Pantoea agglomerans as a new etiological agent of a bacterial necrotic disease of mango trees

Authors: José A. Gutiérrez-Barranquero[1], Francisco M. Cazorla[1], Juan Antonio Torés[2], Antonio de Vicente[1]*

[1] Instituto de Hortofruticultura Subtropical y Mediterránea (IHSM-UMA-CSIC), Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain.

[2] IHSM-UMA-CSIC, Estación Experimental "La Mayora", 29750 Algarrobo-Costa (Málaga), Spain.

*Corresponding author: Antonio de Vicente Moreno
Email: adevicente@uma.es

ABSTRACT

Gutiérrez-Barranquero, J.A., Cazorla, F.M., Torés, J.A., de Vicente, A. 2018. *Pantoea agglomerans* as a new etiological agent of a bacterial necrotic disease of mango trees. Phytopathology: XX:XX-XX.

Bacterial apical necrosis of mango trees, a disease elicited by *Pseudomonas syringae* pv. *syringae*, is a primary limiting factor of the 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 5 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 4 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 to 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.

INTRODUCTION

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-Sauco 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. *mangiferaindicae* (Gagnevin and Pruvost 2001), and bacterial apical necrosis (BAN), caused by *Pseudomonas syringae* pv. *syringae* (Pss) (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 climate, such as the northwest region of Australia (Golzar and Cother 2008) and Pakistan (Naqvi et al. 2016). Unusually, only few reports have described the role of some *Enterobacteriaceae* 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 (Büyükcama et al. 2017; 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. *gypsophilae* 824-1 and *P. agglomerans* pv. *betae* 4188 (Pag 824-1 and Pab 4188 respectively), 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 Pag 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 only restricted to the pv. *gypsophilae* and *betae*, but it has also been described to incite the production of tumors (De Young et al. 1998; Opgenorth et al. 1994), and more frequently, necrotic rot and wilt symptoms in many other plant hosts (Gonzalez et al. 2015; Lee et al. 2010; Lu et al. 2015; Wiriyajitsomboon and Hausbeck 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 5 different years. Bacterial isolation identified bacterial strains other than Pss, the original etiological agent of BAN of mango trees. Thus, the main goal of 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 *P. agglomerans*, and the pathogenicity of 5 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 fruits, 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) $HgCl_2$ 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 10 ml of sterile phosphate buffer (0.1 M, pH = 7.2) per 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 LOPAT tests (levan production, oxidase reaction, potato soft rot, arginine dihydrolase (ADH) and tobacco hypersensitivity response (HR)), excluding the potato soft rot analysis. HR elicitation in resistant and non-host plants such as tobacco is generally-dependent of T3SS in Gram-negative bacterial pathogens (Lindgren 1997), as it has also been reported in *P. agglomerans* (Nizan et al. 1997).

Subsequently, indole production and nitrate reduction assays were performed. All 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 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 if 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. Twenty microliters of 3×10^7 CFU/ml bacterial suspensions in phosphate buffer was 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 (2 bacterial strains per year: Pa UMAF3066, Pa UMAF3067, Pa UMAF3075, Pa UMAF3078, Pa UMAF3084, Pa UMAF3086, Pa UMAF3090, Pa UMAF3093, Pa UMAF3098 and Pa UMAF3101), including one that was negative for HR response (Pa UMAF3090) and one that was isolated from another location, La Palma (Pa UMAF3098) were assayed. Similarly, two strains of *P. agglomerans* (CECT 850 and CFBP 4740), *P. stewartii* subsp. *indologenes* CFBP 3614 (Supplementary Table S2) and phosphate buffer were used as controls. Re-isolation 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 thirty 2-year-old mango plants growing in pots. Buds of mango plants were inoculated with 20 µl of a 3×10^7 CFU/ml of a bacterial suspension in phosphate buffer, with the inoculum forced into buds with a sterile microsyringe as previously described (Cazorla et al. 1998). Five representative *P. agglomerans* strains isolated from mango trees selected by year of isolation from the previous ten bacterial strains used in onion bulbs (Pa UMAF3067, Pa UMAF3075, Pa UMAF3084, Pa UMAF3093 and Pa UMAF3098), were used for this experiment. In addition, two *P. syringae* pv. *syringae* strains (Pss UMAF0048 and UMAF0049), both producers of BAN symptoms in mango trees, were used as positive controls, and *P. 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). Re-isolation 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 LB 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. *gypsophilae* 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).

PCR 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 isolated from mango trees, specific primers designed in this study using the *repA* sequences of Pag 824-1 and Pab 4188 (GenBank accession number: EF173387.1 and EF173388.1, respectively) were used for 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 (Pa UMAF3067, Pa UMAF3075, Pa UMAF3084, Pa UMAF3093, and Pa UMAF3098) as well as Pab 4188 and Pag 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* DH5α cells. Purified plasmids were used to sequence the insert DNA region. Nucleotide sequences were determined at Macrogen. Afterwards, the specific primers designed in this study (hrpJPaF: 5′-GACCTTCGCGGAACAGGTTG-3′ and hrpJPaR: 5′-CGGCAGATGGTAGACCAG-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.

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 Pag 824-1 was included as positive control. Plasmid DNA extraction gels, which included Pag 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, Basel, Switzerland), and immunological detection was performed with an antidigoxigenin antibody conjugated to alkaline phosphatase. The DIG-labeled nucleic acids were detected by chemiluminescence using a Molecular Imager ChemiDoc system (Bio-Rad). Pre-hybridization and hybridization at high-stringency (55°C) was performed for both the *hrpJ* and *repA* genes, and low-stringency (45°C) was conducted 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'-CTACGGCAAGGCGACGCTGACG-3'). PCR amplifications were performed with a C1000 Thermal Cycler (Bio-Rad, UK). PCR amplification conditions are summarized in Supplementary Table S3. The total volume of each PCR reaction (25 µl) were loaded onto a 1.2 % agarose gel and run in 1× 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 (UPGMA). Gel normalization and background subtraction were performed as previously described (Rademaker et al. 1998).

