Pantoea agglomerans as a New Etiological Agent of a Bacterial Necrotic Disease of Mango Trees

José A. Gutiérrez-Barranquero, Francisco M. Cazorla, Juan Antonio Torés, and Antonio de Vicente†

First, second, and fourth authors: Instituto de Hortofruticultura Subtropical y Mediterránea (IHSM-UMA-CSIC), Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain; and third author: IHSM-UMA-CSIC, Estación Experimental “La Mayora”, 29750 Algarrobo-Costa (Málaga), Spain.

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ABSTRACT

Bacterial apical necrosis of mango trees, a disease elicited by *Pseudomonas syringae* pv. *syringae*, is a primary limiting factor of mango crop production in the Mediterranean region. In this study, a collection of bacterial isolates associated with necrotic symptoms in mango trees similar to those produced by bacterial apical necrosis disease were isolated over five consecutive years in orchards from the Canary Islands. The bacterial isolates were characterized and identified as *Pantoea agglomerans*. Pathogenicity tests conducted on onion bulbs and mango plants confirmed that *P. agglomerans* strains isolated from mango trees are a new etiological agent of a bacterial necrotic disease in the Canary Islands. Pathogenicity plasmids of the pPATH family have been previously reported in *P. agglomerans*. The majority of putatively pathogenic ($n = 23$) and pathogenic ($n = 4$) *P. agglomerans* strains isolated from mango trees harbored four plasmids, one of which was close in size to the

135-kb pPATH pathogenicity plasmid. The analysis of the presence of two major genes in pPATH plasmids (*repA* and *hrpJ*) was undertaken in *P. agglomerans* strains isolated from mango trees. The *hrpJ* gene was detected in the 140-kb plasmid of pathogenic *P. agglomerans* strains isolated from mango trees but it showed differences in nucleotide sequences compared with other pathogenic strains. In contrast, the *repA* gene was not detected in any of the putatively pathogenic and pathogenic *P. agglomerans* strains isolated from mango trees. Finally, genetic characterization and phylogenetic analysis using the *hrpJ* gene and the housekeeping genes *gyrB* and *rpoB* showed that almost all *P. agglomerans* strains that were putatively pathogenic and pathogenic on mango trees clustered together, forming a differentiated phylogroup with respect to the other pathogenic *P. agglomerans* strains described from other hosts.

The mango tree (*Mangifera indica* L.) is widely distributed in many tropical and subtropical areas and represents one of the most important subtropical fruit crops worldwide (Galán Saúco 2015). Mango crops are affected by several diseases elicited by bacterial and fungal pathogens. The most severe bacterial diseases are bacterial black spot, caused by *Xanthomonas citri* pv. *mangiferaeindicae* (Gagnevin and Pruvost 2001), and bacterial apical necrosis (BAN), caused by *Pseudomonas syringae* pv. *syringae* (Cazorla et al. 1998), which is the most important limiting factor for the production of this crop in Southern Europe (Cazorla et al. 1998, 2006). BAN has also been described to be present in other countries in the Mediterranean region, such as Israel (Pinkas et al. 1996) Italy (Aiello et al. 2015), and, more recently, Greece (Trantas et al. 2017), but has also been detected in other parts of the world with tropical and subtropical climates such as the northwest region of Australia (Golzar and Cother 2008) and Pakistan (Naqvi et al. 2016). Unusually, only a few reports have described the role of some Enterobacteriaceae species as pathogens of mango crops, such as *Erwinia* spp. (Guevara et al. 1980; McMillan and Wang 1992) and *Pantoea agglomerans* (Lee and Tzeng 2006).

P. agglomerans is a bacterium that is widely distributed in agricultural and natural environments (Delétoile et al. 2009). This species has been observed to be associated with many different plants, frequently as epiphytic and endophytic bacteria, and it has also been widely used as a potential biological control agent against fungal and bacterial plant pathogens (Walterson and Starvinides 2015). However, this bacterium can also cause secondary infections in humans in soft tissues, bones, and joints, which can develop into bacteremia (Büyükcem et al. 2018; Cheng et al. 2013; Cruz et al. 2007). Interestingly, *P. agglomerans* has been naturally transformed into a gall-forming plant pathogen by the acquisition of pathogenicity plasmids belonging to the pPATH family (Manulis and Barash 2003). The two best-studied model of *P. agglomerans* pathogenic strains are *P. agglomerans* pv. *gypsophillae* 824-1 and *P. agglomerans* pv. *betae* 4188, which differ in host range (Burr et al. 1991; Cooksey 1986) and in gall formation in specific plant hosts (Burr et al. 1991; Manulis and Barash 2003). Both strains harbor a similarly sized pPATH plasmid (approximately 135 kb), although the best studied is the plasmid from strain 824-1. This pathogenicity plasmid carries an important 75-kb pathogenicity island (PAI) that encodes *hrp/hrc* type III secretion system genes (T3SS), T3SS protein effector-encoding genes, and genes involved in the production of phytohormones (Barash and Manulis-Sasson 2007, 2009). In the last two decades, several studies have reported that the transformation of *P. agglomerans* into pathogenic strains is not restricted only to the pathogens *gypsophillae* and *betae* but also has been described to incite the production of tumors (DeYoung et al. 1998; Opgenorth et al. 1994) and, more frequently, necrotic rot and wilt symptoms in many other plant hosts (González et al. 2015; Lee et al. 2010; Lu et al. 2015; Tho et al. 2015; Yang et al. 2011).

In this study, necrotic symptoms affecting different mango tissues were observed in mango trees in Canary Islands orchards (Spain) over five different years. Bacterial isolation identified bacterial strains

†Corresponding author: Antonio de Vicente; E-mail: adevicente@uma.es

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This work is dedicated to the memory of Juan A. Torés (1952–2018), a pioneer in the discovery of BAN disease, who passed away during the publication process of this work.

