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A *Pseudomonas syringae* Diversity Survey Reveals a Differentiated Phylotype of the Pathovar *syringae* Associated with the Mango Host and Mangotoxin Production

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ABSTRACT

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Pseudomonas syringae pv. *syringae*, the causal agent of bacterial apical necrosis (BAN) in mango crops, has been isolated in different mango-producing areas worldwide. An extensive collection of 87 *P. syringae* pv. *syringae* strains isolated from mango trees affected by BAN from different countries, but mainly from Southern Spain, were initially examined by repetitive sequence-based polymerase chain reaction (rep-PCR) to analyze the genetic diversity with an epidemiological aim. rep-PCR was powerful in assessing intrapathovar distribution and also allowing clustering of the *P. syringae* pv. *syringae* strains isolated from mango, depending on the isolation area. A clear pattern of clustering was observed for all the *P. syringae* pv. *syringae* strains isolated from mango distinct from strains from other hosts, including strains for the same geographical regions as the mango isolates. For this reason, a representative group of 51 *P. syringae* pv. *syringae* strains isolated from mango and other hosts, as well as some *P. syringae* strains from other pathovars, were further characterized to determine their possible genetic, phenotypic, and phylogenetic relationships. Similar to the rep-PCR results, the randomly amplified polymorphic DNA PCR (RAPD-PCR) and catabolic diversity analysis using the Biolog GN2 profile grouped 90% of the mango isolates together in a unique cluster. Interestingly, the majority of *P. syringae* pv. *syringae* strains isolated from mango produced mangotoxin. The analysis of the phylogenetic distribution using the multilocus sequence typing analysis strongly supports the existence of a differentiated phylotype of the pathovar *syringae* mainly associated with the mango host and characterized by the mangotoxin production.

Pseudomonas syringae is unique in its ability to cause diseases in a wide range of hosts, up to 180 plant species worldwide (13,40). *P. syringae* is taxonomically subdivided into pathogenic varieties known as pathovars, based on the host of isolation (77). Pathovars of the *P. syringae* complex have traditionally been defined both biochemically and by pathogenicity tests. Schaad et al. (65) described biochemical-nutritional tests that differentiate between pathovars of this bacterial species. However, strains of *P. syringae* infecting unreported hosts and that are biochemically similar to *P. syringae* pv. *syringae* strains have been frequently placed into the pathovar *syringae* without establishment of a common host range (77).

The pathovar-based classification of the *P. syringae* complex has a practical interest but it does not reveal the genetic relationship among pathovars. Gardan et al. (26) described nine genomic species within *P. syringae* on the basis of DNA pairing analysis. Meanwhile, Clerc et al. (22) demonstrated that other genetic techniques such as randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) could be used as alternatives to DNA pairing studies and often agree with the classification obtained by polymerase chain reaction (PCR) restriction fragment length polymorphism of the *rrn* operon (49), DNA pairing studies, and analysis of pathogenicity data. In addition to these studies, repetitive sequence-based PCR (rep-PCR) has been successfully used to demonstrate considerable genetic diversity among *P. syringae* pathovars (46, 69). This fingerprinting technique has been widely used in different pathovars of *P. syringae*, highlighting the possibility of its use to observe intraspecific or intrapathovar genetic diversity (25,39,45,51,52). Additionally, multilocus sequence typing (MLST) could be considered as an alternative technique to characterize phylogenetic relationships of bacteria, by analysis of genes sequences that belong to the core genome (37,48,63). Sarkar and Guttman (63) provided the first MLST analysis of this plant-pathogenic bacterium, reporting that *P. syringae* is a highly clonal population. Later, MLST was demonstrated to be highly discriminative, accurately allocating *P. syringae* isolates to their corresponding genomospecies and revealing previously unknown diversity (14).

P. syringae pv. *syringae* is one of the 60 pathovars (77) belonging to the highly heterogeneous species *P. syringae*. Bacterial apical necrosis (BAN) of mango caused by *P. syringae* pv. *syringae* strains is the most limiting disease of mango crops in the Mediterranean area (16,20), as well as in other subtropical areas, such as northwestern Australia (29). BAN disease significantly

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reduces the commercial viability of mango crops and, thus, several attempts have been made to control this disease (16,30). Furthermore, a preliminary study of *P. syringae* pv. *syringae* strains isolated from mango revealed the existence of genetic diversity within this pathovar (31) that could be helpful for a future epidemiological study to determine the origin of the isolated pathogens and their distribution.

During the interaction of *P. syringae* with the host plant, the bacterium produces different virulence factors such as toxins, ice nucleation proteins, antimicrobial resistance, and secreted effectors which are the best studied virulence-associated factors (1,12, 33,44). *P. syringae* produces a powerful arsenal of toxins depending on the pathovar (5,9,75). In 2003, Arrebola et al. (4) reported the preliminary characterization of an antimetabolite toxin produced primarily by *P. syringae* pv. *syringae* strains isolated from mango (thus called "mangotoxin"). Recent studies have since described mangotoxin production in some strains from pvs. *avellanae* (54) and *pisi* (15). The resistance of many *P. syringae* strains to antimicrobial compounds is well known (56). Copper compounds have been used for decades to control *P. syringae* infection of crops and to also control BAN in mango crops (16). Copper resistance has been frequently associated with native plasmids (23,43), even in the *P. syringae* pv. *syringae* strains isolated from mango, where this resistance is mostly associated with 62-kb native plasmids (17,32).

The initial goal of this study was to evaluate the epidemiological structure of the *P. syringae* pv. *syringae* strains isolated from mango using different approaches. This aim was also extended to characterize the phylogenetic position of the mango isolates into pv. *syringae* and *P. syringae* spp. The results, based on genetic and phenotypic evidence, showed that *P. syringae* pv. *syringae* strains isolated from mango formed a differentiated cluster separate from most of the *P. syringae* pv. *syringae* strains isolated from other hosts. Moreover, together with the MLST data, a differentiated phylotype of pv. *syringae* strongly associated with mangotoxin production and adapted mainly to the mango host is proposed and discussed in this study.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A group of 87 *P. syringae* pv. *syringae* strains isolated from mango from different areas, but mainly from Southern Spain, along with 28 strains isolated from different hosts (n = 19), different pathovars (n = 7), and different *Pseudomonas* spp. (n = 2) (Table 1; Supplemental Table 1) were initially selected and used for the genetic diversity study based on the rep-PCR technique. For the subsequent genetic and phenotypic analysis, a representative group of 51 *P. syringae* pv. *syringae* strains isolated from mango, 6 *P. syringae* pv. *syringae* strains isolated from other hosts, and 2 *P. syringae* strains from different pathovars were selected (Table 1). Three *P. syringae* pv. *syringae* strains isolated in Málaga (Spain) from other hosts (*P. syringae* pv. *syringae* strains UMAF4007, UMAF6016, and UMAF6582) (Table 1) were used only for the evaluation of toxins production and for the phylogenetic analysis by MLST. Most of the *P. syringae* pv. *syringae* strains isolated from mango used in this work have been isolated in previous studies from different years (1990–2007) and different geographical locations in Spain (4,20) and, to a lesser extent, Portugal, Italy, Israel, and Australia. The remaining strains used in this work were isolated from other hosts, and most of them were kindly provided by other laboratories. All strains were cultured in King's B (KB) (41) agar and Luria-Bertani (LB) broth media and incubated at 28°C for 24 h.

Molecular fingerprinting techniques. Cultures for DNA extraction were grown overnight in LB broth at 28°C with shaking at 150 rpm, and DNA was extracted using the Jet Flex Extraction Kit (Genomed, Germany) according to the manufacturer's instructions. PCR mixtures with final volumes of 25 µl were prepared as follows: 10 ng of template DNA, 2.5 µl of 10× PCR buffer, and 2 µl of dNTPS at 100 µM each. A total of 2 µl of each primer was used at a concentration of 10 µM and 2.5 U of *Taq* DNA polymerase (GE Healthcare Life Sciences). This mixture was the same for rep-PCR and RAPD-PCR analysis. The primer sets and the PCR amplification conditions for both fingerprinting techniques are summarized in Supplemental Table 2. All PCR amplifications were performed with a C1000 Thermal Cycler (Bio-Rad, UK). rep-PCR and RAPD-PCR patterns were visualized by agarose gel electrophoresis. Aliquots of 25 µl (the volume for all amplifications) were loaded onto 1.2% Agarose D1 Low EEO (Pronadisa Laboratorios, Conda, Spain) gels and run in 1× Tris-acetate EDTA buffer (40 mM Tris-acetate and 1 mM EDTA) at 80 V/cm for 1.5 h. Gels were stained with ethidium bromide at 0.4 µg/ml and visualized using a Gel Doc XR+ imaging system (Bio-Rad). Molecular weight analysis of patterns was performed with Quantity One software (version 4.2.1; Bio-Rad), using the Hyperladder I (1-kb DNA ladder) (Bioline, UK) as a molecular weight marker. Strains were tested in triplicate for both fingerprinting methods.

