallad
HDRB	<i>X. alfalfae</i> pv. <i>rosa</i>	<i>Rosa</i> sp.	Florida	2009	G. Vallad
X1808	<i>X. alfalfae</i> pv. <i>rosa</i>	<i>Rosa</i> sp.	Florida	2004	D. Norman
X1811	<i>X. alfalfae</i> pv. <i>rosa</i>	<i>Rosa</i> sp.	Florida	2004	D. Norman
X1847	<i>X. alfalfae</i> pv. <i>rosa</i>	<i>Rosa</i> sp.	Florida	2010	D. Norman
X1850	<i>X. alfalfae</i> pv. <i>rosa</i>	<i>Rosa</i> sp.	Florida	2010	D. Norman
Tx11	<i>X. alfalfae</i> pv. <i>rosa</i>	<i>Rosa</i> sp.	Texas	2010	K. Ong
Tx12	<i>X. alfalfae</i> pv. <i>rosa</i>	<i>Rosa</i> sp.	Texas	2010	K. Ong
CitN101	<i>X. alfalfae</i> subsp. <i>citrumelonis</i>	<i>Citrus</i> spp.	Florida	Unknown	J. Jones
CitF1	<i>X. alfalfae</i> subsp. <i>citrumelonis</i>	<i>Citrus</i> spp.	Florida	Unknown	J. Jones
Man672	<i>X. axonopodis</i> pv. <i>manihotis</i>	<i>Manihot esculenta</i>	Florida	Unknown	D. Norman
Man678	<i>X. axonopodis</i> pv. <i>manihotis</i>	<i>M. esculenta</i>	Florida	Unknown	D. Norman
X476	<i>X. axonopodis</i> pv. <i>dieffenbachiae</i>	<i>Philodendron</i> spp.	Florida	Unknown	D. Norman
X1750	<i>X. axonopodis</i> pv. <i>dieffenbachiae</i>	<i>Philodendron</i> spp.	Florida	Unknown	D. Norman
Euv69	<i>X. euvesicatoria</i>	<i>Capsicum annuum</i>	Florida	Unknown	P. Roberts
Euv71	<i>X. euvesicatoria</i>	<i>C. annuum</i>	Florida	Unknown	P. Roberts
Euv73	<i>X. euvesicatoria</i>	<i>C. annuum</i>	Florida	Unknown	P. Roberts
XcT4	<i>X. perforans</i>	<i>Solanum lycopersicum</i>	Florida	Unknown	J. Scott

Fig. 2. Symptoms of bacterial spot on *Rosa RADtko* (left) and *RADrazz* (right).

rRNA gene sequencing. A nearly 2-kb 16S-23S rRNA intergenic spacer (ITS) along with flanking portions of the 16S and 23S rRNA genes were sequenced for R07, HDRA, and HDRB. Primers and amplification conditions were used as previously described (40). Amplifications were performed in a Mastercycler ep thermocycler (Eppendorf). Amplicons were sequenced using BigDye Terminator Cycle Sequencing Chemistry and ABI 3730 XL DNA Sequencer (Applied Biosystems) at the Interdisciplinary Center for Biotechnology Research facility, University of Florida, Gainesville. Sequences were edited using Geneious version 5.3.6 (Biomatters Ltd.). The sequences of the rose strains were compared with those from GenBank using BLAST.

MLSA. Ten rose strains (Table 1) were subjected to MLST/MLSA. Primers and amplification conditions for six gene fragments—*fusA*, *gapA*, *gltA*, *gyrB*, *lacF*, and *lepA*—were employed as previously described (1). Methods for DNA sequencing and sequence editing were performed as described above.

Nucleotide sequence accession numbers. The DNA sequence data reported in this study have been deposited in GenBank (<http://www.ncbi.nlm.nih.gov/>) under accession numbers JX013568 to JX013633.

Phylogenetic analysis. Nonredundant sequences of *fusA*, *gapA*, *gltA*, *gyrB*, *lacF*, and *lepA* of *Xanthomonas* were downloaded from PAMDB (1) for alignment with sequences of rose strains in this study. The alignment was performed using Clustal X version 2.0.6 (16) and adjusted by eye using Se-AI version 2.0a10 (copyright 1996; A. Rambaut, Zoology Department, University of Oxford). Gaps were considered missing data. Prior to phylogenetic analyses, sequence diversity, including numbers of haplotypes, numbers of polymorphic sites, and nuclear diversity, was determined among all rose strains of *Xanthomonas* using DnaSP version 5 (17).