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary tables are published online.

other than *Pseudomonas syringae* pv. *syringae*, the original etiological agent of BAN of mango trees. Thus, the main goal this work was to characterize and identify these bacterial isolates associated with necrotic symptoms of mango trees and to demonstrate their pathogenicity on mango trees. Following different biochemical and physiological tests, most of these bacterial isolates were identified as *Pantoea agglomerans*, and the pathogenicity of five selected strains was confirmed in mango adult plants. Moreover, plasmid characterization revealed the presence of plasmids other than those belonging to the pPATH pathogenicity family in pathogenic *P. agglomerans* strains isolated from mango trees. Genetic and phylogenetic analyses revealed a strong clustering of *P. agglomerans* strains isolated from mango trees that may represent a differentiated phylogroup with respect to other pathogenic *P. agglomerans* strains previously described from other hosts.

MATERIALS AND METHODS

Isolation, characterization, and identification of bacterial isolates. Commercial mango orchards in the Canary Islands, Spain, primarily from Tenerife and La Palma, were surveyed in this study for the presence of necrotic symptoms. To isolate the causal agent of the necrotic symptoms, small pieces of affected leaves, buds, stems, flower panicles, and immature fruit were cut from the edges of necrotic lesions, placed in sterile plastic bags, and transported to the laboratory for processing. The samples were processed by two different methods. (i) Some portions were surface disinfected by immersion in a sterile aqueous solution of 0.1% (wt/vol) HgCl₂ for 1 min, rinsed twice in sterile distilled water for 1 min each, and plated onto agar-solidified King's medium B (KB) (King et al. 1954) supplemented with cycloheximide (100 µg/ml). (ii) The remaining samples were homogenized in a lab blender for 3 min with sterile phosphate buffer (0.1 M, pH 7.2) at 10 ml/g of fresh plant material, after which 100 µl was spread onto KB plates supplemented with cycloheximide. All KB plates were incubated at 25°C for 2 days.

The bacterial isolates were identified according to biochemical and physiological tests described previously (Bradbury 1986; Lelliott and Stead 1987), including Gram staining; glucose metabolism; growth on yeast extract-dextrose-calcium carbonate agar; production of fluorescent pigments; and levan production, oxidase reaction, potato soft rot, arginine dihydrolase, and tobacco hypersensitivity response (HR) tests, excluding the potato soft rot analysis. HR elicitation in resistance and nonhost plants such as tobacco is generally dependent on T3SS in Gram-negative bacterial pathogens (Lindgren 1997), as has also been reported in *P. agglomerans* (Nizan et al. 1997).

Subsequently, indole production and nitrate reduction assays were performed. All of the results obtained from these tests are summarized in Table 1. All inoculated media were incubated at 25°C for 2 to 3 days. Duplicate tubes or plates were assayed for each test, and tests were repeated at least twice. All of the bacterial isolates identified in this study are summarized in Supplementary Table S1. Several bacterial strains of different species were used as controls (Supplementary Table S2) for the different biochemical and physiological tests.

Pathogenicity tests on onion bulbs and mango plants. To determine whether the isolated *P. agglomerans* was the causal agent of necrotic symptoms observed in mango trees in Canary Islands, two different pathogenicity tests were performed to fulfill Koch's postulates.

First, a pathogenicity test in onion bulbs following the procedure of Kim et al. (2002) and Jacobs et al. (2008) with minor modifications was carried out. Three different commercial onion bulbs were used per bacterial strain inoculated, and this experiment was repeated twice. Onion bulbs were initially devoid of their outer coverings, and their surfaces were washed with sterile water before and after being disinfected with ethanol 70%. Next, the bacterial strains selected for this experiment were inoculated by injection using sterile syringes. Bacterial suspensions (20 µl of 3×10^7 CFU/ml) in phosphate buffer were used for each onion bulb. Onion bulbs were maintained at 25°C in darkness for 28 days in closed boxes lined with damp blotting paper. The production of symptoms was estimated after this time. A positive score was recorded when bulb rot symptoms were observed in the center of the onion bulbs, and a negative score was recorded when no bulb rot symptoms appeared. Ten representative *P. agglomerans* strains isolated from mango trees were selected by year of isolation (two bacterial strains per year: UMAF3066, UMAF3067, UMAF3075, UMAF3078, UMAF3084, UMAF3086, UMAF3090, UMAF3093, UMAF3098, and UMAF3101), including one that was negative for HR response (UMAF3090) and one that was isolated from another location, La Palma (UMAF3098), and were assayed. Similarly, two strains of *P. agglomerans* (CECT850 and CFBP4740), *P. stewartii* subsp. *indologenes* CFBP3614, and phosphate buffer were used as controls. Reisolation and identification of the etiological agent through biochemical and physiological tests was carried out at the end of the experiment.

Second, to test the pathogenicity of *P. agglomerans*, Koch's postulates were fulfilled using 30 2-year-old mango plants growing in pots. Buds of mango plants were inoculated with 20 µl of a bacterial suspension (3×10^7 CFU/ml) in phosphate buffer, with the inoculum forced into buds with a sterile microsyringe, as previously

TABLE 1. Physiological and biochemical tests performed in this study to identify the bacterial isolates ($n = 44$) from necrotic lesions in different tissues in mango trees in Canary Islands, Spain

Test	Physiological and biochemical profiles of bacterial isolates ^a			
	Bt1	Bt2	Bt3	Bt4
Morphology	Coccobacillus	Coccobacillus	Coccobacillus	Coccobacillus
Gram stain	–	–	–	–
Fluorescence	–	–	–	+
Glucose metabolism ^b	F	F	F	O
YDC ^c	Y	Y	Y	W
Levan production	+	+	+	+
Oxidase	–	–	–	–
Arginine dihydrolase	–	–	–	–
Tobacco hypersensitivity	+	–	–	+
Identification	<i>Pantoea</i> spp.	<i>Pantoea</i> spp.	<i>Pantoea</i> spp.	<i>Pseudomonas syringae</i>
Indole production	–	–	+	–
Nitrate reduction	+	+	–	–
Number of isolates	29	4	5	6
Identification	<i>Pantoea agglomerans</i>	<i>P. agglomerans</i>	<i>P. ananatis</i>	<i>Pseudomonas syringae</i>

^a Bt = bacterial type.