Polymorphic band patterns from rep-PCR (n = 115 strains) (Table 1) and RAPD-PCR (n = 59 strains) (Table 1) were individually identified by their specific electrophoretic migration rates. Once bands were identified, binary matrices (0/1) were constructed to compare the patterns. The Jaccard's similarity coefficient was generated using InfoQuest FP software (Bio-Rad), and cluster analyses, along with their corresponding dendrograms, were generated by the unweighted pair-group method with average linkages (UPGMA) (67). Gel normalization and background subtraction were performed as previously described (31,61).

Copper resistance and plasmid profile. Plasmid DNA from the selected strains (Table 1) was isolated according to a modified alkaline lysis method (17,78) and separated by electrophoresis on 0.6% agarose gels. Plasmid size was estimated by comparison with the control strain *P. syringae* pv. *tomato* PT23 that harbors four plasmids (pPT23A, pPT23B, pPT23C, and pPT23D that are 100, 83, 65, and 36 kb in size, respectively) (55). Copper resistance was determined by using mannitol-glutamate-yeast extract (MGY) agar medium (10) amended with $CuSO_4 \cdot 5H_2O$. Bacteria previously grown for 24 h at 28°C on LB agar were used as inoculum. Strains growing on MGY + 0.8 mM $CuSO_4$ after 72 h at 28°C were considered resistant to copper.

Antibiotics resistance profile. Few antibiotics are appropriate for use in agriculture, and antibiotic-resistant strains are easily generated from their repetitive use. The selected strains (Table 1) were tested to determine their resistance or sensitivity to different antibiotics. These strains were freshly streaked from a single colony onto LB agar medium containing the following antibiotics: ampicillin (100 µg/ml), chloramphenicol (25 µg/ml), gentamicin (10 µg/ml), kanamycin (50 µg/ml), nitrofurantoin (100 µg/ml), spectinomycin (50 µg/ml), streptomycin (100 µg/ml), and tetracycline (15 µg/ml). Absence of bacterial growth after 24 and 48 h was considered sensitivity to the assayed antibiotic concentration.

Catabolic profile. The catabolic activities of the representative group of strains (Table 1) on 95 substrates were tested using a Biolog GN2 (Biolog) analysis according to the manufacturer's protocol, with minor modifications. Briefly, strains were grown on KB plates for 24 h and then colonies were scraped and suspended in NaCl solution at 4 g/liter until reaching an optical density (OD) of 0.15 to 0.2 at 590 nm. Aliquots of 150 µl were added to each well, color changes were measured at 590 nm using a microplate reader (Multiskan Ascent; Thermo Fisher Scientific) after 24 and 48 h of incubation at 28°C, and the resulting data were corrected using the no-substrate control. The threshold value for a positive result was an OD of ≥0.350 at 590 nm. A binary matrix was constructed for cluster analysis by assigning a value for each substrate as follows: 0 for negative result (no catabolism)

and 1 for positive result (catabolism). Two independent plates per each strain were used. The Jaccard's similarity coefficient was generated by the InfoQuest FP software (Bio-Rad), and cluster analysis along with its corresponding dendrogram was generated by UPGMA (67).

Antimetabolite toxin bioassay and dot blot hybridization analysis. The production of mangotoxin and phaseolotoxin was assayed by the indicator technique as previously described (26), with minor modifications (4). This method involves the growth inhibition of *Escherichia coli* on *Pseudomonas* minimal medium (PMS) (27). Briefly, a double layer of indicator microorganism was made using the *E. coli* strain CECT831. After solidification, the strains to be tested (Tables 1 and 2) were stabbed, and the plates were incubated at 22°C for 24 h and at 37°C for an addi-

TABLE 1. Representative group of *Pseudomonas syringae* strains used in this study

P. syringae pathovar and strain	Geographical origin	Host plant	Year of isolation	Reference or source[a]
phaseolicola 1448A	Unknown (Ethiopia)	Bean	1985	71
tomato DC3000	Guernesey (United Kingdom)	Tomato	1960	24
syringae (other hosts)				
1444-5	Madrid (Spain)	Laurel	1995	4
2676	Lesotho (South Africa)	Bean	1990	4
B728a	Wisconsin (United States)	Bean		35
EPS17A	Gerona (Spain)	Pear	1987	4
EPSMV3	Gerona (Spain)	Pear	1990	4
FF5	Oklahoma (United States)	Pear	1988	68
UMAF4002	Málaga (Spain)	Tomato	1994	4
UMAF6016	Málaga (Spain)	Chestnut	1994	4
UMAF6582	Málaga (Spain)	Peach	1994	4
syringae (mango)				
UMAF0001	Málaga (Spain)	Mango	1992	18
UMAF0005	Málaga (Spain)	Mango	1996	18
UMAF0048	Málaga (Spain)	Mango	1992	4
UMAF0049	Málaga (Spain)	Mango	1992	17
UMAF0081	Málaga (Spain)	Mango	1992	17
UMAF0092	Málaga (Spain)	Mango	1993	UMA-LC
UMAF0100	Málaga (Spain)	Mango	1990	4
UMAF0114	Málaga (Spain)	Mango	1991	4
UMAF0115	Málaga (Spain)	Mango	1991	4
UMAF0119	Málaga (Spain)	Mango	1991	18
UMAF0122	Málaga (Spain)	Mango	1991	4
UMAF0123	Málaga (Spain)	Mango	1991	18
UMAF0128	Málaga (Spain)	Mango	1992	18
UMAF0139	Málaga (Spain)	Mango	1995	4
UMAF0158	Málaga (Spain)	Mango	1993	17
UMAF0162	Málaga (Spain)	Mango	1997	UMA-LC
UMAF0166	Málaga (Spain)	Mango	1994	4
UMAF0167	Málaga (Spain)	Mango	1994	4
UMAF0170	Málaga (Spain)	Mango	1993	17
UMAF0171	Málaga (Spain)	Mango	1993	4
UMAF0176	Málaga (Spain)	Mango	1994	4
UMAF0186	Málaga (Spain)	Mango	2003	UMA-LC
UMAF0187	Málaga (Spain)	Mango	2003	UMA-LC
UMAF0209	Málaga (Spain)	Mango	2006	15
UMAF0214	Málaga (Spain)	Mango	2008	15
UMAF0511	Málaga (Spain)	Mango	1992	4
UMAF1012	Málaga (Spain)	Mango	1997	17
UMAF3028	Málaga (Spain)	Mango	1990	4
UMAF1003	Huelva (Spain)	Mango	1997	4
UMAF1029	Huelva (Spain)	Mango	1997	17
UMAF1051	Huelva (Spain)	Mango	1997	4
UMAF1060	Huelva (Spain)	Mango	1998	31
UMAF1094	Huelva (Spain)	Mango	2003	17
UMAF1105	Huelva (Spain)	Mango	2003	UMA-LC
UMAF1110	Huelva (Spain)	Mango	2003	17
UMAF1118	Huelva (Spain)	Mango	2003	UMA-LC
UMAF1128	Huelva (Spain)	Mango	2003	17
1559-1	Huelva (Spain)	Mango	1996	4
1559-9	Huelva (Spain)	Mango	1996	4
UMAF2007	Almansil (Portugal)	Mango	1997	4
UMAF2025	Almansil (Portugal)	Mango	1998	17
UMAF2026	Almansil (Portugal)	Mango	1998	17
UMAF2700	Sicily (Italy)	Mango	2002	UMA-LC
UMAF2702	Sicily (Italy)	Mango	2002	31
UMAF2801	Canary Islands (Spain)	Mango	2000	31
UMAF2802	Canary Islands (Spain)	Mango	2000	4
UMAF2808	Canary Islands (Spain)	Mango	2006	UMA-LC
DAR77787	Dandaragan (Australia)	Mango	2008	76
DAR77789	Perth (Australia)	Mango	2008	76
Ps10	Bet Dagan (Israel)	Mango	1999	4
Ps35	Bet Dagan (Israel)	Mango	1999	4

[a] UMA-LC: IHSM-UMA-CSIC, Microbiology and Plant Pathology laboratory collection (Málaga, Spain).

tional 24-h period. To assess the biochemical step that is the putative target of the toxin, the same plate bioassay was performed but 100 µl of a 6-mM solution of the corresponding amino acid or intermediate (N-acetyl-Ornithine for mangotoxin and Ornithine for phaseolotoxin) was added to the double layer. Inhibition zones were observed for the mangotoxin- or phaseolotoxin-producing strains.

In addition, dot blot analysis was performed to confirm the presence of representative genes involved in toxin production (mangotoxin, phaseolotoxin, tabtoxin, and coronatine). The bacterial strains used as controls for toxin determination are listed in Table 2. The primers used to amplify the different toxins genes and their relevant characteristics are summarized in Supplemental Table 3. To generate the DNA probes for the different toxins, the PCR amplicons were cloned into the pCR2.1 vector (Invitrogen Corporation) and transformed into competent *E. coli* DH5α cells. For dot blot assays, genomic DNA was isolated from single colonies grown overnight in LB broth in wells of a microtiter plate. In all, 200 µl of 0.4 M NaOH and 10 mM EDTA was added in each well. The plate was sealed with autoclave tape and incubated at 60°C for 15 min; then, the plate was chilled on ice for 5 min. Cell lysates from each strain were transferred into a nylon membrane using a Bio-Dot apparatus (Bio-Rad). Dot blot hybridizations were carried out using a DIG DNA labeling and detection kit (Roche Applied Science), following the manufacturer's instructions. The presence of signal indicated a positive result (presence of the representative genes involved in the toxins production).