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 (Seoul, Korea) directly from purified PCR products amplified with the primer sets gyrB3i (5′-AACGCWATCGACGAAGC-3′) and gyrB4i (5′-TGGAAVCCRTCRTTCCAC-3′) and Vic3 (5′-GGCGAA ATGGCWGAGAACCA-3′) and Vic2 (5′-GAGTCTTCGAAGTTGTAACC-3′) for *gyrB* and *rpoB*, respectively (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 1000 bootstrap replicates. Pag 824-1, Pab 4188, and other *P. agglomerans* control strains used 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 NCBI under the following accession numbers: *gyrB* sequences (MH479140 - MH479183, MH643873, MH643874 and NC_017554.1), *rpoB* sequences (MH479184 - MH479227, MH643875, MH643876 and NC_017554.1), *hrpJ* sequences (MH479228 - MH479258) and *repA* sequences (MH479259 - MH479260).

RESULTS

Description of symptomatology. From different samplings carried out in the Canary Islands, between February and March (2005- 2009), the presence of necrotic symptoms was not primarily associated with Pss 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 Pss. The presence of random necrotic spots through all the surface of the flower panicles (Fig. 1A) and a slight increase in the presence of necrosis of immature fruits in comparison with BAN symptoms (Fig. 1B and 1C) was observed. Necrosis on branches and random necrotic spots in leaves were also reported (Fig. 1D and 1E).

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 *P. agglomerans*. The remaining 5 yellow isolates were identified as *Pantoea ananatis* (Bt3, from Pan UMAF3070 to Pan UMAF3074). With the exception of 4 strains isolated from mango trees (Bt2, Pa UMAF3090, Pa UMAF3094, Pa UMAF3097, and Pa UMAF3102), the five *P. ananatis* strains and the control strains Pag 824-1 and Pab 4188, the rest of *P. agglomerans* strains produced a positive HR response at 24 h post-inoculation in tobacco leaves (Bt1, n= 29). The HR elicitation suggests the pathogenic potential of these bacterial strains. Furthermore, 6 fluorescent strains on KB under UV light were also isolated (Bt4), characterized and identified as *Pseudomonas syringae*.

Pathogenicity tests revealed the pathogenic potential of *P. 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 *P. agglomerans* strains were selected to perform the pathogenicity assay on onion bulbs. The selected *P. agglomerans* strains (with the exception of the strain Pa UMAF3090) produced bulb rot symptoms in onion bulbs (Fig. 2) at 28 days post-inoculation. *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 non-pathogenic 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 Pss 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, G). Pss control strains produced visible necrotic symptoms just one month after the inoculation, reaching disease incidence values of 42.8 %. Two months after inoculation, Pss showed the highest disease incidence values (92.8 %). Similarly, the first necrotic symptoms induced by *P. 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 to the Pss strains. A 100 % disease incidence was reached at 3.5 months after the inoculation with *P. 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 out 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 Pag 824-1 control strain used in this study. The other two strains, putatively pathogenic Pa UMAF3092 and pathogenic Pa UMAF3093, harbored 4 plasmids but with some differences in sizes (approximately 90, 140, 160 and 170 kb). The four putatively non-pathogenic *P. agglomerans* strains isolated from mango trees harbored two different plasmid profiles; Pa UMAF3090 and Pa UMAF3094 contain two plasmids (110 and 140 kb approximately), and Pa UMAF3097 and Pa UMAF3102 carry only one plasmid (150 kb approximately) (Fig. 3A).

PCR using specific primers to amplify *repA* were designed in this study onto the sequences of *repA* from Pag 824-1 and Pab 4188 as mentioned above. Amplicon electrophoresis revealed that the replication gene *repA* was only present in the putatively non-pathogenic mango strains Pa UMAF3090 and Pa UMAF3094, and the amplicon sequencing showed a high level of nucleotide identity (over 96 % in comparison with *repA* from Pag 824-1 and Pab 4188). Finally, southern-blot experiments using plasmid profile gels of selected strains confirmed that the *repA* gene was only detected in Pa 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 4 putatively non-pathogenic *P. agglomerans* strains tested were negative. This PCR experiment was carried out using the primers designed by Weinthal et al. (2007). Because amplification of non-specific bands was observed, an amplicon of the expected size was difficult to recover from agarose gels for all the assayed strains. Some of the amplicons were recovered, purified, cloned, transformed in *E. coli* DH5α 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 non-pathogenic 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 Pa UMAF3092 and Pa 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 *hprJ* sequence from the 27 putatively pathogenic and pathogenic *P. agglomerans* strains from mango trees compared with the pathogenic strains Pag 824-1 and Pab 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 (Pa UMAF3067 and Pa 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 Pag 824-1 control strain, revealing the presence of this gene in the 140 kb plasmid in two selected pathogenic strains of *P. agglomerans* isolated from mango trees (Pa UMAF3067 and Pa UMAF3084). In pathogenic Pa 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 BOXA1R 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 Pa UMAF3092, pathogenic Pa UMAF3093, and putatively non-pathogenic (Pa UMAF3090, Pa UMAF3094, Pa UMAF3097 and Pa UMAF3102) *P. agglomerans* strains isolated from mango trees were distributed randomly along with the pathogenic and non-pathogenic *P. agglomerans* control strains (Fig. 4A).

Next, a phylogenetic analysis based on the *hrpJ* gene sequences was carried out, taking into account that the Pa UMAF3090, Pa UMAF3094, Pa UMAF3097, Pa UMAF3102 and *P. agglomerans* non-pathogenic controls strains could not be utilized since 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 sub-phylogroups 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, Pa UMAF3092 and Pa UMAF3093 respectively. Both sub-phylogroups, contained different variants of the *hrpJ* gene. The hrpII phylogroup was composed of Pag 824-1 and Pab 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 the bacterial strains isolated from mango trees (Supplementary Table S1) 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 non-pathogenic *P. agglomerans* strains (Pa UMAF3090 and Pa 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 (Pag 824-1, Pab 4188, Pa UMAF3093), putatively pathogenic (Pa UMAF3092) and non-pathogenic and putatively non-pathogenic (Pa CECT850, Pa CFPB 4740, Pa UMAF3097 and Pa 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. Pss 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; Gutierrez-Barranquero et al. 2012). The necrotic symptoms produced by this new etiological agent are similar to those produced by Pss, with the main difference being that this bacterium can also affect immature fruits and flower panicles (Fig. 1). The non-fluorescent and yellow colonies isolated from diseased mango tissues from different years and orchards in the Canary Islands were consistently identified as *P. agglomerans* on the basis of their physiological and biochemical profiles (Table 1) (Gavini et al. 1989).