^b Metabolism of glucose: O = oxidative and F = fermentative.

^c Growth on yeast extract-dextrose-calcium carbonate agar (YDC): W= white colonies and Y= yellow mucoid colonies.

described (Cazorla et al. 1998). In all, 5 representative *P. agglomerans* strains isolated from mango trees selected by year of isolation from the previous 10 bacterial strains used in onion bulbs (UMAF3067, UMAF3075, UMAF3084, UMAF3093, and UMAF3098) were used for this experiment. In addition, two *Pseudomonas syringae* pv. *syringae* strains (UMAF0048 and UMAF0049), both producers of BAN symptoms in mango trees, were used as positive controls, and *Pantoea agglomerans* CFBP4740 and phosphate buffer were used as negative controls. Each assayed strain was injected into seven buds of different mango plants in a random manner. After bacterial inoculations, the trees were maintained under field conditions from February to June 2010 at “La Mayora” Experimental Station (Algarrobo, Spain). The disease incidence was measured by the percentage of necrotic symptoms in buds compared with the total inoculated buds ($n = 35$). Reisolation from necrotic symptoms and

identification through biochemical and physiological tests of bacterial isolates was carried out at the end of the experiment.

Plasmid profile characterization. Plasmid DNA from the *P. agglomerans* strains isolated from mango trees was isolated by extracting total uncut plasmid DNA from Lysogeny broth cultures grown overnight (Moulton et al. 1993) and separated by electrophoresis on 0.8% agarose gels. The plasmid sizes were estimated by comparison with those isolated from *P. agglomerans* pv. *gyssophila* 824-1, which harbors two plasmids, a pathogenicity pPATH plasmid of approximately 135 kb, and another plasmid of approximately 60 kb (Manulis et al. 1991).

Polymerase chain reaction detection and sequencing of the plasmid-encoded *hrpJ* and *repA* genes. The *repA* gene is required for the replication of pPATH plasmids (Weinthal et al. 2007). To detect the presence of this gene in *P. agglomerans* strains

Fig. 1. Necrotic symptoms on mango trees in Canary Islands. **A**, Initial necrosis of flower panicles; **B**, advanced necrosis of flower panicles; **C**, necrosis of immature fruit; **D**, necrotic symptoms in branches; **E**, necrotic spots in leaves; **F**, detail of necrotic symptoms in mango bud, and **G**, necrotic symptoms in stems, both experimentally inoculated with representative strains of *Pantoea agglomerans*.

isolated from mango trees, specific primers designed in this study using the *repA* sequences of strains 824-1 and 4188 (GenBank accession numbers EF173387.1 and EF173388.1, respectively) were used for polymerase chain reaction (PCR) amplification and sequencing (*repAFsp*: 5'-GCTGACTGAAGCTGCTTATC-3' and *repARsp*: 5'-AGACTCAGGCAGCATGAATC-3'), which generate a predicted amplicon of 499 bp. Nucleotide sequences were determined at Macrogen (Seoul, Korea).

The *hrpJ* gene is a key gene of the T3SS. A PCR experiment using specific primers designed previously for the *hrpJ* gene sequence present in pPATH plasmids (GenBank accession number: X99768.2 and primers *hrpJ5n1*: 5'-CGCCAGGATGAAAATTGCTC-3' and *hrpJ3n1335*: 5'-CGCAGCCGATTCAACGCC-3') was carried out (Weinthal et al. 2007) to assess its presence in *P. agglomerans* strains isolated from mango trees. Five representative bacterial strains (UMAF3067, UMAF3075, UMAF3084, UMAF3093, and UMAF3098) as well as strains 4188 and 824-1 were used to determine the partial nucleotide sequence of the *hrpJ* gene. DNA amplicons were purified from an agarose gel, cloned into the pGEM-T vector (Promega Biotech, Ibérica, Spain), and transformed into chemically competent *Escherichia coli* DH5 α cells. Purified plasmids were used to sequence the insert DNA region. Nucleotide sequences were determined at Macrogen. Afterward, the specific primers designed in this study (*hrpJPaF*: 5'-GACCTTCGCGGAACAGGTTG-3' and *hrpJPaR*: 5'-CGGCA GATGGTAGACCAG-3') based on the *hrpJ* sequences, obtained as described above, were used to amplify and sequence a partial region of this gene (803 bp) for the *P. agglomerans* strains isolated in this study.

Fig. 2. Pathogenicity test in onion bulbs using selected *Pantoea agglomerans* strains. Ten strains of *P. agglomerans* isolated from mango were tested (UMAF3066, UMAF3067, UMAF3075, UMAF3078, UMAF3084, UMAF3086, UMAF3090, UMAF3093, UMAF3098, and UMAF3101). Just five representative strains were included in this figure. Positive control = *P. stewartii* subsp. *indologenes* (Pst) CFBP3614. Negative controls = *P. agglomerans* (Pa) CFBP4740 and phosphate buffer.

PCR amplification conditions for the different amplifications described previously are summarized in Supplementary Table S3.