Virulence assay on tomato leaflets. The pathogenicity tests using mango tissues are difficult and show low reproducibility. Therefore, in this study, the analysis of virulence on *P. syringae* pv. *syringae* strains isolated from mango was carried out using tomato leaflets to develop the disease symptoms (3,6). To determine the virulence level of the studied strains (Table 1), inoculation experiments were performed using 'Hellfrucht Frühstamm' tomato (*Lycopersicum esculentum* L.) plants. The tomato plants were grown in a greenhouse under natural light. Tomato leaflets from 6- to 8-week-old plants were detached, disinfected in a 0.1% (wt/vol) $HgCl_2$ solution, and placed in petri dishes with their petioles immersed in Murashige and Skoog (MS) agar medium (Sigma-Aldrich) (59). To determine differences in the virulence level, bacterial suspensions from exponential cultures were adjusted to 10^6 CFU/ml. Leaflets were inoculated by placing six 10-µl droplets of the bacterial suspension on the principal lateral veins, and inoculations were carried out by piercing through the droplets with an entomological pin, with a final inoculation dose of $\approx 10^4$ CFU per inoculation point. The leaflets were maintained at 25°C under a 16-h photoperiod. Four tomato leaflets were used to evaluate symptoms for each assayed strain. Leaflets treated with sterile water and maintained in MS medium were included in all the experiments as a control. This experiment was repeated twice. Therefore, 48 inoculation points were analyzed per strain, and all the data were analyzed together. The necrotic lesions produced on tomato leaflets by the inoculated strains were evaluated at 5 days postinoculation. A necrotic area >1 mm surrounding the inoculation point was considered positive. The virulence degree was the percentage of necrotic points (>1 mm in diameter) of each strain versus the total number of inoculated points. *P. syringae* pv. *tomato* DC3000 was used as positive control because it is a natural pathogen of tomato plants (*Lycopersicum esculentum* L.), and *P. syringae* pv. *phaseolicola* 1448A was used as negative control because it is not a pathogen of tomato, that should only produce a hypersensitive response. The virulence level was evaluated using the following scale: <15%, not pathogenic; ≥15 to <30%, low virulence; ≥30 to <60%, intermediate virulence; and ≥60%, high virulence.

MLST analysis. For this experiment, a group of 55 *P. syringae* strains was selected, including 24 *P. syringae* pv. *syringae* strains isolated from mango and coinciding with the maximum diversity showed in this work by genetic and phenotypic traits; 3 *P. syringae* pv. *syringae* strains isolated from pear; 12 *P. syringae* pv. *syringae* and *P. syringae* pv. *phaseolicola* 1448A strains isolated from bean; 10 *P. syringae* pv. *syringae* strains isolated from cherry; 1 *P. syringae* pv. *syringae* strain each isolated from laurel, tomato, peach, and chestnut; and *P. syringae* pv. *tomato* DC3000 isolated from tomato were analyzed by MLST. Partial sequences of the housekeeping genes *rpoD* and *gyrB* were obtained from a National Center for Biotechnology Information database or directly from purified PCR products amplified with primer sets rpoDFor2 (ACCGATCCCGTTCGTATGTA) and rpoDRev2 (TGGTGTACTTCTTGGCGATG) and gyrBFor2 (GTCATCAT GACCGTGCTCCA) and gyrBRev2 (CCCTTCCACCAGGTAC AGTT), respectively, as previously described (54). Partial sequences of *rpoD* (807 nucleotides [nt]) and *gyrB* (890 nt) were concatenated for each strain and treated as a single sequence for multiple alignments using ClustalW2 (42). Phylogenetic trees were generated using MEGA 5.05 (70) with neighbor-joining, minimum evolution, and the option of complete deletion to eliminate positions containing gaps. Confidence levels of the branching points were determined using 10,000 bootstrap replicates. Nucleotide sequences were determined at Macrogen (Seoul, Korea) directly from purified PCR products. The accession numbers of the determined sequences used in this study are included in Supplemental Table 4.

RESULTS

rep-PCR revealed strong clustering depending on the host and area of isolation. DNA fingerprinting analysis was initially carried out using four different primer sets (ERIC, BOX, GTG-5, and CAG-5) on the complete group of selected strains (n = 115) (Table 1). The distribution tree generated (Fig. 1) was the result of analyzing all the fingerprinting data from the four different primers together. Individual analysis for each primer showed similar results (data not shown). This combined analysis revealed a strong clustering of *P. syringae* pv. *syringae* strains isolated from mango as well as a clear distribution by area of isolation (Fig. 1). The strains from two different *Pseudomonas* spp. and pathovars of *P. syringae* different from pv. *syringae* clustered separately. The group of strains marked in light gray was formed primarily by different subclusters of *P. syringae* pv. *syringae* strains isolated from hosts other than mango and two strains from different pathovars but belonging to the genomospecies 1 (*P. syringae* pv. *pisi* P123R6 and *P. syringae* pv. *avellanae* ISPaVe2056).

TABLE 2. Bacterial strains used as control for toxin production

Pseudomonas syringae pvs.	Relevant characteristics[a]	Reference or source
glycinea 49a/90	*Glycine max*, 1990, Germany; coronatine producer	73
phaseolicola 1448A	*Phaseolus vulgaris*, 1985, Ethiopia; phaseolotoxin producer	71
syringae UMAF0158	*Mangifera indica*, 1993, Nf^r wild type strain, Spain; mangotoxin producer	17
syringae CFBP3388	*Vicia sativa*, 1995, France; phaseolotoxin and mangotoxin producer	72
tabaci BR2R	*Phaseolus vulgaris*, 1979, Rif^r isolate, Brazil; tabtoxin producer	62
tomato DC3000	*Lycopersicum esculentum*, 1960, Rif^r derivative of NCPPB1106, United Kingdom; coronatine producer	24

[a] Nf^r and Rif^r indicate resistant to nitrofurantoin and rifampicin, respectively.

Finally, a main cluster, marked in dark gray, consisted almost exclusively of *P. syringae* pv. *syringae* strains isolated from mango, and it was subsequently divided into different subclusters associated basically with the geographical origin of the mango isolates. Thus, we can distinguish three different subcluster groupings: the *P. syringae* pv. *syringae* strains isolated from mango in Málaga (Spain), those isolated from Huelva (a separate region of Spain), and those isolated from different regions worldwide (Australia, Israel, Italy, Portugal, and Canary Islands). Furthermore, only three *P. syringae* pv. *syringae* strains (UMAF1198, UMAF0005, and Ps-6) were located outside of the main clusters of *P. syringae* pv. *syringae* isolated from mango, and these strains differed greatly from the rest of *P. syringae* pv. *syringae* strains isolated from this host. Finally, the rep-PCR analysis was also conducted with the representative group of selected strains (Table 1), and a similar distribution was obtained (Supplemental Figure 1), maintaining the main clustering by host and an internal subclustering by geographical isolation.

TABLE 3. Plasmid profile and copper resistance of the representative group of strains