Parsimony analyses were conducted using MEGA5 (44). The most parsimonious tree was obtained using the close-neighbor-interchange algorithm with search level 1 in which initial trees were obtained with the random addition of sequences (1,000 replicates). Clade stability was evaluated using 1,000 bootstrap replicates. For maximum likelihood (ML) and Bayesian analysis, jMODELTEST version 2.1.1 (30) was used to determine appropriate models of nucleotide substitution. The general time reversible (GTR) model with γ -distributed rate variation across sites and a proportion of invariable sites was chosen based on the Akaike Information Criterion (12). ML was analyzed using GARLI version 2.0 (54), in which a stochastic genetic algorithm-like approach was used to simultaneously estimate the topology, branch lengths,

and substitution model parameters that maximize the log-likelihood. A bootstrap analysis was performed using 1,000 replicates. Bayesian analysis was performed using MrBayes version 3.2.1 (35). The Markov chain Monte Carlo (MCMC) analysis was run with four chains for 10,000,000 generations, sampling every 100 generations and starting with a random tree. The first 25,000 trees with low likelihoods were discarded because the burn-in of the chain since stationarity was reached after approximately generation 2,500,000. The remaining 75,000 trees were imported to PAUP* 4.0b10 (43) to generate a 90% majority-rule consensus tree. Phylogenetic trees derived from the three methods were rooted with the outgroup, *Stenotrophomonas maltophilia* R551.

A similarity matrix of concatenated sequence data representing *Xanthomonas* spp. grouped with rose strains into the same clade was prepared using MEGA5. Numbers of nucleotide differences with standard errors for all pairwise relationships were obtained using the distance estimation with 1,000 bootstrap replicates.

To test correlation between pathogenicity profile and MLSA, distance matrices were generated from the concatenated sequence of the strains used in cross-pathogenicity tests and from the pathogenicity profile (Table 2) using MEGA5 and GenAIE, respectively. Correlations between two distance matrices were determined using the Mantel test (21) implemented in GenAIE with 9,999 permutations.

Results

Pathogenicity and cross-pathogenicity tests. Foliar symptoms of shrub rose samples exhibited small angular black lesions from vein restriction and could often be observed along the leaf margins surrounding hydathodes (Fig. 2). Slight chlorosis was associated with lesions, except in cases where lesions coalesced, causing blighting and premature defoliation. Excised lesions exhibited bacterial streaming upon microscopic examination of wet mounts and yielded yellow mucoid colonies characteristic of xanthomonads when streaked on nutrient agar. Results of Koch's postulates confirmed that a *Xanthomonas* sp. was the causal agent of leaf spot of rose. Based on pathogenicity tests via leaf infiltration, rose strains of *Xanthomonas* were clearly more aggressive on rose and Indian Hawthorn than the other hosts tested (Table 2). Moreover, other groups of *Xanthomonas* strains were also more virulent on the host where they were originally isolated. Although *Xanthomonas* spp. used in cross-pathogenicity tests showed some extent of cross-pathogenicity, PCA results showed that the rose strains formed a distinct cluster from the other *Xanthomonas* spp. based on virulence phenotypes (Fig. 3).

Table 2. Cross-pathogenicity of xanthomonads across several plant families

<i>Xanthomonas</i> spp.	Isolate	Plants by family ^a										
		Rosaceae		Solanaceae		Rutaceae			Euphorbiaceae		Araceae	
		Rose	Hawth	Tom	Pep	SO	CLEO	SWG	Crown	Jatro	Lily	Poth
<i>alfalfae</i> pv. <i>rosa</i>	R07	3	3	1	1	1	1	1	1	1	0	1
<i>alfalfae</i> pv. <i>rosa</i>	R09	3	3	0	0	0	1	0	1	0	1	1
<i>alfalfae</i> pv. <i>rosa</i>	HDRA	3	2	2	0	0	0	1	1	1	0	1
<i>alfalfae</i> pv. <i>rosa</i>	HDRB	3	2	1	1	0	1	1	1	1	0	1
<i>perforans</i>	XcT4	0	0	3	2	1	1	0	1	1	2	1
<i>euvesicatoria</i>	Euv69	0	0	3	3	1	1	2	1	1	0	1
<i>euvesicatoria</i>	Euv71	0	0	3	3	2	1	0	0	1	0	0
<i>euvesicatoria</i>	Euv73	0	0	3	3	0	2	3	1	1	0	0
<i>alfalfae</i> subsp. <i>citrumelonis</i>	CitN101	1	0	3	1	3	3	3	1	1	2	1
<i>alfalfae</i> subsp. <i>citrumelonis</i>	CitF1	1	2	1	0	3	3	3	0	2	1	0
<i>axonopodis</i> pv. <i>manihotis</i>	Man672	0	0	2	1	0	1	0	1	2	0	2
<i>axonopodis</i> pv. <i>manihotis</i>	Man678	1	0	2	0	0	0	0	1	2	0	0
<i>axonopodis</i> pv. <i>dieffenbachiae</i>	X476	0	0	0	0	0	1	2	0	1	2	2
<i>axonopodis</i> pv. <i>dieffenbachiae</i>	X1750	0	0	3	0	0	1	1	0	1	3	3