Nucleotide sequence analysis of the 16S rRNA 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* species (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. Twenty nine out of the 33 *P. agglomerans* mango tree isolates were positive for the HR response, suggesting their potential as pathogenic bacteria, as it has been previously reported in *P. agglomerans* pv. *gypsophilae* (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; Wiriyajitsomboon and Hausbeck 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, but 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 onions 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 Pa UMAF3090) exhibited bulb rot symptoms, confirming that these isolates are pathogenic against onions (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 Pss 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 *P. agglomerans* with respect to Pss during the pathogenicity test in mango trees, because it was performed in Malaga (mainland Spain). Moreover, this could explain the presence of *P. agglomerans* rather than Pss 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 *P. 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 was evaluated (Fig. 3). Some of *P. agglomerans* strains isolated from mango trees that exhibited a positive HR response in tobacco leaves, caused bulb rot in onions (n=10) and produce necrotic symptoms in mango buds (n=4) harbored the same plasmid profile consisting of 4 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 PCR approach, those putatively pathogenic and pathogenic strains of *P. agglomerans* were all positive for the presence of the T3SS *hrpJ* gene. Interestingly, the pathogenic *P. agglomerans* strains from mango Pa UMAF3067 and Pa 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 (Pa UMAF3090 and Pa UMAF3094), which were negative for the HR response, negative for the presence of *hrpJ* gene, and specifically Pa UMAF3090 failed to elicit bulb rot in onions, resulting in these strains being considered putatively non-pathogenic. Despite the presence of *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 completely 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 that rep-PCR provided an important sub-group differentiation, as 27 out 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* species, including *P. agglomerans* (Kido et al. 2008), and was recently used to reveal limited genetic diversity among *P. ananatis* strains causing bulb rot in onions (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 comprised of 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, that could be leading to the adaption 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 sub-cluster 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 sub-cluster and were different between them as 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 Pa UMAF3092 and pathogenic Pa 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 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

ACKNOWLEDGMENTS

This work has been supported by grants from CICE-Junta de Andalucía, Proyecto de Excelencia (P07-AGR-02471; P12-AGR-1473) cofinanced by FEDER (EU). The authors would like to thank Shulamit Manulis-Sasson and Isaac Barash for kindly providing the pathogenic strains *P.*

agglomerans pv. gypsophilae 824-1 and *P. agglomerans* pv. betae 4188. The authors also thank specially Domingo Fernández Galván and Eduardo García for their help in sampling.

LITERATURE CITED

Aiello, D., Ferrante, P., Vitale, A., Polizzi, G., Scortichini, M., and Cirvilleri, G. 2015. Charactertization of *Pseudomonas syringae* pv. *syringae* isolated from mango in Sicily and occurrence of copper-resistant strains. J. Plant Pathol. 97: 273-282.

Barash, I., and Manulis-Sasson, S. 2007. Virulence mechanisms and host specificity of gall forming *Pantoea agglomerans*. Trends Microbiol. 15: 538-545.

Barash, I., and Manulis-Sasson, S. 2009. Recent evolution of bacterial pathogens: the gall forming *Pantoea agglomerans* case. Annu. Rev. Phytopathol. 47: 133-152.

Bradbury, J.F. 1986. Guide to Plant Pathogenic Bacteria. CAB International, Farnham Royal, Slough, UK.

Brady, C., Cleenwerck, I., Venter, S., Vancanneyt, M., Swings, J., and Coutinho, T. 2008. Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Syst. Appl. Microbiol. 31: 447-460.

Burr, T.J., Katz, B.H., Abawi, G.S., and Crosier, D.C. 1991. Comparison of tumorigenic strains of *Erwinia hebicola* isolated from table beet with *E. h. gypsophilae*. Plant Dis. 75: 855-858.

Büyükcam, A., Tuncer, Ö., Gür, D., Sancak, B., Ceyhan, M., Cengiz, A.B., and Kara, A. 2018. Clinical and microbiological characteristics of *Pantoea agglomerans* infection in children. J. Infect. Public Health. 11: 304-309.

Cazorla, F.M., Arrebola, E., Olea, F., Velasco, L., Hermoso, J.M., Pérez-García, A., Torés, J.A., Farré, J.M. and de Vicente, A. 2006. Field evaluation of treatments for the control of the bacterial apical necrosis of mango (*Mangifera indica*) caused by *Pseudomonas syringae* pv. *syringae*. Eur. J. Plant Pathol. 116:279-288.

Cazorla, F.M., Torés, J.A., Olalla, L., Pérez-García, A., Farré, J.M., and deVicente, A. 1998. Bacterial apical necrosis of mango in Southern Spain: a disease caused by *Pseudomonas syringae* pv. *syringae*. Phytopathology. 88:614-620.

Cheng, A., Liu, C.Y., Tsai, H.Y., Hsu, M.S., Yang, C.J., Huang, Y.T., Liao, C.H., and Hsueh, P.R. 2013. Bacteremia caused by *Pantoea agglomerans* at a medical center in Taiwan, 2000-2010. J. Microbiol. Immunol. Infect. 46: 187-194.

Cooksey, D.A. 1986. Galls of *Gypsophila paniculata* caused by *Erwinia herbicola*. Plant Dis. 70: 4648.

Cruz, A.T., Cazacu, A.C., and Allen, C.H. 2007. *Pantoea agglomerans*, a plant pathogen causing human disease. J. Clin. Microbiol. 45: 1989-1992.

De Young, R.M., Copeman, R.J., and Hunt, R.S. 1998. Two strains in the genus *Erwinia* cause galls on Douglas fir in Southern British Columbia. Can. J. Plant Pathol. 20: 194-200.

Delétoile, A., Decré, D., Courant, S., Passet, V., Audo, J., Grimont, P., Arlet, G., and Brisse, S. 2009. Phylogeny and identification of *Pantoea* species and typing of *Pantoea agglomerans* strains by multilocus gene sequencing. J. Clin. Microbiol. 47: 300-310.

Edens, D.G., Gitaitis, R.D., Sanders, F.H., and Nischwitz, C. 2006. First report of *Pantoea agglomerans* causing a leaf blight and bulb rot of onions in Georgia. Plant Dis. 90: 1551.

Gagnevin, L., and Pruvost, O. 2001. Epidemiology and control of mango bacterial black spot. Plant Dis. 85: 928-935.

Galán-Saúco, V. 2015. Current situation and future prospects of worldwide mango production and market. Acta Hortic. 1066: 69-84.

Gavini, F., Mergaert, J., Beji, A., Mielcarek, C., Izard, D., Kersters, K., and De Ley, J. 1989. Transfer of *Enterobacter agglomerans* (Beijerinck 1888) Ewing & Fife 1972 to *Pantoea* gen. nov. as *Pantoea agglomerans* comb. nov. and description of *Pantoea dispersa* sp. nov. Int. J. Syst. Bacteriol. 39: 337-345.