Southern blot experiments. The genomic location of the *repA* and *hrpJ* genes was assessed by Southern blot using plasmid DNA extractions that were separated by electrophoresis in 0.8% agarose gels. DNA probes were obtained by PCR amplification using the specific primers designed in this study for both genes (*repAFsp*-*repARsp* and *hrpJPaF*-*hrpJPaR*). The DNA probes obtained from the purified PCR amplicons were labeled with digoxigenin (DIG) using a DIG-High Prime labeling kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Strain 824-1 was included as positive control. Plasmid DNA extraction gels, which included strain 824-1 as positive control, were transferred to nylon membranes and crosslinked in a UV chamber. For membrane hybridization, a DIG Easy Hyb kit was used (Roche), and immunological detection was performed with an anti-DIG antibody conjugated to alkaline phosphatase. The DIG-labeled nucleic acids were detected by chemiluminescence using a Molecular Imager ChemiDoc system (Bio-Rad, UK). Prehybridization and hybridization at high stringency (55°C) were performed for both the *hrpJ* and *repA* genes and at low stringency (45°C) only for the *hrpJ* gene.

Genetic analysis. To perform a DNA fingerprinting of *P. agglomerans* strains isolated from mango trees, a repetitive element sequence-based PCR (rep-PCR) method was selected, using specifically BOXA1R primer (5'-CTACGGCAAGGC GACGCTGACG-3'). PCR amplifications were performed with a C1000 Thermal Cycler (Bio-Rad). The total volume of each PCR (25 μ l) was loaded onto a 1.2% agarose gel and run in 1 $\times$ Tris-acetate EDTA buffer (40 mM Tris-acetate and 1 mM EDTA) at 80 V/cm for 1.5 h. Polymorphic band patterns were individually identified by their specific electrophoretic migration rates. Once bands were identified, binary matrices (0/1) were constructed to compare those patterns. The Jaccard's similarity coefficient was generated using InfoQuest FP software (Bio-Rad), and a cluster analysis, along with the corresponding dendrogram, was generated by the unweighted pair-group method with average linkages. Gel normalization and background subtraction were performed as previously described (Rademaker et al. 2004).

Phylogenetic analysis. Partial sequences of the *hrpJ* gene and the housekeeping genes *gyrB* and *rpoB* were used to analyze the phylogenetic distribution of the *P. agglomerans* strains isolated from mango trees in comparison with some other *Pantoea* spp. strains. Nucleotide sequences were determined at Macrogen directly from purified PCR products amplified with the primer sets *gyrB*3i (5'-AACGCWATCGACGAAGC-3') and *gyrB*4i (5'-TGGAAVCCRTCTTCCAC-3') for *gyrB*, and *Vic3* (5'-GGCGAAATGGCWGAGAACCA-3') and *Vic2* (5'-GAGTCTTC GAAGTTGTAACC-3') for *rpoB* (Delétoile et al. 2009). The partial sequences of *gyrB* (511 bp) and *rpoB* (801 bp) were concatenated for each strain and treated as a single sequence for multiple alignments using Clustal Omega. Phylogenetic trees were generated using MEGA 6 (Tamura et al. 2013) with neighbor joining, Jukes-Cantor model, minimum evolution, and the option of complete deletion to eliminate positions containing gaps. Confidence levels of the branching points were determined using 1,000 bootstrap replicates. Strains 824-1, 4188, and other *P. agglomerans* control strains used

TABLE 2. Pathogenicity test in 2-year-old mango plants using selected *Pantoea agglomerans* strains isolated from mango

Bacterial isolates	Disease incidence (%)					
	28 days	42 days	56 days	70 days	84 days	98 days
<i>Pseudomonas syringae</i> pv. <i>syringae</i> (n = 2) ^a	42.8	64.2	92.8	92.8	92.8	92.8
<i>Pantoea agglomerans</i> (n = 5) ^b	0	0	17.1	42.8	80	100
<i>P. agglomerans</i> CFBP4740 ^c	0	0	0	0	0	0
Phosphate buffer	0	0	0	0	0	0

^a Bacterial strains inoculated: UMAF0048 and UMAF0049.

^b Strains isolated from mango. Bacterial strains inoculated: UMAF3067, UMAF3075, UMAF3084, UMAF3093, and UMAF3098.

^c CFBP4740 = nonpathogenic control strain.

in this study (CFBP4740 and CECT850) were included, as well as *P. stewartii* subsp. *indologenes* CFBP3614.

Nucleotide accession numbers. The nucleotide sequences determined in this study are deposited at the National Center for Biotechnology Information under the following accession numbers: *gyrB* sequences (MH479140 to MH479183, MH643873, MH643874, and NC_017554.1), *rpoB* sequences (MH479184 to MH479227, MH643875, MH643876, and NC_017554.1), *hrpJ* sequences (MH479228 to MH479258), and *repA* sequences (MH479259 to MH479260).

RESULTS

Description of symptomatology. From different samplings carried out in the Canary Islands between February and March 2005 to 2009, the presence of necrotic symptoms was not primarily associated with *Pseudomonas syringae* pv. *syringae* isolation. The symptomatology observed in mango trees in the Canary Islands, Spain, was similar to that symptomatology produced by BAN disease described in southern Spain elicited by *P. syringae* pv. *syringae*. The presence of random necrotic spots throughout the surface of the flower panicles (Fig. 1A) and a slight increase in the presence of necrosis of immature fruit in comparison with BAN symptoms (Fig. 1B and C) was observed. Necrosis on branches and random necrotic spots in leaves were also reported (Fig. 1D and E).