^a Common and scientific names (in parentheses) of plants used in pathogenicity test are CLEO = Mandarin orange 'Cleopatra' (*Citrus reshni*), Crown = Crown of thorns (*Euphorbia millii*), Hawth = Indian Hawthorn (*Raphiolepis indica*), Jatro = Jatropha (*Jatropha integerrima*), Lily = Peace Lily (*Spathiphyllum* sp.), Poth = Pothos (*Epipremnum aureum*), Rose = Rosa Knock Out 'Radrazz' (*Rosa* sp.), SO = Sour orange (*Citrus aurantium*), and SWG = Trifoliate hybrid orange 'Swingle' (*Citrus paradisi* × *Poncirus trifoliata*). Scores: 0 = no response, 1 = minor chlorosis or necrosis at injection site, 2 = minor to moderate chlorosis and minor necrosis, mostly confined to injection site, and 3 = moderate-severe chlorosis and necrosis, expanding lesion. Scores are a summation of results from two to three independent experiments.

FAME analysis. FAME analysis showed that the three selected strains had similar fatty acid profiles, which matched the strains to *X. alfalfae* subsp. *citrumelonis* with similarity indices of 0.73 to 0.93. Similarity indices greater than 0.5 were also observed as compared with other pathovars of *X. axonopodis* such as *dieffenbachiae* (0.64 to 0.80) and *manihotis* (0.67 to 0.77) (data not shown).

rRNA gene sequencing. The nearly 2-kb sequences of the entire ITS along with portions of 16S rRNA and 23S rRNA genes were identical among the three selected rose strains sequenced. BLAST results showed that the closest match for the rose strains was *X. axonopodis* pv. *dieffenbachiae*, with 100% identity. Other *Xanthomonas* spp. (i.e., *X. perforans*, *X. alfalfae* subsp. *citrumelonis*, *X. gardneri*, and *X. vesicatoria*) also demonstrated high percent identity matches, with as few as one to three bases different, confirming that the rose strains were members of the genus *Xanthomonas*.

Fig. 3. Principal component analysis plot of virulence phenotypes of 14 *Xanthomonas* strains based on differences in virulence on 11 plant species belonging to five families. Note that rose strains formed a distinct cluster from the other *Xanthomonas* spp.

Table 3. Sequence diversity estimates for rose strains of *Xanthomonas*

Gene	Sequence length (bp)	Number of polymorphic sites	Nucleotide diversity ^a
<i>fusA</i>	591	0	0
<i>gapA</i>	444	25	0.01126
<i>gltA</i>	501	0	0
<i>gyrB</i>	411	0	0
<i>lacF</i>	408	0	0
<i>lepA</i>	390	0	0
Combined	2,745	25	0.00182

^a Nucleotide diversity was calculated based on the average number of nucleotide differences per site between two sequences (26).

Phylogenetic analyses. Analyzed sequence lengths ranged from 390 bp (*lepA*) to 591 bp (*fusA*), concatenating a total of 2,745 bp for the six genes sequenced. The sequence diversity of the 10 rose strains was small, with only two haplotypes identified (Fig. 3). Of these 10 rose strains, R07, R09, HDRA, X1808, X1811, X1847, X1850, Tx11, and Tx12 shared the same haplotype. HDRB was a unique haplotype. The two haplotypes differed by 25 nucleotides (nt) in the 444-nt *gapA* sequence, with a nucleotide diversity value of 0.01126. Variable sites were not detected in the other genes sequenced from the rose strains (Table 3).