Bacterial strains	Isolated from	Plasmid profile (kb)	$CuSO_4 \cdot 5H_2O$ (R or S 0.8 mM)[a]
Pseudomonas syringae pvs.			
phaseolicola 1448A	Unknown, Ethiopia	131, 51	S
tomato DC3000	Guernesey, United Kingdom	73, 67	S
P. syringae pv. *syringae* (other hosts)			
1444-5	Madrid, Spain	62	R
2676	Lesotho, South Africa	Plasmidless	S
B728a	Wisconsin, United States	Plasmidless	R
EPS17A	Gerona, Spain	Plasmidless	R
EPSMV3	Gerona, Spain	Plasmidless	R
FF5	Oklahoma, United States	Plasmidless	S
P. syringae pv. *syringae* (mango)			
UMAF0001	Málaga, Spain	88, 62	R
UMAF0005	Málaga, Spain	Plasmidless	**R**
UMAF0048	Málaga, Spain	62	R
UMAF0049	Málaga, Spain	88, 62	R
UMAF0081	Málaga, Spain	62	R
UMAF0092	Málaga, Spain	62	R
UMAF0100	Málaga, Spain	Plasmidless	S
UMAF0114	Málaga, Spain	120	R
UMAF0115	Málaga, Spain	Plasmidless	**R**
UMAF0119	Málaga, Spain	88, 62	R
UMAF0122	Málaga, Spain	88, 62	R
UMAF0123	Málaga, Spain	88, 62	R
UMAF0128	Málaga, Spain	62	R
UMAF0139	Málaga, Spain	88, 62	R
UMAF0158	Málaga, Spain	62	S
UMAF0162	Málaga, Spain	62	R
UMAF0166	Málaga, Spain	120	R
UMAF0167	Málaga, Spain	120	R
UMAF0170	Málaga, Spain	62	R
UMAF0171	Málaga, Spain	Plasmidless	**R**
UMAF0176	Málaga, Spain	62	S
UMAF0186	Málaga, Spain	62	R
UMAF0187	Málaga, Spain	62	R
UMAF0209	Málaga, Spain	Plasmidless	S
UMAF0214	Málaga, Spain	62	S
UMAF0511	Málaga, Spain	62	R
UMAF1012	Málaga, Spain	62	R
UMAF3028	Málaga, Spain	45	R
UMAF1003	Huelva, Spain	Plasmidless	S
UMAF1029	Huelva, Spain	62	S
UMAF1051	Huelva, Spain	Plasmidless	**R**
UMAF1060	Huelva, Spain	62	S
UMAF1094	Huelva, Spain	62	S
UMAF1105	Huelva, Spain	62	S
UMAF1110	Huelva, Spain	62	R
UMAF1118	Huelva, Spain	62	R
UMAF1128	Huelva, Spain	62, 45	R
1559-1	Huelva, Spain	62	S
1559-9	Huelva, Spain	62	S
UMAF2007	Almansil, Portugal	Plasmidless	**R**
UMAF2025	Almansil, Portugal	Plasmidless	**R**
UMAF2026	Almansil, Portugal	45	R
UMAF2700	Sicily, Italy	Plasmidless	**R**
UMAF2702	Sicily, Italy	62	S
UMAF2801	Canary Islands, Spain	62	S
UMAF2802	Canary Islands, Spain	Plasmidless	S
UMAF2808	Canary Islands, Spain	Plasmidless	S
DAR77787	Dandaragan, Australia	Plasmidless	**R**
DAR77789	Perth, Australia	62	R
Ps10	Bet Dagan, Israel	Plasmidless	**R**
Ps35	Bet Dagan, Israel	62	R

[a] Strains able to grow at 0.8 mM are considered resistant to copper. R = resistant, S = sensitive, and bold = plasmidless copper-resistant strains.

RAPD-PCR cluster of the *P. syringae* pv. *syringae* strains isolated from mango. Three primers (OPA-04, OPA-10, and OPA-13) were selected in a preliminary setup to assess the genetic diversity in the representative group of strains (Table 1). The different fingerprinting patterns obtained with the different primers were analyzed individually, and then all combined (Fig. 2). The combined analysis of the three different fingerprinting patterns resulted in a more robust dendrogram with a similar ten-

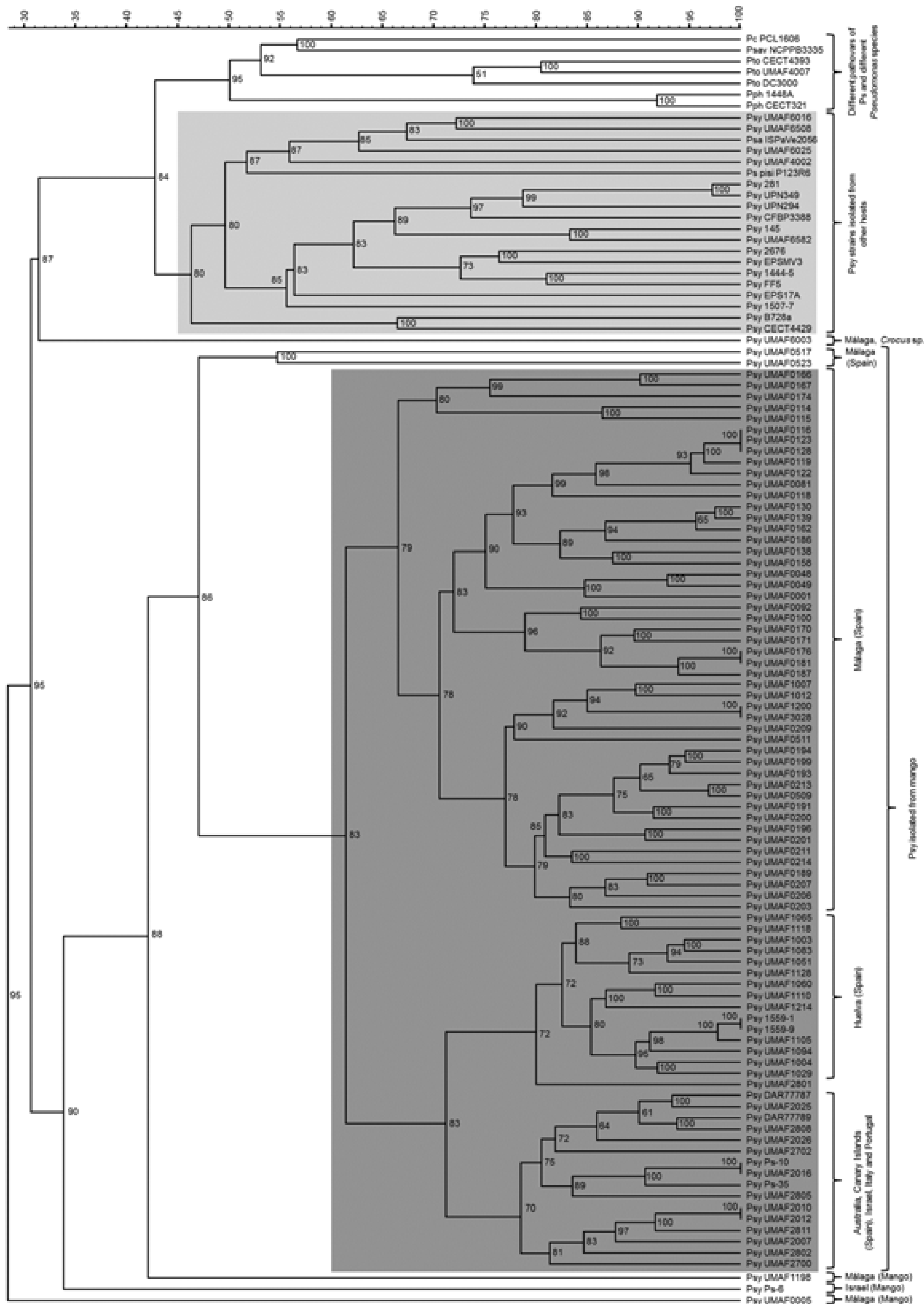

Fig. 1. Dendrogram cluster analysis using combined data obtained from the four different repetitive sequence-based polymerase chain reaction fingerprinting methods (ERIC, BOX, GTG-5, and CAG-5), showing genetic relationships of *Pseudomonas syringae* pv. *syringae* isolated from mango trees, with isolates from other hosts and with other pathovars of *P. syringae*. Jaccard's similarity coefficient was used to generate a consensus tree with InfoQuest FP software (Bio-Rad), using the unweighted pair-group method with average linkages. Light gray box: *P. syringae* pv. *syringae* strains isolated from other hosts than mango, and dark gray box: *P. syringae* pv. *syringae* strains isolated from mango forming different subgroups based on the area of isolation. Table 1 provides detailed information of the strains ($n = 115$).

dency to each separate one (data not shown). The resulting RAPD-PCR dendrogram (Fig. 2) showed a number of clusters. The main cluster, marked in gray, consisted primarily of the *P. syringae* pv. *syringae* strains isolated from mango (n = 48, representing the 94.1% of the strains isolated from mango), excluding the three *P. syringae* pv. *syringae* strains isolated from mango in Portugal. This cluster also included a *P. syringae* pv. *syringae* strain isolated from pear (*P. syringae* pv. *syringae* EPS17A). The rest of the *P. syringae* pv. *syringae* strains isolated from other hosts (n = 5) and different pathovars of *P. syringae* (*P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *phaseolicola* 1448A), along with the three *P. syringae* pv. *syringae* strains isolated from mango in Portugal, were grouped separately from the main cluster of the mango isolates.