Bacterial isolation, characterization, and identification. Samples from different symptomatic mango tissues (leaf, stem, flower panicle, and immature fruit) were collected and analyzed and bacterial isolates were recovered. Bacterial isolation from diseased mango tissues yielded almost pure cultures of yellow colonies that were not fluorescent on KB under UV light. These yellow bacterial isolates ($n = 38$) showed similar biochemical and physiological features that are typical of species belonging to the genus *Pantoea* (Table 1). The indole production and nitrate reduction tests were used to discriminate among the species of the *Pantoea* isolates, identifying 33 as *Pantoea agglomerans*. The remaining five yellow isolates were identified as *P. ananatis* (Bt3; UMAF3070 to UMAF3074). With the exception of four strains isolated from mango trees (Bt2; UMAF3090, UMAF3094, UMAF3097, and UMAF3102), the five *P. ananatis* strains and the control strains 824-1 and 4188, the rest of the *P. agglomerans* strains produced a positive HR response at 24 h postinoculation in tobacco leaves (Bt1; $n = 29$). The HR elicitation suggests the pathogenic potential of these bacterial strains. Furthermore, six fluorescent strains on KB under UV light were also isolated (Bt4), characterized and identified as *Pseudomonas syringae*.

Pathogenicity tests revealed the pathogenic potential of *Pantoea agglomerans* strains isolated from mango trees. In both pathogenicity experiments, onion bulbs and apical buds from mango trees were selected and bacterial isolation and identification using biochemical and physiological tests was performed, which fulfilled Koch's postulates.

Ten *Pantoea agglomerans* strains were selected to perform the pathogenicity assay on onion bulbs. The selected *P. agglomerans* strains (with the exception of the strain UMAF3090) produced bulb rot symptoms in onion bulbs (Fig. 2) at 28 days postinoculation. *P. stewartii* subsp. *indologenes* CFBP3614 was included as positive control. No symptoms were detected in onion bulbs inoculated with the negative controls (phosphate buffer and two nonpathogenic strains of *P. agglomerans*, CECT850 and CFBP4740).

To fulfill Koch's postulates on mango trees, a pathogenicity test on 2-year-old mango plants was carried out with five selected *P. agglomerans* strains that were isolated from mango trees. Similar to the *Pseudomonas syringae* pv. *syringae* control strains used in this experiment, all of the inoculated strains induced necrotic symptoms in mango buds, and some did so on stems (Fig. 1F and G). *P. syringae* pv. *syringae* control strains produced visible necrotic

symptoms just 1 month after the inoculation, reaching disease incidence values of 42.8%. Two months after inoculation, *P. syringae* pv. *syringae* showed the highest disease incidence values (92.8%). Similarly, the first necrotic symptoms induced by *Pantoea agglomerans* strains appeared 2 months after the inoculation, reaching disease incidence values of 17.1%, indicating that there was a delay in the onset of necrotic symptoms with respect

Fig. 3. Plasmid profile and detection of *repA* and *hrpJ* genes by Southern blot hybridization. **A**, Plasmid profile of some *Pantoea agglomerans* strains isolated from mango that are representative of the diversity of plasmids found in the *P. agglomerans* population. *P. agglomerans* pv. *gypsophilae* control strain (Pag 824-1) that harbors two plasmids: a 60-kb plasmid and a plasmid belonging to the pPATH family of 135 kb. UMAF3067 and UMAF3084 harbor four plasmids: approximately 90, 140, 150, and 160 kb. UMAF3090 harbors two plasmids: approximately 110 and 140 kb. UMAF3093 harbors four plasmids: approximately 90, 140, 160, and 170 kb. UMAF3097 harbors one plasmid: approximately 150 kb. PCS = pathogenic control strain, Pal = phylogroup Pal, Pall = phylogroup Pall, and CDNA = chromosomal DNA. **B**, Southern blot hybridization of *repA* gene at high stringency, 55°C. **C**, Southern blot hybridization of *hrpJ* gene at low stringency, 45°C.

to the *Pseudomonas syringae* pv. *syringae* strains. A 100% disease incidence was reached at 3.5 months after the inoculation with *Pantoea agglomerans*. Apical buds inoculated with *P. agglomerans* CFBP4740 and phosphate buffer did not show any necrotic symptoms (Table 2).

Plasmids of *P. agglomerans* strains from mango trees do not belong to the pPATH family. *P. agglomerans* was previously described as having evolved to be a pathogen by the acquisition of pathogenicity plasmids from the pPATH family (Manulis and Barash 2003). Thus, the presence of plasmids was analyzed in the

P. agglomerans strains isolated from mango trees. Four plasmids were identified in 27 of 29 putatively pathogenic ($n = 23$) and pathogenic ($n = 4$) *P. agglomerans* strains (with approximate sizes of 90, 140, 150, and 160 kb), one of which was close in size to the pPATH pathogenicity plasmid (135 kb) of the control strain 824-1 used in this study. The other two strains, putatively pathogenic UMAF3092 and pathogenic UMAF3093, harbored four plasmids but with some differences in sizes (approximately 90, 140, 160, and 170 kb). The four putatively nonpathogenic *P. agglomerans* strains isolated from mango trees harbored two different plasmid profiles; UMAF3090 and UMAF3094 contain two plasmids (approximately 110 and 140 kb) and UMAF3097 and UMAF3102 carry only one plasmid (approximately 150 kb) (Fig. 3A).

PCR using specific primers to amplify *repA* were designed in this study onto the sequences of *repA* from strains 824-1 and 4188, as mentioned above. Amplicon electrophoresis revealed that the replication gene *repA* was only present in the putatively nonpathogenic mango strains UMAF3090 and UMAF3094, and the amplicon sequencing showed a high level of nucleotide identity (over than 96% in comparison with *repA* from strains 824-1 and 4188). Finally, Southern blot experiments using plasmid profiles gels of selected strains confirmed that the *repA* gene was only detected in UMAF3090 in the 140-kb plasmid (Fig. 3B).