Golzar, H., and Cother, E. 2008. First report of bacterial necrosis of mango caused by *Pseudomonas syringae* pv. *syringae* in Australia. Australas. Plant Dis. Notes. 3:107-109.

González, A.D., Franco, M.A., Contreras, N., Galindo-Castro, I., Jayaro, Y., and Graterol, E. 2015. First report of *Pantoea agglomerans* causing rice leaf blight in Venezuela. Plant Dis. 99: 552.

Guevara, Y., Rondón, A., and Solórzano, R. 1980. Bacteriosis del mango (*Mangifera indica* L.) en Venezuela. I. Sintomatología e identificación. Agronomía Tropical. 30: 65-76.

Gutiérrez-Barranquero, J.A., Arrebola, E., Bonilla, N., Sarmiento, D., Cazorla, F.M., and de Vicente A. 2012. Environmentally friendly treatment alternatives to Bordeaux mixture for controlling bacterial apical necrosis (BAN) of mango. Plant Pathol. 61: 665-676.

Jacobs, J.L., Fasi, A.C., Ramette, A., Smith, J.J., Hammerschmidt, R., and Sundin, G.W. 2008. Identification and onion pathogenicity of *Burkholderia cepacia* complex isolates from the onion rhizosphere and onion field soil. Appl. Environ. Microbiol. 74: 3121-3129.

Kido, K., Adachi, R., Hasegawa, M., Yano, K., Hikichi, Y., Takeuchi, S., Atsuchi, T., and Takikawa, Y. 2008. Internal fruit rot of netted melon caused by *Pantoea ananatis* (=*Erwinia ananas*) in Japan. J. Gen. Plant Pathol. 74: 302-312.

Kim, Y., Lee, S., Choi, C., Lee, S., and Lee, S. 2002. Soft rot of onion bulbs caused by *Pseudomonas marginalis* under low temperature storage. Plant Pathol. J. 18: 199-203.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.

Lee, H.B., Hong, J.P., and Kim, S.B. 2010. First report of leaf blight caused by *Pantoea agglomerans* on rice in Korea. Plant Dis. 94: 1372.

Lee, M.H., and Tzeng, D.D.S. 2006. *Pantoea agglomerans* MB-9 is a potential pathogen causing necrosis on mango leaf. Plant Pathol. Bull. 15: 63-68.

Lelliott, R.A., and Stead, D.E. 1987. Methods for the diagnosis of bacterial diseases of plants. Pages 44-56 In: Methods in Plant Pathology, vol 2. Preece T.F., ed. Blackwell Scientific Publications, Oxford, UK.

Lindgren, P.B. 1997. The role of *hrp* genes during plant-bacterial interactions. Annu. Rev. Phytopathol. 35: 129-152.

Lu, B.H., Jia, M.J., and Gao, J. 2015. First report of *Pantoea agglomerans* causing Bacterial leaf blight on *Vigna angularis* in Jilin Province, China. Plant Dis. 2015 99: 1269.

Manulis, M., and Barash, I. 2003. *Pantoea agglomerans* pvs. *gypsophilae* and *betae*, recently evolved pathogens?. Mol. Plant Pathol. 4: 307-314.

Manulis, M., Gafni, Y., Clark, E., Zutra, D., Ophir, Y., and Barash, I. 1991. Identification of a plasmid DNA probe for detection of strains of *Erwinia herbicola* pathogenic on *Gypsophila paniculata*. Phytopathology. 81: 54-57.

McMillan, R.T., and Wang, A. 1992. A new disease of mango in Costa Rica caused by *Erwinia*-like bacteria. Proc. Fl. St. Hortic. Soc. 105: 288-289.

Moulton, J.L., Vivian, A., Hunter, P.J., and Taylor, J.D. 1993. Changes in cultivar-specificity toward pea can result from transfer of plasmid RP4 and other incompatibility group P1 replicons to *Pseudomonas syringae* pv. *pisi*. J. Gen. Microbiol. 139: 3149-3155.

Naqvi, S.A.H., Perveen, R., Rehman, A.U., Khan, T., Malik, M.T., Chohan, S., Tariq, A., and Abbas Pak, S.H. 2016. Outbreak of bacterial apical necrosis of mango in Multan, Punjab, Pakistan. Pak. J. Phytopathol. 28: 107-113.

Nizan, R., Barash, I., Valinsky, L., Litcher, A., and Manulis, S. 1997. The presence of *hrp* genes on the pathogenicity associated plasmid of the tumorigenic bacterium *Erwinia herbicola* pv. *gypsophilae*. Mol. Plant-Microbe Interact. 5: 667-682.

Nizan-Koren, R., Manulis, S., Mor, H., Iraki, N., and Barash, I. 2003. The regulatory cascade that activates the Hrp regulon in *Erwinia herbicola* pv. *gypsophilae*. Mol. Plant-Microbe Interact. 15: 1249-1260.

Opgenorth, D.C., Hendson, T.M., and Clark, E. 1994. First report of a bacterial gall of a *Wisteria sinensis* caused by *Erwinia herbicola* pv. *milletiae* in California. Plant Dis. 78: 1217.

Pinkas, Y., Maymon, M., and Smolewich, Y. 1996. Bacterial black blight of mango. Alon Hanotea 50:475.

Rademaker, J.L.W., Louws, F.J., Versalovic, J., and de Bruijn, F.J. 2004. Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting. Pages 1-33 In: Molecular Microbial Ecology Manual, Second Edition. Kowalchuk, G.A., de Bruijn, F.J., Head, I.M., Akkermans, A.D., van Elsas, J.D., eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Rezzonico, F., Smits, T.H., Montesinos, E., Frey, J. E., and Duffy, B. 2009. Genotypic comparison of *Pantoea agglomerans* plant and clinical strains. BMC Microbiol. 9: 204.

Romero, D., de Vicente, A., Olmos, J.L., Davila, J.C., and Pérez-García, A. 2007. Effect of lipopeptides of antagonistic strains of *Bacillus subtilis* on the morphology and ultrastructure of the cucurbit fungal pathogen *Podosphaera fusca*. J. Appl. Microbiol. 103: 969-976.

Samson, J.A. 1986. Tropical fruits. Second edition. Logman Scientific & Technical, Essex, New York.