The plasmid and copper resistance distribution. The distribution pattern of the native plasmids in the representative group of strains and their possible relationship with copper resistance (Table 3) was studied. Different plasmid profiles, such as the

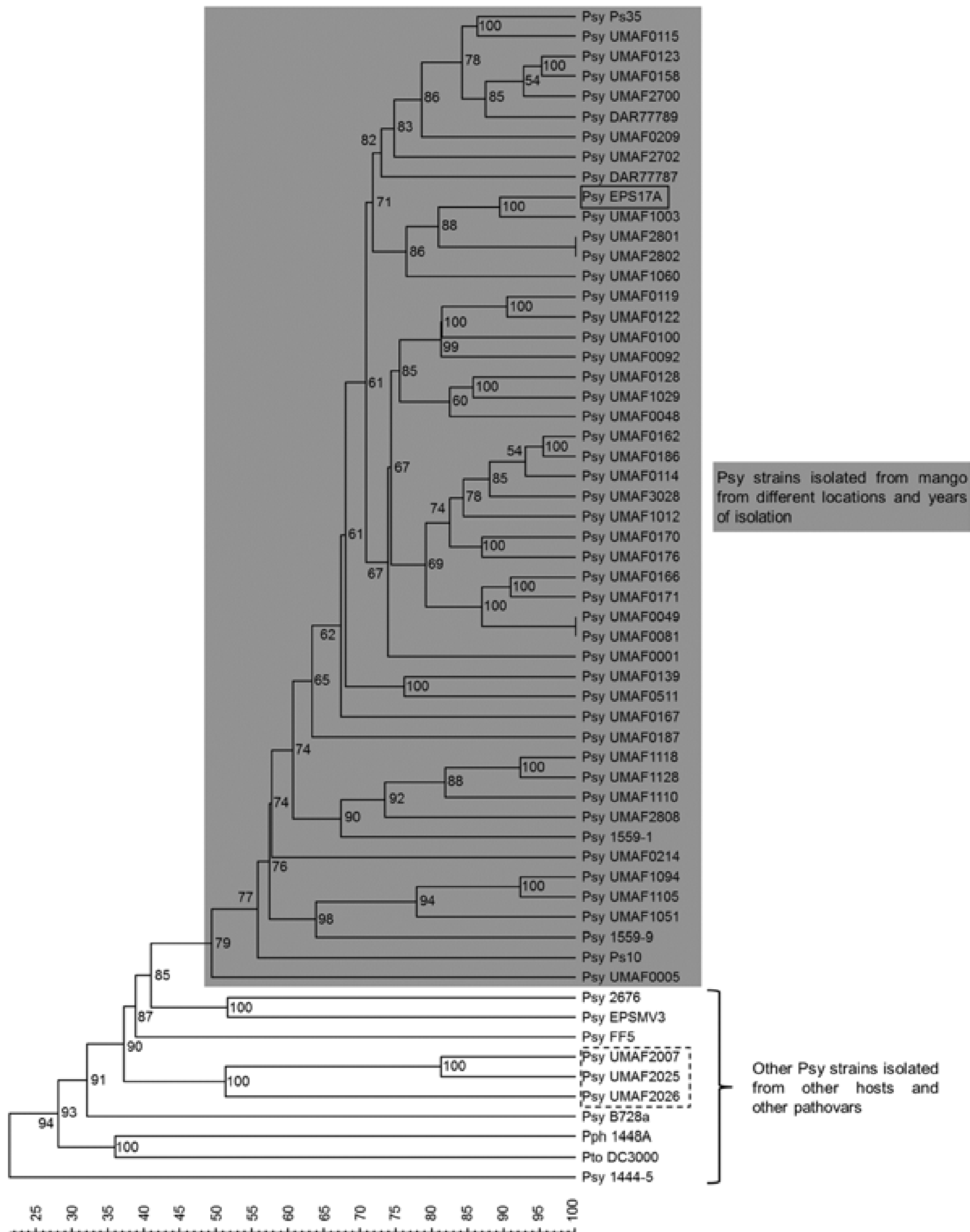

Fig. 2. Dendrogram produced by cluster analysis of the representative group of the strains based on the data obtained from the three different randomly amplified polymorphic DNA polymerase chain reaction fingerprints analyzed (OPA-04, OPA-10, and OPA-13). Banding patterns were compared between strains using the Jaccard's similarity coefficient and unweighted pair-group method with average linkages to generate a consensus tree with InfoQuest FP software (Bio-Rad). Gray box: *Pseudomonas syringae* pv. *syringae* strains isolated from mango. Black box: *P. syringae* pv. *syringae* EPS17A strain isolated from pear. Dashed box: *P. syringae* pv. *syringae* strains isolated from mango in Portugal (UMAF2007, UMAF2025, and UMAF2026). Table 1 provides detailed information of the strains.

different 62-kb plasmids, some of which are related to copper resistance, were observed and consistent with previous reports (17). Only 14 of the 51 strains isolated from mango were plasmidless; 37 harbored plasmids and 32 of those contained a 62-kb plasmid, either alone or together with other plasmids. The 62-kb plasmids were found in 21 copper-resistant and 11 copper-sensitive strains. The presence of a 120-kb plasmid was also observed in three *P. syringae* pv. *syringae* strains (UMAF0114, UMAF0166, and UMAF0167), which was previously associated with copper resistance (17,18). In summary, of the 51 *P. syringae* pv. *syringae* strains isolated from mango, 32 of them (≈62.7%) contained a 62-kb plasmid and 21 of these (≈66%) were copper-resistant strains.

Heterogeneous distribution of antibiotic resistance. The antibiotic resistance of the selected strains (Table 1) is summarized in Table 4. All the strains tested showed resistance to nitrofurantoin. Similarly, a high number of *P. syringae* pv. *syringae* strains isolated from mango were resistant to ampicillin (62.7%), distributed randomly in different clusters obtained by the genetic diversity analysis. In contrast, none of these strains were able to grow in kanamycin. Interestingly, two multi-resistant antibiotic strains were detected, UMAF0005 and UMAF3028. Both were able to grow in ampicillin, chloramphenicol, nitrofurantoin, spectinomycin, streptomycin, and tetracycline. In addition, strain UMAF0005 was also resistant to gentamicin, showed genetic traits different from other *P. syringae* pv. *syringae* strains isolated from mango, and was more divergent from the other mango isolates when analyzed by RAPD and even more so by rep-PCR analysis.

Catabolic diversity supports the genetic distribution. The catabolic activities of the *P. syringae* pv. *syringae* strains on 95 substrates were determined using a Biolog GN2 (Biolog), and these results were used to construct a dendrogram based on the ability of the bacterial strains to grow using these substrates as unique carbon sources. This phenotypic distribution tree (Fig. 3) again showed an interesting association of the majority of the *P. syringae* pv. *syringae* strains isolated from mango in contrast with the *P. syringae* pv. *syringae* strains from other hosts and with other pathovars of *P. syringae*. This phenotypic analysis generated a number of separate clusters, of which the main cluster grouped together the majority of the *P. syringae* pv. *syringae* strains isolated from mango (45 *P. syringae* pv. *syringae* strains, 88% of the mango isolates). The rest of the *P. syringae* pv. *syringae* strains isolated from other hosts and the five *P. syringae* pv. *syringae* strains isolated from mango from different countries (three strains from Portugal and one strain each from Spain and Italy) were grouped together separately from the main cluster. *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *phaseolicola* 1448A, strains used as external controls, and *P. syringae* pv. *syringae* UMAF0005 were located completely outside from these clusters.

Mangotoxin is a specific phenotypic trait of *P. syringae* pv. *syringae* strains isolated from mango. Mangotoxin is an antimetabolite toxin produced by *P. syringae* pv. *syringae* strains isolated from mango (4). Mangotoxin production was tested by an indicator technique based on the growth inhibition bioassay of *E. coli* in PMS and by dot blot detection of an amplicon of the mangotoxin biosynthetic operon (*mbo*) operon involved in the biosynthesis of mangotoxin. Most of the *P. syringae* pv. *syringae* strains isolated from mango were positive for mangotoxin production with both indicator and dot blot techniques (Table 5). *P. syringae* pv. *syringae* strain UMAF3028 was the unique exception because it did not produce *mbo* amplicon and was negative for mangotoxin production. The *mbo* amplicon was also detected in five strains isolated from other hosts: *P. syringae* pv. *syringae* 1444-5, FF5, EPS17A, CFBP3388, and UMAF4002. Strains EPS17A isolated from pear, CFBP3388 isolated from *Vicia sativa*, and UMAF4002 isolated from tomato were able to produce mangotoxin (15,54). Production of the other toxins analyzed in this study (phaseolotoxin, tabtoxin, and coronatine) was only observed in the expected reference strains of the corresponding pathovar. However, none of the *P. syringae* pv. *syringae* strains isolated from mango produce any of these toxins, which confirms that *P. syringae* pv. *syringae* strains isolated from mango specifically produce mangotoxin (Table 5).

Virulence level on tomato leaflets. The virulence level on tomato leaflets of the representative strains is represented in Figure 4. The number of necrotic spots >1 mm in diameter was recorded at 5 days postinoculation. The results from the different inoculated tomato leaflets were represented in percentages, following the scale described in Materials and Methods (Fig. 4). Seventeen *P. syringae* pv. *syringae* strains isolated from mango (33.3%) showed a low degree of virulence (≥15 and <30% of necrotic spots). This virulence group included two strains not belonging to the *P. syringae* pv. *syringae* strains isolated from mango (B728a and 1444-5). As expected, the negative control strain *P. syringae* pv. *phaseolicola* 1448A was considered not pathogenic together with the *P. syringae* pv. *syringae* strain UMAF0005 isolated from mango, a strain that was also separated from the *P. syringae* pv. *syringae* mango group by other traits. The majority of *P. syringae* pv. *syringae* strains isolated from mango (60.8%) showed an intermediate degree of virulence (≥30 to <60%). In addition, this virulence group included three *P. syringae* pv. *syringae* strains isolated from other hosts (FF5, 2676, and EPS17A). Finally, the highly virulent group (>60%) included only three *P. syringae* pv. *syringae* strains isolated from mango (DAR77789, UMAF0171, and UMAF0214; 5.9%) from different geographical areas, a *P. syringae* pv. *syringae* strain isolated from pear (EPSMV3), and the positive control strain *P. syringae* pv. *tomato* DC3000 (a tomato pathogen). The virulence level was distributed heterogeneously among the *P. syringae* pv. *syringae* strains isolated from mango and was not associated with any of the genetic or biochemical grouping in this work.