Because pPATH plasmids have been described as harboring an important PAI that encodes the T3SS (Manulis and Barash 2003), among other genes, *P. agglomerans* strains isolated from mango trees were analyzed to investigate the presence of the *hrpJ* gene. The PCR analysis showed that all putatively and confirmed pathogenic *P. agglomerans* strains from mango trees ($n = 29$) tested were positive for the presence of an amplicon of the expected size, and the result with confirmed pathogenic strains correlates with their positive HR response on tobacco leaves. In contrast, the four putatively nonpathogenic *P. agglomerans* strains tested were negative. This PCR experiment was carried out using the primers designed by Weinthal et al. (2007). Because amplification of nonspecific bands was observed, an amplicon of the expected size was difficult to recover from agarose gels for all of the assayed strains. Some of the amplicons were recovered, purified, cloned, transformed in *E. coli* DH5 α , and sequenced. By using the specific primers designed in this study, which were based on previously obtained sequences, we obtained partial *hrpJ* gene-specific sequences for the 29 putative pathogenic and pathogenic strains of *P. agglomerans*. Again, the PCR amplification was negative for the four putatively nonpathogenic strains of *P. agglomerans*. Almost all *P. agglomerans* strains from mango trees yielded a positive PCR result for the presence of the *hrpJ* gene, showing a high degree of identity for the partial *hrpJ* gene sequence, except for UMAF3092 and UMAF3093, which showed small differences but were identical to one another (91.4% of identity). In addition, a greater difference was observed regarding the nucleotide identity of the partial *hrpJ* sequence from the 27 putatively pathogenic and pathogenic *P. agglomerans* strains from mango trees compared with

the pathogenic strains 824-1 and 4188 (77.1% of identity). Because the Southern blot analysis at 55°C only detected the presence of the *hrpJ* gene in the two selected pathogenic *P. agglomerans* strains isolated from mango trees (UMAF3067 and UMAF3084), we could not confirm in which of the four plasmids this gene was present. Southern blot experiments at 45°C confirmed the presence of the *hrpJ* gene in the 135-kb pPATH plasmid of the control strain 824-1, revealing the presence of this gene in the 140-kb plasmid in two selected pathogenic strains of *P. agglomerans* isolated from mango trees (UMAF3067 and UMAF3084). In pathogenic UMAF3093, this gene was detected in the 160-kb plasmid (Fig. 3C).

Genetic and phylogenetic analyses revealed a differentiated phylogroup of *P. agglomerans* strains isolated from mango trees. By using a DNA fingerprinting rep-PCR method with the BOXAIR primer, we observed that the generated dendrogram displayed a primary cluster with 27 of the putatively pathogenic and pathogenic *P. agglomerans* strains isolated from mango trees. The remaining putatively pathogenic UMAF3092, pathogenic UMAF3093, and putatively nonpathogenic (UMAF3090, UMAF3094, UMAF3097, and UMAF3102) *P. agglomerans* strains isolated from mango trees were distributed randomly along with the pathogenic and nonpathogenic *P. agglomerans* control strains (Fig. 4A).

Next, a phylogenetic analysis based on the *hrpJ* gene sequences was carried out, taking into account that the UMAF3090, UMAF3094, UMAF3097, UMAF3102, and *P. agglomerans* nonpathogenic control strains could not be utilized because they were negative for the presence of this gene by PCR. Two main phylogroups were generated, *hrpI* and *hrpII* (Fig. 4B). It is noteworthy that, inside the *hrpI* phylogroup, two subphylogroups arose, one that was composed of almost all *P. agglomerans* strains isolated from mango trees (the same 27 strains that clustered together using rep-PCR) and a second that was composed of the remaining putatively pathogenic and pathogenic strains, UMAF3092 and UMAF3093, respectively. Both subphylogroups contained different variants of the *hrpJ* gene. The *hrpII* phylogroup was composed of strains 824-1 and 4188, the control pathogenic strains included in this study.

After performing the genetic diversity and phylogenetic distribution analyses based on the *hrpJ* gene sequences, a phylogenetic analysis based on the *gyrB* and *rpoB* housekeeping gene sequences was performed that included all of the bacterial strains isolated from mango trees and all *Pantoea* control strains used in this work (Fig. 4C). The phylogenetic distribution generated two primary phylogroups, PaI and PaII. The PaI phylogroup was composed of 27 of the 29 putatively pathogenic and pathogenic (only 5 confirmed in mango) *P. agglomerans* strains from mango trees that were positive for the HR response and for the presence of the *hrpJ* gene. In addition, two putatively nonpathogenic *P. agglomerans* strains (UMAF3090 and UMAF3094), both positive for the presence of *repA* gene but negative for the HR response and the presence of *hrpJ* gene, were included in this phylogroup but in a separate branch. A second phylogroup (PaII) was primarily composed of pathogenic (824-1, 4188, and UMAF3093), putatively pathogenic (UMAF3092), and