Stice, S.P., Stumpf, S.D., Gitaitis, R.D., Kvitko, B.H., and Dutta, B. 2018. *Pantoea ananatis* genetic diversity analysis reveals limited genomic diversity as well as accessory genes correlated with onion pathogenicity. Front. Microbiol. 9: 184.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30: 2725-2729.

Tho, K.E., Wiriyajitsomboon, P., and Hausbeck, M.K. 2015. First report of *Pantoea agglomerans* causing onion leaf blight and bulb rot in Michigan. Plant Dis. 2015 99: 1034.

Trantas, E.A., Mpalantinaki, E., Pagoulatou, M., Markakis, E., Sarris, P.F., Ververidis, F., and Goumas, D.E. 2017. First report of bacterial apical necrosis of mango caused by *Pseudomonas syringae* pv. *syringae* in Greece. Plant Dis. 101: 1541.

Walterson, A.M., and Starvinides, J. 2015. *Pantoea*: insights into a highly versatile and diverse genus within the Enterobacteriaceae. FEMS Microbiol. Rev. 39: 968-984.

Weinthal, D.M., Barash, I., Panijel, M., Valinsky, L., Gaba, V., and Manulis-Sasson, S. 2007. Distribution and replication of the pathogenicity plasmid pPATH in diverse populations of the gall-forming bacterium *Pantoea agglomerans*. Appl. Environ. Microbiol. 73:7552-7561.

Yang, Q.K., Qu, W.W., Liu, X., Liu, H.X., and Hou, L.Q. 2011. First report of *Pantoea agglomerans* causing brown apical necrosis of Walnut in China. Plant Dis. 95: 773.

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. For isolation details of bacterial isolates see Supplementary Table S1

	Physiological and biochemical profiles of bacterial isolates			
Test	Bt 1[a]	Bt2	Bt 3	Bt 4
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 (ADH)	-	-	-	-
Tobacco hypersensitivity	+	-	-	+
Identification	*Pantoea* spp.	*Pantoea* spp.	*Pantoea* spp.	*Pseudomonas syringae*
Indole production	-	-	+	-
Nitrate reduction	+	+	-	-
Number of isolates	29	4	5	6
Identification	*P. agglomerans*	*P. agglomerans*	*P. ananatis*	*Pseudomonas syringae*

[a] Bt= Bacterial type.

[b] Metabolism of glucose: O = oxidative, and F = fermentative.

[c] Growth on yeast extract-dextrose-calcium carbonate agar: W= white colonies, and Y= yellow mucoid colonies.

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

	Disease incidence (%)					
Bacterial isolates	28 days	42 days	56 days	70 days	84 days	98 days
Pss (n=2)[a]	42.8	64.2	92.8	92.8	92.8	92.8
Pa (n=5)[b]	0	0	17.1	42.8	80	100
Pa CFBP4740[c]	0	0	0	0	0	0
Phosphate buffer	0	0	0	0	0	0

[a] Pss: *Pseudomonas syringae* pv. *syringae*. Bacterial strains inoculated: UMAF0048 and UMAF0049.

[b] Pa: *P. agglomerans* strains isolated from mango. Bacterial strains inoculated: Pa UMAF3067, Pa UMAF3075, Pa UMAF3084, Pa UMAF3093 and Pa UMAF3098.

[c] *P. agglomerans* CFBP 4740, non-pathogenic control strain.

FIGURE LEGENDS

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 fruits; **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*.

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

Fig. 3. Plasmid profile and detection of *repA* and *hrpJ* genes by southern-blot hybridization. **A,** Plasmid profile of some *P. agglomerans* strains isolated from mango that are representative of the diversity of plasmids found in the *P. agglomerans* population. Pag 824-1: *P. agglomerans* pv. *gypsophilae* control strain that harbors two plasmids: 60 kb plasmid and a plasmid belonging to the pPATH family of 135 kb. Pa UMAF3067 and Pa UMAF3084 harbor 4 plasmids: 90 kb, 140 kb, 150 kb and 160 kb approximately. Pa UMAF3090 harbors two plasmids: 110 kb and 140 kb approximately. Pa UMAF3093 harbors 4 plasmids: 90 kb, 140 kb, 160kb and 170 kb approximately. Pa UMAF3097 harbors one plasmid: 150 kb approximately; **B,** Southern blot hybridization of *repA* gene at high-stringency, 55 ºC; **C,** Southern blot hybridization of *hrpJ* gene at low-stringency, 45 ºC. PCS: Pathogenic control strain. PaI: Phylogroup PaI. PaII: Phylogroup PaII. CDNA: Chromosomal DNA.

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 fingerprinting method using a BOXA1-R primer, showing genetic relationships of *P. agglomerans* strains isolated from mango trees along with pathogenic and

non-pathogenic *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. *gypsophilae* 824-1 and *P. agglomerans* pv. *betae* 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 non-pathogenic *P. agglomerans* strains isolated from other hosts, *P. stewartii* subsp. *indologenes* CFBP3614, 2 *P. ananatis* strains isolated from mango trees (Pan UMAF3071 and Pan UMAF3074), *P. ananatis* PA13 from the database, and the 6 *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, *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 (hrpj5n1 and hrpj3n1335) designed using the pPATH plasmid sequence from Pag 824-1. Pa: *Pantoea agglomerans*; Pan: *Pantoea ananatis*; Pst: *Pantoea stewartii* subsp. *indologenes*; Pag 824-1: *Pantoea agglomerans* pv. *gypsophilae* 824-1; and Pab 4188: *Pantoea agglomerans* pv. *betae* 4188.