Phylogenetic analysis reveals a differentiated phylotype of *P. syringae* pv. *syringae* strains isolated from mango. Four main MLST phylogroups were clearly generated (Fig. 5), showing a similar tendency, consistent with results previously observed by rep-PCR, RAPD, and Biolog GN2 analysis. The different isolates were clearly grouped based on the host of isolation, being highly relevant to mangotoxin production, and do not show any relation

TABLE 4. Distribution of the antibiotic resistance on the representative group of strains

	Antibiotics resistance[b]							
P. syringae pvs.[a]	Amp	Cm	Gm	Km	Nf	Sp	Sm	Tet
syringae (mango, *n* = 51)	32	4	1	NR	51	2	2	2
syringae (other hosts, *n* = 6)	3	2	NR	NR	6	NR	1	NR
phaseolicola 1448A (bean, *n* = 1)	NR	NR	NR	NR	1	NR	NR	NR
tomato DC3000 (tomato, *n* = 1)	NR	NR	NR	NR	1	NR	NR	NR

[a] Total number (*n*) of strains tested shown in parentheses.
[b] Number of resistant strains to each antibiotic tested. Amp = ampicillin (100 µg/ml), Cm = chloramphenicol (25 µg/ml), Gm = gentamicin (10 µg/ml), Km = kanamycin (50 µg/ml), Nf = nitrofurantoin (100 µg/ml), Sp = spectinomycin (50 µg/ml), Sm = streptomycin (100 µg/ml), and Tet = tetracycline (15 µg/ml). NR = no resistance (0 strains resistant).

with the area of isolation. The different MLST phylogroups were named as follows: Psy4 for *P. syringae* pv. *syringae* strains isolated from cherry; Psy3 for *P. syringae* pv. *syringae* strains isolated from bean and also including two *P. syringae* pv. *syringae* strains isolated from chestnut and peach in Málaga; Psy2 for *P. syringae* pv. *syringae* strains isolated from other hosts than mango (bean, pear, cherry, and laurel) that were mangotoxin producers or harbored the *mbo* genes related to its production detected by dot blot; and the Psy1 group, which included all the *P. syringae* pv. *syringae* mango isolates, mainly mangotoxin producers, with the exception of three *P. syringae* pv. *syringae* strains. Within the Psy1 group, strain UMAF3028 does not produce mangotoxin, although it was isolated from mango, and EPS17A isolated from pear and UMAF4002 isolated from tomato do produce mangotoxin.

DISCUSSION

P. syringae pv. *syringae* is distinguished from many other plant pathogens by its ability to infect a large variety of woody and

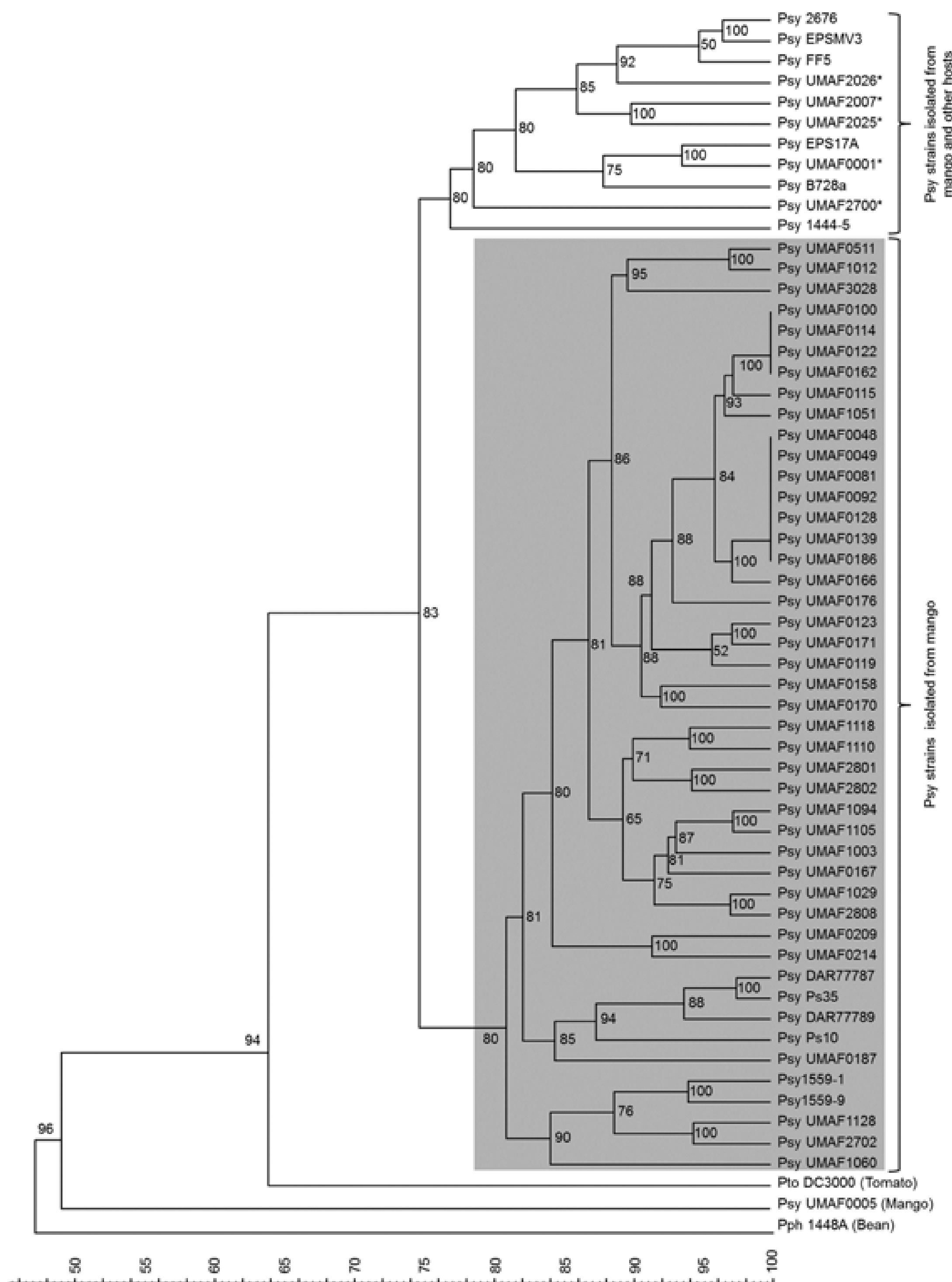

Fig. 3. Cluster analysis of the representative group of the strains ($n = 59$) based on their catabolic activities on 95 different substrates (Biolog GN2). Jaccard's similarity coefficient was used to generate a dendrogram using the InfoQuest FP software (Bio-Rad), and unweighted pair-group method with average linkages; * indicates five *Pseudomonas syringae* pv. *syringae* strains isolated from mango (UMAF0001, UMAF2700, UMAF2007, UMAF2025, and UMAF2026). Gray box: *P. syringae* pv. *syringae* strains isolated from mango.

TABLE 5. Toxins production analyzed by *Escherichia coli* inhibition growth bioassay and dot-blot hybridization analysis[a]