Results of phylogenetic analyses obtained from maximum parsimony, ML, and Bayesian inference were congruent for rose strains, which grouped rose strains into the 9.2 clade identified by Rade- marker and colleagues (32; Fig. 1). Interestingly, these rose strains formed a subclade of clade 9.2 based on maximum parsimony and ML analyses, although Bayesian analysis did not support the subclade stability. Bootstrap values inferred by maximum parsimony and ML for the 9.2 clade were 55 and 65%, respectively. Moreover, the posterior probability inferred by Bayesian analysis was 100% for the clade, supporting the clade reliability. The rose strains were more phylogenetically close to *X. alfalfae* subsp. *citrumelonis*, *X. alfalfae* subsp. *alfalfae*, and *X. perforans* of the 9.2 clade than the other *Xanthomonas* spp. The internal similarity value of DNA sequences was 99.1% for the rose strains (Table 4). Similarity values for strains of the 9.2 clade were 98.3 to 99.7%, with the lowest value between the rose strain HDRB and *X. alfalfae* subsp. *alfalfae*. Overall, the rose strains shared the highest similarity values (98.8 to 99.7%) with *X. alfalfae* subsp. *citrumelonis* (Table 4). The correlation value ($R^2 = 0.127$, $P = 0.130$) obtained from the Mantel test for the relationship between cross-pathogenicity and MLSA suggested that the strains of the clades 9.2 and 9.4 used in our cross-pathogenicity test might have a wide host range that was not significantly associated with genetic relatedness revealed by MLSA.

Discussion

In this study, we confirmed that bacterial spot of rose was caused by *Xanthomonas* spp. based on pathogenicity, FAME, and DNA sequence analyses. Koch's postulate showed that rose strains of *Xanthomonas* were pathogenic on rose and another member of the family Rosaceae, Indian Hawthorn, and that *Xanthomonas* spp. were consistently reisolated. Results of FAME and rRNA gene sequencing confirmed that the rose strains were members of *Xanthomonas*, although species identification was not conclusive. MLSA was used to further characterize the rose strains, suggesting that they had considerably low genetic variation, with only two haplotypes, and fell into *X. axonopodis* subgroup 9.2 (31,32). The final taxonomic rank and species attribution of this pathogen cannot be determined at this point. However, because host range is distinct from the most similar pathovars used in the host range test, this pathogen is suggested to be a new pathovar. The pathovar epithet *rosa* is proposed according to the nomenclatural decision described previously (6).

Pathogenicity tests are the imperative part in pathovar classification (52). Strains of *X. axonopodis* subgroups are generally grouped into pathovars based on host range and disease symptoms

Table 4. Similarity matrix values (%) with standard errors derived from concatenated nucleotides between rose strains and their phylogenetically close *Xanthomonas* spp.

Species	1	2	3	4	5	6	7	8
1 HDRA (nine rose strains)	...	...	...	...	...	...	...	...
2 HDRB (one rose strain)	99.1 ± 0.16	...	...	...	...	...	...	...
3 <i>Xanthomonas alfalfae</i> subsp. <i>alfalfae</i> ATCC 11765	99.1 ± 0.17	98.3 ± 0.23	...	...	...	...	...	...
4 <i>X. alfalfae</i> subsp. <i>citrumelonis</i> 1887	99.3 ± 0.14	98.6 ± 0.19	99.0 ± 0.19	...	...	...	...	...
5 <i>X. alfalfae</i> subsp. <i>citrumelonis</i> 1902	99.7 ± 0.10	98.8 ± 0.19	99.2 ± 0.17	99.3 ± 0.15	...	...	...	...
6 <i>X. alfalfae</i> subsp. <i>citrumelonis</i> LMG 9325	99.5 ± 0.12	98.7 ± 0.20	99.2 ± 0.17	99.1 ± 0.18	99.5 ± 0.13	...	...	...
7 <i>X. perforans</i> ICMP 16690	99.5 ± 0.13	98.5 ± 0.22	99.0 ± 0.19	98.9 ± 0.18	99.4 ± 0.13	99.4 ± 0.14	...	...
8 <i>X. euvesicatoria</i> ICPB 10461	99.3 ± 0.15	98.4 ± 0.21	98.9 ± 0.20	98.9 ± 0.19	99.2 ± 0.19	99.1 ± 0.18	99.0 ± 0.20	...