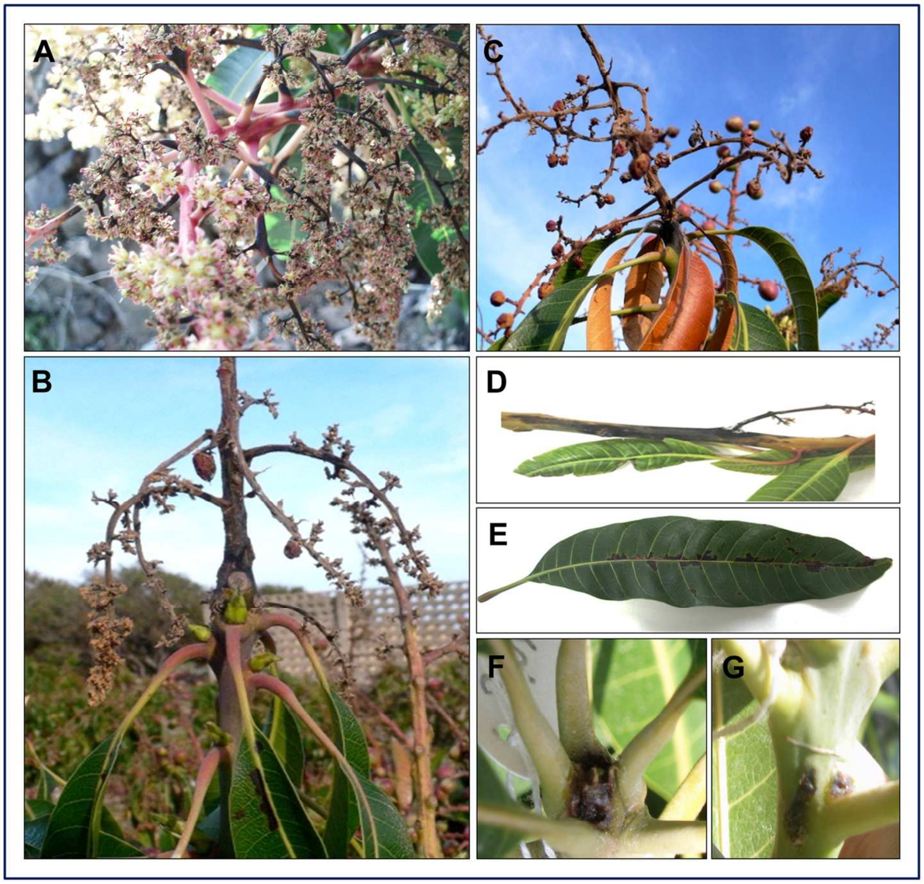

Fig. 1. Necrotic symptoms on mango trees in Canary Islands. A, Initial necrosis of flower panicles; B, Advanced necrosis of flower panicles; C, Necrosis of immature fruits; D, Necrotic symptoms in branches; E, Necrotic spots in leaves; F, Detail of necrotic symptoms in mango bud, and G, Necrotic symptoms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

143x137mm (300 x 300 DPI)

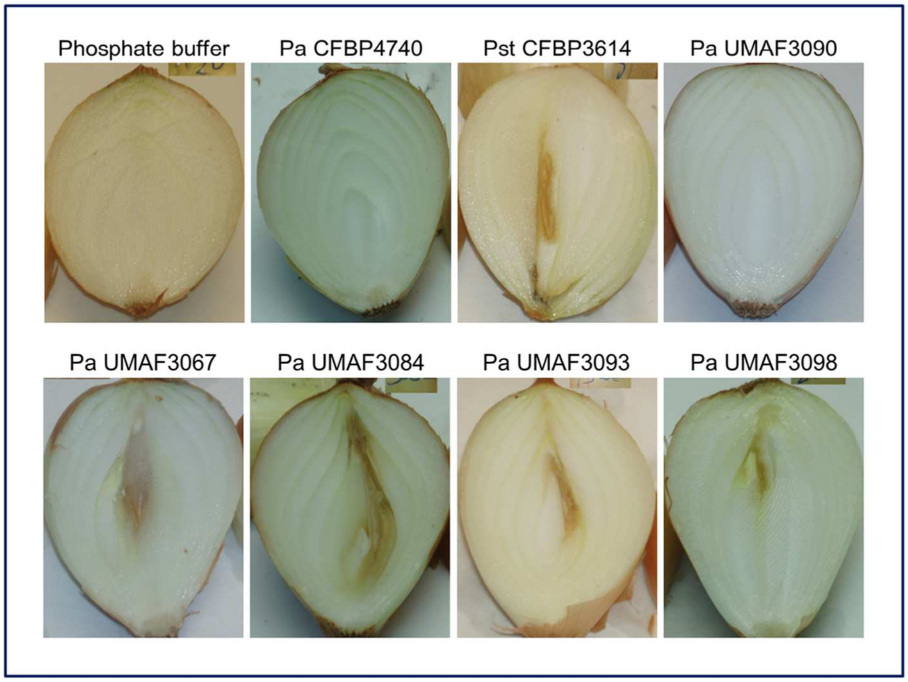

Fig. 2. Pathogenicity test in onion bulbs using selected *Pantoea agglomerans* strains. Ten strains of *P. agglomerans* isolated from mango were tested (Pa UMAF3066, Pa UMAF3067, Pa UMAF3075, Pa UMAF3078, Pa UMAF3084, Pa UMAF3086, Pa UMAF3090, Pa UMAF3093, Pa UMAF3098 and Pa UMAF3101). Just five representative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

115x88mm (300 x 300 DPI)