Bacterial strains	Mangotoxin		Phaseolotoxin		Tabtoxin	Coronatine
	Bioassay	Dot blot	Bioassay	Dot blot	Dot blot	Dot blot
Pseudomonas syringae pvs.						
glycinea 49a/90	–	–	–	–	–	**+**
phaseolicola 1448A	–	–	**+**	**+**	–	–
tabaci BR2R	–	–	–	–	**+**	–
tomato DC3000	–	–	–	–	–	**+**
tomato PT23	–	–	–	–	–	**+**
syringae (other hosts)						
1444-5	**–**	**+**	–	–	–	–
2676	–	–	–	–	–	–
B728a	–	–	–	–	–	–
CFBP3388	**+**	**+**	**+**	**+**	–	–
EPS17A	**+**	**+**	–	–	–	–
EPSMV3	–	–	–	–	–	–
FF5	**–**	**+**	–	–	–	–
UMAF4002	**+**	**+**	–	–	–	–
UMAF6016	–	–	–	–	–	–
UMAF6582	–	–	–	–	–	–
syringae (mango)						
UMAF0001	+	+	–	–	–	–
UMAF0005	+	+	–	–	–	–
UMAF0048	+	+	–	–	–	–
UMAF0049	+	+	–	–	–	–
UMAF0081	+	+	–	–	–	–
UMAF0092	+	+	–	–	–	–
UMAF0100	+	+	–	–	–	–
UMAF0114	+	+	–	–	–	–
UMAF0115	+	+	–	–	–	–
UMAF0119	+	+	–	–	–	–
UMAF0122	+	+	–	–	–	–
UMAF0123	+	+	–	–	–	–
UMAF0128	+	+	–	–	–	–
UMAF0139	+	+	–	–	–	–
UMAF0158	**+**	**+**	–	–	–	–
UMAF0162	+	+	–	–	–	–
UMAF0166	+	+	–	–	–	–
UMAF0167	+	+	–	–	–	–
UMAF0170	+	+	–	–	–	–
UMAF0171	+	+	–	–	–	–
UMAF0176	+	+	–	–	–	–
UMAF0186	+	+	–	–	–	–
UMAF0187	+	+	–	–	–	–
UMAF0209	+	+	–	–	–	–
UMAF0214	+	+	–	–	–	–
UMAF0511	+	+	–	–	–	–
UMAF1012	+	+	–	–	–	–
UMAF3028	**–**	**–**	–	–	–	–
UMAF1003	+	+	–	–	–	–
UMAF1029	+	+	–	–	–	–
UMAF1051	+	+	–	–	–	–
UMAF1060	+	+	–	–	–	–
UMAF1094	+	+	–	–	–	–
UMAF1105	+	+	–	–	–	–
UMAF1110	+	+	–	–	–	–
UMAF1118	+	+	–	–	–	–
UMAF1128	+	+	–	–	–	–
1559-1	+	+	–	–	–	–
1559-9	+	+	–	–	–	–
UMAF2007	+	+	–	–	–	–
UMAF2025	+	+	–	–	–	–
UMAF2026	+	+	–	–	–	–
UMAF2700	+	+	–	–	–	–
UMAF2702	+	+	–	–	–	–
UMAF2801	+	+	–	–	–	–
UMAF2802	+	+	–	–	–	–
UMAF2808	+	+	–	–	–	–
DAR77787	+	+	–	–	–	–
DAR77789	+	+	–	–	–	–
Ps10	+	+	–	–	–	–
Ps35	+	+	–	–	–	–

[a] Bold with gray shading indicates strains used as control of toxin production. Bold without shading indicates interesting strains and their relevant characteristics. *P. syringae* pv. *syringae* FF5 and 1444-5 are non-mangotoxin-producing strains but showed hybridization signal by dot blot. Strain CFBP3388 produces mangotoxin and phaseolotoxin. Strains EPS17A and UMAF 4002, isolated from pear and tomato, respectively, are mangotoxin producers and showed hybridization signal by dot blot. UMAF3028 is the only strain isolated from mango that does not produce mangotoxin and does not present hybridization signal.

herbaceous plant species (40). This fact suggests a high degree of genetic variability in this pathovar that can provide understanding of the development of this plant pathogen in different hosts. In the current study, we evaluated the genetic, phenotypic, and phylogenetic diversity in a large collection of *P. syringae* pv. *syringae* strains isolated from mango (and in comparison with isolates from other hosts) that encompasses a wide range of geographical origins but is primarily from southern Spain.

rep-PCR is a commonly used technique because of its sensitivity and reproducibility, making it a highly discriminatory technique for assessing bacterial diversity at the strain and pathovar level with an epidemiological aim (25,51,52,58,66,69). A previous study demonstrating the usefulness of this molecular fingerprinting technology was performed (31), showing the ability of some primers tested to produce repetitive fingerprinting patterns in *P. syringae* pv. *syringae* strains isolated from mango. When the 115 strains used in this study were genotyped by rep-PCR, a main cluster was observed composed almost exclusively of *P. syringae* pv. *syringae* strains isolated from mango, forming a distinct group associated with host of isolation and clearly separate from other *P. syringae* pv. *syringae* strains isolated from other hosts and other pathovars of *P. syringae* (Fig. 1). In contrast, a previous study using different *P. syringae* pv. *syringae* strains isolated from various woody and herbaceous plants did not show a clear relationship between the host plant and genomic fingerprinting (66). The rep-PCR technique also provides subcluster differentiation of the *P. syringae* pv. *syringae* mango isolates based on geographical origins (Fig. 1) but these data are not consistent with the recent results observed for *P. syringae* pv. *syringae* strains isolated from pea (52). Notably, clustering of the *P. syringae* pv. *syringae* mango isolates worldwide is stronger among them than with others isolates from different hosts that are closer geographically (for instance, southern Spain). Although few exceptions were observed, the reliability and robustness of the rep-PCR technique to cluster nearly all *P. syringae* pv. *syringae* strains from mango and to determine subgroups based on the area of isolation was confirmed.

Similarly, the RAPD-PCR fingerprinting method was able to differentiate the majority of the *P. syringae* pv. *syringae* strains isolated from mango from the *P. syringae* pv. *syringae* strains isolated from other hosts and other pathovars (Fig. 2). This technique was successfully used to characterize pathogenic races of *P. syringae* pv. *pisi* (2) and to distinguish pv. *tomato* from pv. *maculicola* (22), both of which belong to the genomospecies 3. In this study, RAPD-PCR grouped pv. *syringae* strains based on the host of isolation. Despite the fact that these techniques (rep-PCR and RAPD-PCR) showed different resolution levels, both clustered together the *P. syringae* pv. *syringae* strains based on the host of isolation. These results suggest a possible genetic specialization of *P. syringae* pv. *syringae* strains, particularly in the mango host, as has been previously observed for the same pathovar in stone fruit (45) and in different bean pathovars (7).

MLST analysis has been previously performed to compare the phylogeny of *P. syringae* pathovars (8,28,37,63). To determine whether the genetic clustering is consistent with the phylogeny of *P. syringae*, a MLST analysis using different housekeeping genes was performed that included *P. syringae* pv. *syringae* strains isolated from mango and other hosts (Fig. 5). This analysis generated four different phylogroups that were mainly clustered by the host of isolation. A similar result was previously observed for *P. syringae* pv. *actinidiae* isolated from kiwifruit, which allocated all the *P. syringae* pv. *actinidiae* strains in the same phylogroup with respect to other pathovars isolated from other hosts (21). In our study, the cluster named Psy1 comprised all of the *P. syringae* pv. *syringae* strains isolated from mango worldwide, the majority of which are mangotoxin producers. Another interesting cluster is formed primarily by *P. syringae* pv. *syringae* strains isolated from different herbaceous and woody hosts (Psy2), and these strains are also mangotoxin producers, as was previously reported for pv. *syringae* and others pathovars belonging to the genomospecies 1 (15,54). Therefore, mangotoxin production is a highly specific phenotypic trait for the *P. syringae* pv. *syringae* strains isolated from mango. Thus, mangotoxin production may be one of the most important traits, following a similar evolution-

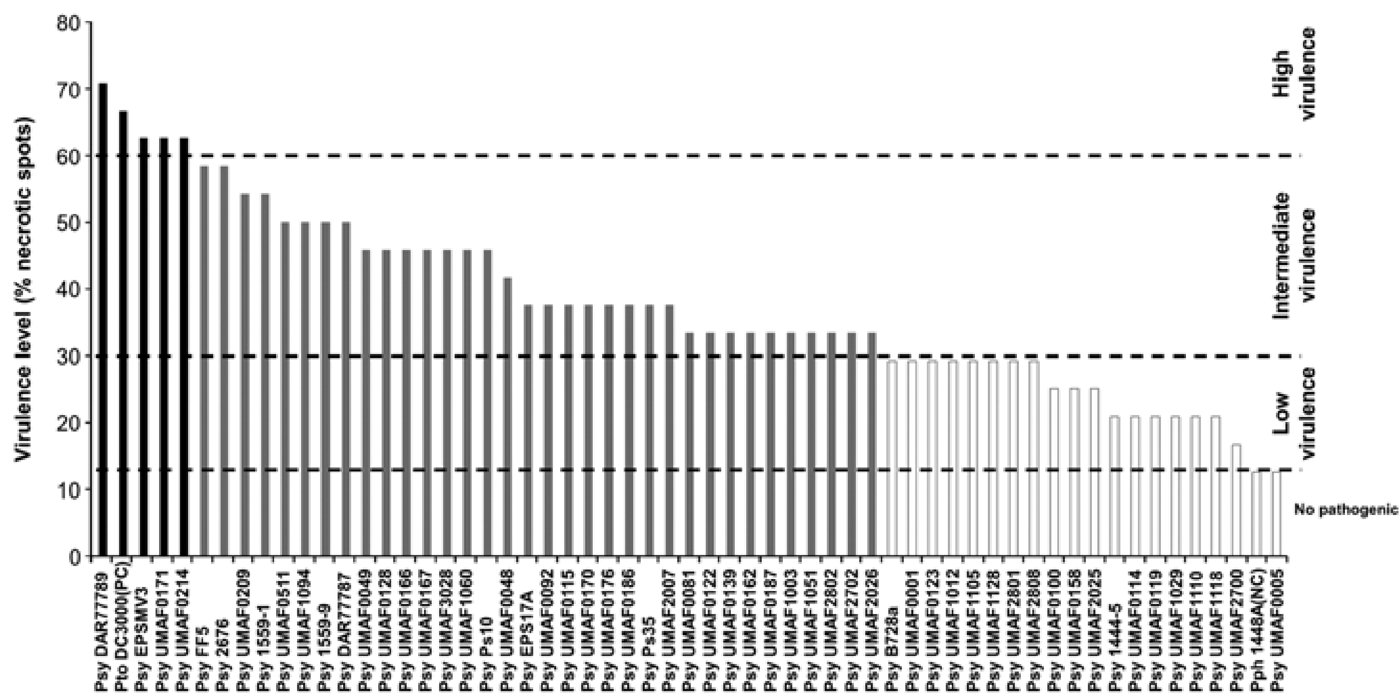

Fig. 4. Virulence level analysis of the representative group of the strains on tomato leaflets was determined as percentage of inoculated points that developed necrotic symptoms (>1 mm) at 5 days postinoculation. The black bar represents the high virulence level, the gray bar the intermediate virulence level, and the white bar the low virulence level. *Pseudomonas syringae* pv. *tomato* DC3000 (PC) is the positive control and a natural pathogen of tomato plants. *P. syringae* pv. *phaseolicola* 1448A (NC) is the negative control and not a pathogen of tomato.