Fig. 4. Genetic diversity and phylogenetic analysis of *Pantoea agglomerans* strains isolated from mango trees. **A**, Dendrogram cluster analysis obtained by a repetitive sequence-based polymerase chain reaction (PCR) fingerprinting method using a BOXA1-R primer, showing genetic relationships of *P. agglomerans* (Pa) strains isolated from mango trees along with pathogenic and nonpathogenic *P. agglomerans* strains isolated from other hosts. Jaccard's similarity coefficient was used to generate a tree with InfoQuest FP software (Bio-Rad), using the unweighted pair-group method with average linkages. The dotted-line box contains almost all pathogenic *P. agglomerans* strains isolated from mango trees. **B**, Phylogenetic analysis based on a partial sequence of the *hrpJ* gene obtained from *P. agglomerans* strains isolated from mango trees, including the pathogenic control strains *P. agglomerans* pv. *gypsophila* (Pag) 824-1 and *P. agglomerans* pv. *betae* (Pab) 4188. The neighbor-joining tree was constructed using MEGA 6, and bootstrap values (1,000 repetitions) are shown on branches. Evolutionary distances are in units of nucleotide substitutions per site. **C**, Phylogenetic analysis using partial sequences of the housekeeping genes *gyrB* and *rpoB*, including all *P. agglomerans* strains isolated from mango trees, pathogenic and nonpathogenic *P. agglomerans* strains isolated from other hosts, *P. stewartii* subsp. *indologenes* (Pst) CFBP3614, two *P. ananatis* strains isolated from mango trees (Pan UMAF3071 and Pan UMAF3074), *P. ananatis* PA13 from the database, and the six *Pseudomonas syringae* strains also isolated from mango trees in this study. The neighbor-joining tree was constructed with MEGA 6 using the concatenated sequences of both housekeeping genes. Bootstrap values (1,000 repetitions) are shown on branches and evolutionary distances are in units of nucleotide substitutions per site. HR = hypersensitive response, *hrpJ*sp (PCR) = PCR of the *hrpJ* gene with the specific primers designed in this study, and *repA*sp (PCR) = PCR of the *repA* gene with the specific primers designed in this study. Black box = positive result, white box = negative result, and gray box = positive result obtained with the *hrpJ* gene-specific primers (*hrpJ*5n1 and *hrpJ*3n1335) designed using the pPATH plasmid sequence from *P. agglomerans* pv. *gypsophila* 824-1.

nonpathogenic and putatively nonpathogenic (CECT850, CFPB 4740, UMAF3097, and UMAF3102) *P. agglomerans* strains isolated from mango trees and other hosts.

DISCUSSION

In this study, a new etiological agent of a bacterial necrotic disease has been described in mango crops in the Canary Islands. *Pseudomonas syringae* pv. *syringae* is the described causal agent of BAN disease of mango trees worldwide (Cazorla et al. 1998), which is the major limiting factor for the productivity of mango crop yields in the Mediterranean region (Cazorla et al. 1998, 2006; Gutiérrez-Barranquero et al. 2012). The necrotic symptoms produced by this new etiological agent are similar to those produced by *P. syringae* pv. *syringae*, with the main difference being that this bacterium can also affect immature fruit and flower panicles (Fig. 1). The nonfluorescent and yellow colonies isolated from diseased mango tissues from different years and orchards in the Canary Islands were consistently identified as *Pantoea agglomerans* on the basis of their physiological and biochemical profiles (Table 1) (Gavini et al. 1989).

Nucleotide sequence analysis of the 16S ribosomal RNA gene has been used to reveal taxonomic discrepancies in the *Pantoea* genus (Rezzonico et al. 2009) but the use of housekeeping genes has shown a better resolution to classify and identify different *Pantoea* spp. (Delétoile et al. 2009). In this study, the use of nucleotide sequences of the housekeeping genes *gyrB* and *rpoB* conclusively confirmed that the bacterial isolates from mango trees belonged to *P. agglomerans* species. Of the 33 *P. agglomerans* mango tree isolates, 29 were positive for the HR response, suggesting their potential as pathogenic bacteria, as was previously reported in *P. agglomerans* pv. *gypsophylae* (Nizan et al. 1997). Although *P. agglomerans* is a frequent epiphytic and endophytic bacterium (Walterson and Starvinides 2015), it has been reported to cause diseases in different plant hosts (Lu et al. 2015; Tho et al. 2015). In particular, it has been demonstrated that *P. agglomerans* has evolved to be a plant pathogen through the acquisition of pathogenicity plasmids of the so-called pPATH family (Manulis and Barash 2003). Plasmids in this family share the major replication protein RepA (Weinthal et al. 2007) with a high identity (over 98%) and are characterized by the presence of a PAI that includes genes involved in the production of phytohormones, the T3SS, and different pools of T3 effectors (Barash and Manulis-Sasson 2007, 2009; Manulis and Barash 2003). Interestingly, this bacterium has been previously reported as a possible mango pathogen in China, where it was observed to only produce necrotic lesions on leaves; however, the pathogenicity mechanisms remain to be elucidated (Lee and Tzeng 2006).

After the bacterial isolates in this study were identified as *P. agglomerans*, their role in the induction of necrotic symptoms in mango trees was analyzed. *P. agglomerans* has been previously described to cause bulb rot in onion in Georgia and Michigan (Edens et al. 2006; Tho et al. 2015). Thus, this pathosystem can serve as an initial and simpler approach to unravel the pathogenicity of *P. agglomerans* strains isolated from mango trees. All assayed *P. agglomerans* isolates from mango trees ($n = 10$, with the exception of UMAF3090) exhibited bulb rot symptoms, confirming that these isolates are pathogenic against onion (Fig. 2A). Subsequently, a pathogenicity assay in mango plants confirmed the role of a number of selected *P. agglomerans* strains in the induction of necrotic symptoms in mango buds similar to those produced by BAN (Fig. 2B). These results support *P. agglomerans* as a new etiological agent of a bacterial necrotic disease of mango trees in the Canary Islands. It was previously described that *Pseudomonas syringae* pv. *syringae* populations and disease severity are higher during cool and wet seasons (autumn and winter) and are lower in warm seasons in southern Spain (Cazorla et al. 1998). These data could support the delay and the development of necrotic symptoms produced by *Pantoea agglomerans* with respect to *Pseudomonas syringae* pv.

syringae during the pathogenicity test in mango trees, because it was performed in Malaga (mainland Spain). Moreover, this could explain the presence of *Pantoea agglomerans* rather than *Pseudomonas syringae* pv. *syringae* in the Canary Islands but not in southern Spain due to the milder environmental conditions. In particular, mango trees reach a dormancy period when the temperature is below 15°C (Samson 1986), which correlates with the minimum temperature in winter in southern Spain (specifically in Malaga), giving rise to the most destructive BAN symptoms. In the Canary Islands, the minimum temperature is rarely lower than 15°C. Thus, the development of BAN symptoms is unusual in the Canary Islands, and this could promote the emergence of alternative pathogens such as *Pantoea agglomerans*.