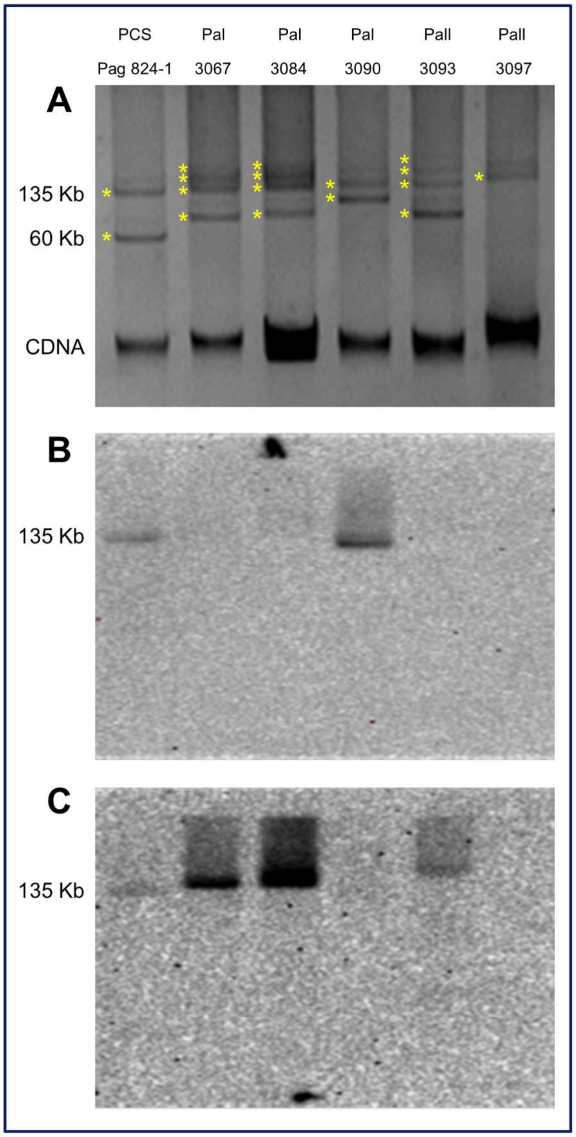

Fig. 3. Plasmid profile and detection of *repA* and *hrpJ* genes by southern-blot hybridization. A, Plasmid profile of some *P. agglomerans* strains isolated from mango that are representative of the diversity of plasmids found in the *P. agglomerans* population. Pag 824-1: *P. agglomerans* pv. gypsophilae control strain that harbors two plasmids: 60 kb plasmid and a plasmid belonging to the pPATH family of 135 kb. Pa UMAF3067 and Pa UMAF3084 harbor 4 plasmids: 90 kb, 140 kb, 150 kb and 160 kb approximately. Pa UMAF3090 harbors two plasmids: 110 kb and 140 kb approximately. Pa UMAF3093 harbors 4 plasmids: 90 kb, 140 kb, 160kb and 170 kb approximately. Pa UMAF3097 harbors one plasmid: 150 kb approximately; B, Southern blot hybridization of *repA* gene at high-stringency, 55 ºC; C, Southern blot hybridization of *hrpJ* gene at low-stringency, 45 ºC. PCS: Pathogenic control strain. PaI: Phylogroup PaI. PaII: Phylogroup PaII. CDNA: Chromosomal DNA.

168x316mm (300 x 300 DPI)

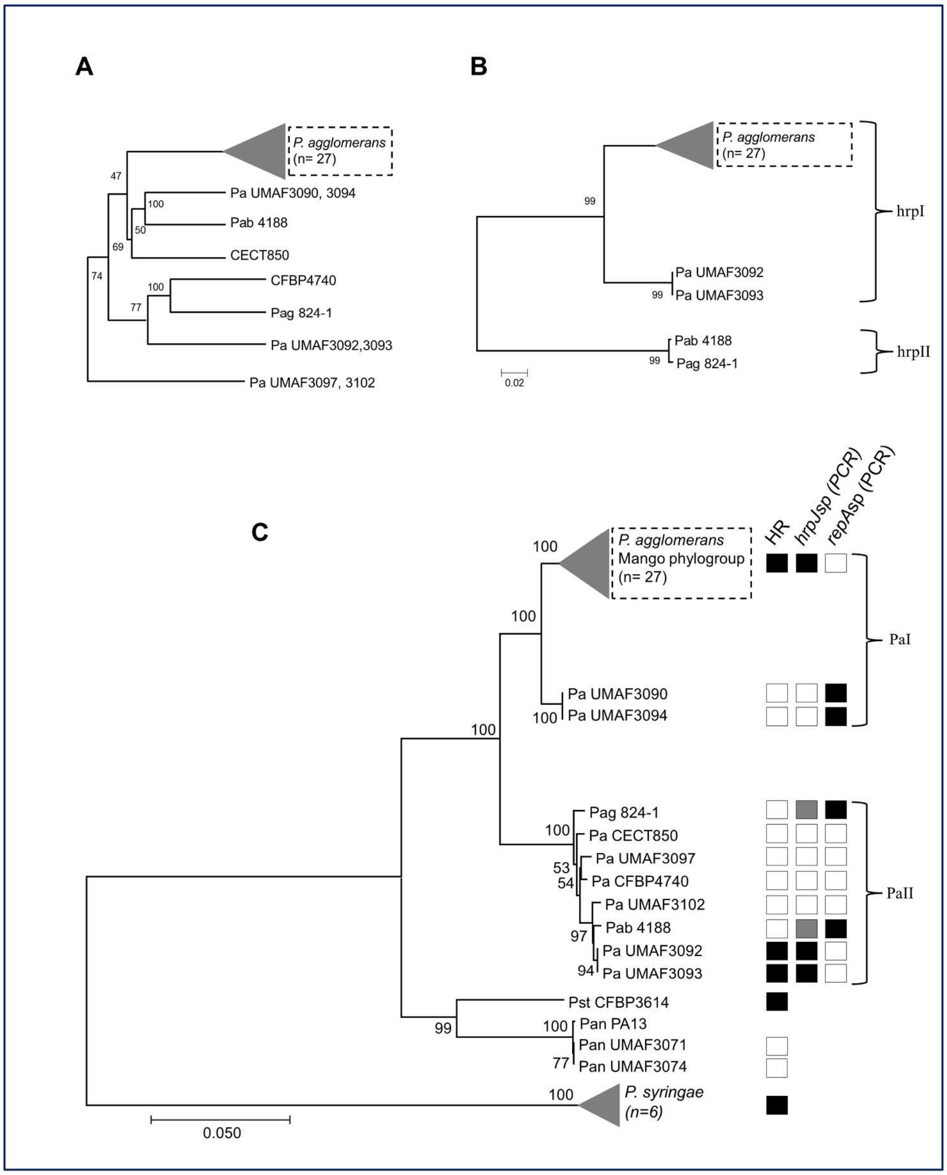

Fig. 4. Genetic diversity and phylogenetic analysis of Pantoea agglomerans strains isolated from mango trees. A, Dendrogram cluster analysis obtained by a repetitive sequence-based polymerase chain reaction fingerprinting method using a BOXA1-R primer, showing genetic relationships of P. agglomerans strains isolated from mango trees along with pathogenic and non-pathogenic 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. gypsophilae 824-1 and P. agglomerans pv. betae 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 non-pathogenic P. agglomerans strains isolated from other

hosts, P. stewartii subsp. indologenes CFBP3614, 2 P. ananatis strains isolated from mango trees(Pan UMAF3071 and Pan UMAF3074), P. ananatis PA13 from the database, and the 6 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, hrpJsp (PCR): PCR of the hrpJ gene with the specific primers designed in this study, repAsp (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 (hrpj5n1 and hrpj3n1335) designed using the pPATH plasmid sequence from Pag 824-1. Pa: Pantoea agglomerans; Pan: Pantoea ananatis; Pst: Pantoea stewartii subsp. indologenes; Pag 824-1: Pantoea agglomerans pv. gypsophilae 824-1; and Pab 4188: Pantoea agglomerans pv. betae 4188.