ary history together with the adaptation to the determined host, such as mango. The presence of two other distinct groups of *P. syringae* pv. *syringae* strains isolated from bean and cherry (Fig. 5) that were non-mangotoxin producers suggests that *P. syringae* pv. *syringae* strains isolated from mango, which are mangotoxin producers, may constitute a differentiated phylotype within the heterogeneous pv. *syringae*.

Conversely, the phenotypic approaches to study the diversity of *P. syringae* pv. *syringae* strains isolated from mango also support their host specialization and separated lineage. The catabolic

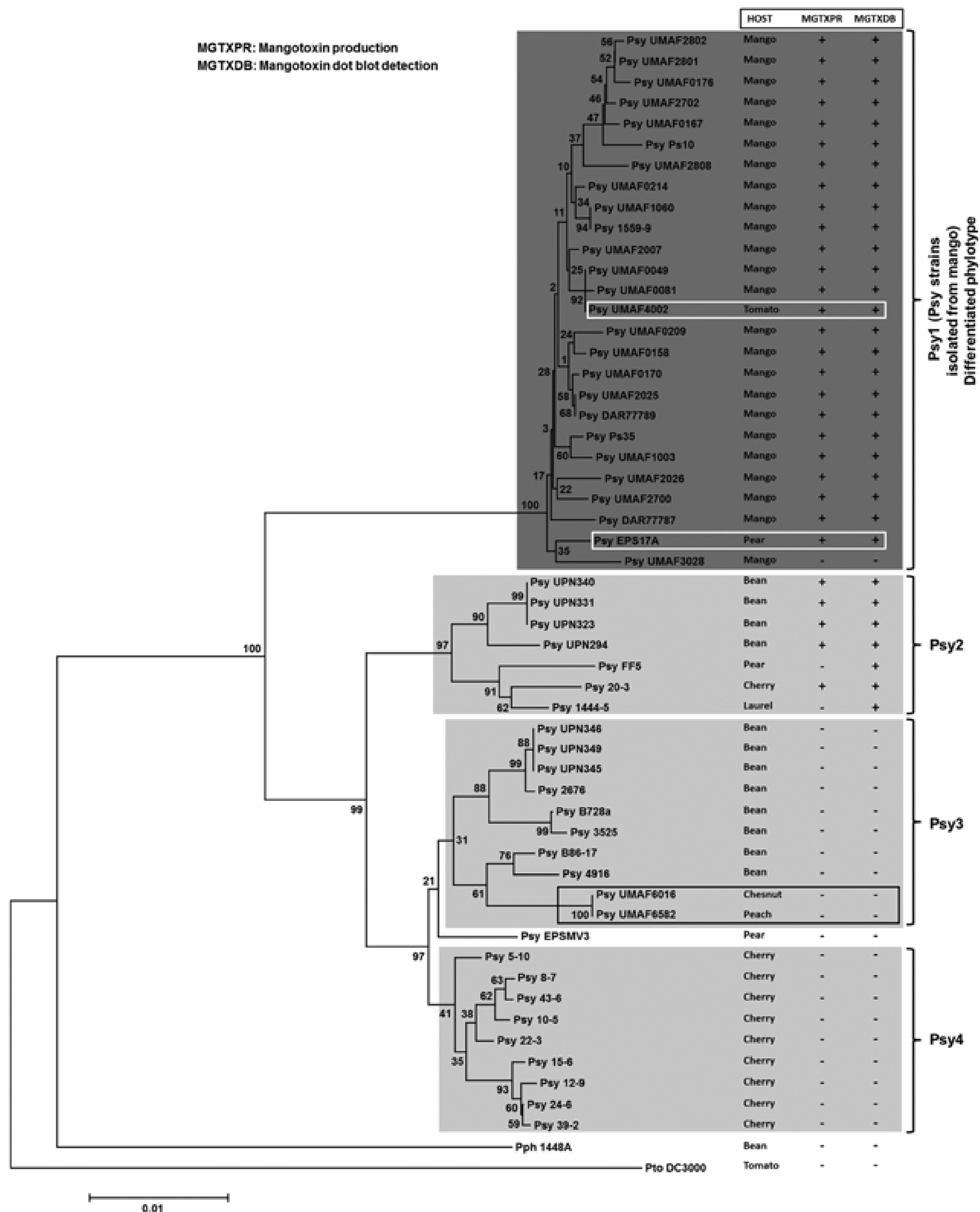

Fig. 5. Multilocus sequence typing analysis produced a phylogeny of *Pseudomonas syringae* strains. The neighbor-joining tree was constructed with combined partial sequences of *rpoD* and *gyrB* genes using MEGA 5.0.5. The host of isolation, the mangotoxin production, and the presence of genes related to its production are represented in the right column beside each strain number. Bootstrap values (10,000 repetitions) are shown on branches and evolutionary distances are in units of nucleotide substitutions per site. Dark gray box: *P. syringae* pv. *syringae* strains isolated from mango and mangotoxin producer. White box: *P. syringae* pv. *syringae* strains isolated from other hosts and mangotoxin producer (UMAF4002 and EPS17A). Black box: *P. syringae* pv. *syringae* strains isolated in Málaga (Spain) from other hosts (UMAF6016 and UMAF6582) and not mangotoxin producer. Supplemental Table 4 provides detailed information of the strains and accession numbers of the DNA sequences used.

diversity determined by Biolog GN2 analysis has been used to characterize different pathovars of *P. syringae*, clustering all of them together while internally subclustering each different pathovar (38). This system has also been used to separate pv. *phaseolicola* from other pathovars (34). In our study, Biolog GN2 analysis (Fig. 3) was able to discern *P. syringae*, even at an intrapathovar level, clustering the majority of *P. syringae* pv. *syringae* strains isolated from mango together and separating them from other *P. syringae* pv. *syringae* strains isolated from other hosts and pathovars. Furthermore, the presence of 62-kb plasmids (Table 3) in *P. syringae* pv. *syringae* strains isolated from mango and their possible relationship with copper resistance could be revealing the important role that these plasmids have in the ecological fitness of this bacterium (17,18,32). Finally, the virulence level for the *P. syringae* pv. *syringae* strains isolated from mango is heterogeneous and shows no correlation with any other distribution pattern, a situation similar to that previously described for *P. syringae* pv. *syringae* strains isolated from woody and herbaceous hosts (66). This heterogeneity was also observed for *P. syringae* pv. *lachrymans* isolates from cucumber leaves (58).

Interestingly, the different genetic and phenotypic approaches used in this work reveal that strain UMAF0005 could be wrongly classified as *P. syringae* pv. syringae. This strain could not be part of the *P. syringae* pv. *syringae* mango group, although it displays mangotoxin production. This suggests that it probably could belong to another pathovar of a *P. syringae* mangotoxin producer, as has been previously described (15).

Overall, our results based on the MLST and genetic and phenotypic diversity analysis strongly suggest that *P. syringae* pv. *syringae* strains isolated from mango constitute a differentiated phylotype that is specifically adapted to the mango host and distributed worldwide in the different subtropical areas where mango is grown and BAN is a relevant disease (20,29). This phylotype associated with mango is separate from other phylogroups of pv. *syringae* isolated from others hosts and it is characterized mainly by the production of mangotoxin. Additionally, some genetic diversity techniques, such as rep-PCR, are useful in differentiating among strains of the differentiated phylotype isolated from various geographical areas, revealing their usefulness in epidemiological studies.

As mentioned above, the pathovar-based classification of the *P. syringae*-complex does not reveal the wide genetic diversity among pathovars previously observed in many studies (8,22, 49,63). The *P. syringae* complex shows a very heterogeneous genetic background, and an extensive revision of this group may be needed in the near future to better understand this group of phytopathogenic bacteria.

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