To shed light on the possible mechanisms involved in the pathogenicity of *P. agglomerans* strains isolated from mango trees, the presence of plasmids and the detection of relevant genes present on pPATH family plasmids were evaluated (Fig. 3). Some *P. agglomerans* strains isolated from mango trees that exhibited a positive HR response in tobacco leaves caused bulb rot in onion ($n = 10$) and produce necrotic symptoms in mango buds ($n = 4$) harbored the same plasmid profile, consisting of four different plasmids. The T3SS system present in pPATH plasmids is one of the major virulence factors of *P. agglomerans* (Nizan-Koren et al. 2003). By a PCR approach, those putatively pathogenic and pathogenic strains of *P. agglomerans* were all positive for the presence of the *hrpJ* gene. Interestingly, the pathogenic *P. agglomerans* strains from mango UMAF3067 and UMAF3084 were positive for the presence of the T3SS *hrpJ* gene in the 140-kb plasmid. However, these strains were negative for the presence of a unique gene shared by all pPATH plasmids, the replication gene *repA*. Thus, our results suggest that the 140-kb plasmid harbored by the pathogenic *P. agglomerans* strains isolated in this study was different than that belonging to the previously described pPATH pathogenicity plasmids, and the presence of the T3SS *hrpJ* gene could suggest a role for this plasmid in their pathogenicity. The presence of the pPATH *repA* gene was only demonstrated in the 140-kb plasmid of two strains (UMAF3090 and UMAF3094), which were negative for the HR response and negative for the presence of *hrpJ* gene; and UMAF3090, specifically, failed to elicit bulb rot in onion, resulting in these strains being considered putatively nonpathogenic. Despite the presence of the *repA* gene in these two bacterial strains, in pPATH plasmids, the T3SS is located in a pathogenicity island (Barash and Manulis-Sasson 2007) which could be complete or partially lost during their evolutionary history.

Subsequently, a genetic diversity analysis of *P. agglomerans* strains isolated from mango trees was performed using rep-PCR. Three different groups were generated that were composed of different *P. agglomerans* strains isolated from mango trees and other hosts. It is important to highlight the fact that rep-PCR provided an important subgroup differentiation, because 27 of the 29 putatively pathogenic and pathogenic *P. agglomerans* strains that share the same *hrpJ* gene variant clustered together (Fig. 4A). The band pattern obtained for these strains was identical, suggesting a clonal origin of this population associated potentially with the induction of necrotic symptoms in mango plants on the Canary Islands. rep-PCR was successfully used to differentiate the melon-infecting *P. ananatis* from other *Pantoea* spp., including *P. agglomerans* (Kido et al. 2008), and was recently used to reveal limited genetic diversity among *P. ananatis* strains causing bulb rot in onion (Stice et al. 2018). To determine whether the observed genetic clustering is consistent with the evolutionary history of putatively pathogenic and pathogenic *P. agglomerans* strains isolated from mango trees, a phylogenetic analysis was performed using the housekeeping *gyrB* and *rpoB* genes (Fig. 4C). Despite a previous report describing that the phylogenetic distribution of *P. agglomerans* was indistinguishable between clinical and environmental *P. agglomerans* isolates (Brady et al. 2008; Delétoile et al. 2009; Rezzonico et al. 2009), in this study, a primary phylogroup comprising 27 of the 29 putatively

pathogenic and pathogenic *P. agglomerans* strains was observed. This result showed a similar tendency to that observed previously in a genetic diversity analysis, suggesting a specific and different evolutionary history of these isolates than pathogenic and non-pathogenic *P. agglomerans* strains isolated from different hosts, which could be determined by the adaptation of this group to the mango host and the environmental conditions of this crop in the Canary Islands. In addition, a phylogenetic analysis based on the *hrpJ* gene sequences generated a main mango phylogroup that again included a subcluster containing 27 of the 29 putatively pathogenic and pathogenic *P. agglomerans* isolates. It is important to note that the *hrpJ* gene sequences were practically identical for each subcluster and were different between them, as was highlighted above. These results again show an identical tendency obtained previously with genetic and phylogenetic analyses. Interestingly, this may suggest that plasmids harbored by *P. agglomerans* strains isolated from mango trees came from a common ancestor and, after this acquisition event, they followed the same evolutionary history as their bacterial hosts, likely influenced by other factors during their adaptation to the mango host. In addition, it is of interest to note that the two remaining *P. agglomerans* strains isolated from mango (putatively pathogenic UMAF3092 and pathogenic UMAF3093) were different from the main 27 *P. agglomerans* strains at different levels, being more phylogenetically related to the original *P. agglomerans* cluster PaII.

Overall, these data support the idea that *P. agglomerans* is a new etiological agent that causes a bacterial necrotic disease associated with the mango tree host in the Canary Islands. Impressively, different levels of genetic characterization and phylogenetic distribution analyses revealed that these strains mostly form a differentiated and clonal phylogroup with respect to other pathogenic *P. agglomerans* strains that were previously described from other hosts

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