(32,46). The subgroups encompass a group of phytopathogenic strains that cause water-soaked lesions that further develop into necrotic spots and foliar blights across a diverse array of hosts. This phenotypic difference may be associated with genetic heterogeneity (51). MLSA clustered rose strains in *X. axonopodis* subgroup 9.2 that comprises more than 50 identified pathovars along with *X. alfalfae*, *X. euvesicatoria*, and *X. perforans* based on the *gyrB* phylogeny (28). Members of the subgroup are considered pathogenic to a wide range of unrelated plant genera (53). Virulence phenotypes of the rose strains on 10 plant species belonging to five plant families were distinct from the other members of the subgroup, forming a unique cluster based on PCA. Moreover, the rose strains were highly aggressive on rose. These findings indicate host-specific characteristics, supporting the pathovar nomenclature for the rose strains.

Similarity data based on concatenated sequences provide a criterion for delimitating species differentiated by DDH. It has been suggested that bacterial species unequivocally assigned by DNA-DNA reassociation are depicted by sequences with similarities greater than 99% (51). In contrast, strains sharing concatenated sequence similarities greater than 98% can also be considered members of the same species (53). The internal similarity value of concatenated DNA sequences for the rose strains of *X. axonopodis* subgroup 9.2 was 99.1% but those for members of the subgroup were 98.3 to 99.7%, which were slightly less than similarities obtained from concatenated sequences of *dnaK*, *fyuA*, *gyrB*, and *rpoD* gene fragments (51). Therefore, species or subspecies identity may be assigned for the subgroup. The rose strains shared the highest similarity values (98.8 to 99.7%) with *X. alfalfae* subsp. *citrumelonis*, suggesting that they are phylogenetically closer to *X. alfalfae*. However, the virulence phenotype of the rose strains is distinct from *X. alfalfae* subsp. *citrumelonis* (Fig. 3). Strains of *X. alfalfae* subsp. *citrumelonis* cause water-soaked lesions on citrus but do not infect other hosts (38). Compared with *X. alfalfae* subsp. *citrumelonis*, causing poor symptoms, the rose strains resulted in severe chlorosis and necrosis at the injection site of the rose leaf in this study. Although a monophyletic taxon was confirmed for the rose strains based on phylogenetic classifications, polyphasic taxonomy, including phenotypic, chemotaxonomic, genotypic, and biochemical tests, should be used to name a new species or subspecies (52). Further studies need to be performed to determine whether the rose strains are a new species or subspecies. Because the taxonomic revision of *X. axonopodis* subgroups is undergoing, we do not suggest any species name but only a pathovar epithet based on host range tests.

Rose strains have little genetic diversity because they represent only two haplotypes and show 25 polymorphic sites only in the *gapA* sequence. The 25 mutations in *gapA* can most easily be explained by a recombination event involving *gapA* because it is very unlikely that *gapA* would mutate so fast. The *gapA* allele was probably acquired by recombination from another strain. To test this hypothesis, we further built a neighbor-net network for members of *X. axonopodis* subgroup 9.2 based on a distance method using SplitsTree v.4 (13) (*data not shown*). Reticulate evolution was detected, suggesting that recombination between rose strains and other members of the subgroup is possible (14).

The occurrence of the same genotype of bacterial plant pathogens in different regions and populations has been attributed to gene flow or migration (4,49). Nucleotide identity between the two Texas and seven Florida isolates was 100% for the six gene fragments sequenced, suggesting that migration of this pathogen might have occurred between two rose-growing states. Moreover, the rose strains showing low genetic diversity may result from the recent introduction of this pathogen to the two states. Florida and Texas are 2 of the 15 states growing potted rose plants in the United States., and Florida ranks second nationally, accounting for \$2.57 million of market value (25). Although the origin of this pathogen is unknown, it has been demonstrated that humans can move pathogens far beyond their natural disseminated restrictions through agricultural practices and trade activities (22). Therefore, the bacterium

may have migrated between the two states by moving infected rose plants. In addition, exported rootstocks from the two states may be a source inoculum because it is not uncommon to graft a rose variety onto a rootstock against plant diseases. Conversely, the idea that this pathogen evolved from the local population of *Xanthomonas* spp. cannot be completely ruled out because changes in virulence genes may result in host adaptation and, in turn, a new pathovar (3,10,18,36,39,41,42,48).

In conclusion, MLSA results and pathogenicity tests confirmed that a new pathovar (pv. *rosa*) of a *Xanthomonas* sp. was the causal agent of bacterial spot of rose. Although this pathovar shared a high level of DNA sequence similarity with other members of *X. axonopodis* subgroup 9.2, it displayed a virulence phenotype distinct from other similar pathovars of the subgroup used in this study. The species name of the rose strains cannot be determined until more adequate data are established. However, the pathovar epithet *rosa* is suggested based on its distinct host range from tests in this study.

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