212x265mm (300 x 300 DPI)

SUPPLEMENTARY TABLE S1. Bacterial strains isolated from necrotic symptoms in mango trees in Canary Islands and their main virulence-related features

Bacterial strains	Location (Canary Islands, Spain)	Isolated from	Year of isolation	HR response	*hrpJ* gene[a]	*repA* gene[b]	Plasmid profile[d]	Pathogenicity onion bulbs	Pathogenicity mango buds
P. agglomerans									
UMAF3064	Tenerife	Leaf	2005	+	+	-	I	NT	NT
UMAF3065	Tenerife	Leaf	2005	+	+	-	I	NT	NT
UMAF3066	Tenerife	Stem	2005	+	+	-	I	+	NT
UMAF3067	Tenerife	Flower panicle	2005	+	+	-	I	+	+
UMAF3068	Tenerife	Leaf	2005	+	+	-	I	NT	NT
UMAF3069	Tenerife	Leaf	2005	+	+	-	I	NT	NT
UMAF3075	Tenerife	Leaf	2006	+	+	-	I	+	+
UMAF3076	Tenerife	Leaf	2006	+	+	-	I	NT	NT
UMAF3077	Tenerife	Flower panicle	2006	+	+	-	I	NT	NT
UMAF3078	Tenerife	Flower panicle	2006	+	+	-	I	+	NT
UMAF3079	Tenerife	Bud	2007	+	+	-	I	NT	NT
UMAF3080	Tenerife	Leaf	2007	+	+	-	I	NT	NT
UMAF3081	Tenerife	Stem	2007	+	+	-	I	NT	NT
UMAF3082	Tenerife	Stem	2007	+	+	-	I	NT	NT
UMAF3083	Tenerife	Leaf	2007	+	+	-	I	NT	NT
UMAF3084	Tenerife	Stem	2007	+	+	-	I	+	+
UMAF3085	Tenerife	Flower panicle	2007	+	+	-	I	NT	NT
UMAF3086	Tenerife	Immature fruit	2007	+	+	-	I	+	NT
UMAF3087	Tenerife	Immature fruit	2007	+	+	-	I	NT	NT
UMAF3090	Tenerife	Flower panicle	2008	-	-	+	III	-	NT
UMAF3091	Tenerife	Leaf	2008	+	+	-	I	NT	NT
UMAF3092	La Palma	Leaf	2008	+	+	-	II	NT	NT
UMAF3093	La Palma	Leaf	2008	+	+	-	II	+	+
UMAF3094	La Palma	Leaf	2008	-	-	+	III	NT	NT
UMAF3095	Tenerife	Leaf	2008	+	+	-	I	NT	NT
UMAF3096	La Palma	Flower panicle	2008	+	+	-	I	NT	NT
UMAF3097	Tenerife	Leaf	2009	-	-	-	IV	NT	NT
UMAF3098	Tenerife	Flower panicle	2009	+	+	-	I	+	+
UMAF3099	Tenerife	Flower panicle	2009	+	+	-	I	NT	NT
UMAF3100	Tenerife	Stem	2009	+	+	-	I	NT	NT
UMAF3101	Tenerife	Stem	2009	+	+	-	I	+	NT
UMAF3102	Tenerife	Stem	2009	-	-	-	IV	NT	NT
UMAF3103	Tenerife	Stem	2009	+	+	-	I	NT	NT
P. ananatis									
UMAF3070	Tenerife	Leaf	2005	-	NT[c]	NT	NT	NT	NT
UMAF3071	Tenerife	Leaf	2005	-	NT	NT	NT	NT	NT
UMAF3072	Tenerife	Stem	2005	-	NT	NT	NT	NT	NT
UMAF3073	Tenerife	Leaf	2005	-	NT	NT	NT	NT	NT
UMAF3074	Tenerife	Leaf	2005	-	NT	NT	NT	NT	NT

P. syringae									
UMAF2805	Tenerife	Leaf	2005	+	NT	NT	NT	NT	NT
UMAF2808	Tenerife	Stem	2006	+	NT	NT	NT	NT	NT
UMAF2811	Tenerife	Petiole	2007	+	NT	NT	NT	NT	NT
UMAF2812	Tenerife	Leaf	2008	+	NT	NT	NT	NT	NT
UMAF2813	La Palma	Leaf	2008	+	NT	NT	NT	NT	NT
UMAF2814	Tenerife	Bud	2009	+	NT	NT	NT	NT	NT

[a] *hrpJ* gene detection by PCR.

[b] *repA* gene detection by PCR

[c] Non-tested.

[d] Plasmid profile. I: 90, 140, 150 and 160 Kb; II: 90, 140, 160 and 170 Kb; III: 110 and 140 Kb; IV: 150 Kb.

SUPPLEMENTARY TABLE S2. Bacterial strains used as controls for the different biochemical, physiological and pathogenicity tests performed in this study

Bacterial strains	Reference or source
Bacillus subtilis UMAF6639	Romero et al. 2007
Escherichia coli CECT831	CECT[a]
Pantoea agglomerans pv. *betae* 4188[b]	Burr et al., 1991
Pantoea agglomerans pv. *gypsophilae* 824-1[b]	Manulis et al., 1991
Pantoea agglomerans CECT850	CECT
Pantoea agglomerans CFBP4740	CFBP[c]
Pantoea stewartii subsp. *indologenes* CFBP3614	CFBP
Pseudomonas syringae pv. *syringae* UMAF0048	Cazorla et al. 1998
Pseudomonas syringae pv. *syringae* UMAF0049	Cazorla et al. 1998
Pseudomonas syringae pv. *syringae* UMAF0158	Cazorla et al. 2002

[a] CECT: Spanish Type Culture Collection.

[b] Bacterial strains kindly provided by Shulamit Manulis-Sasson and Isaac Barash.

[c] CFBP: French Collection of Plant Associated Bacteria.

SUPPLEMENTARY TABLE S3. Primers and PCR conditions used in this study

PCR amplifications	Primer name and sequence	PCR cycles	Source or reference
repA-PCR	repAFsp: 5'-GCTGACTGAAGCTGCTTATC-3' repARsp: 5'-AGACTCAGGCAGCATGAATC-3'	2´-94ºC; 45´´-94ºC, 45´´-58ºC, 30´´-72ºC (X 30 cycles); 5´-72ºC	Weinthal et al. 2007
hrpJ-PCR	hrpj5n1: 5´-CGCCAGGATGAAAATTGCTC-3´ hrpj3n1335: 5´-CGCAGCCGATTCAACGCC-3´	2´-94ºC; 45´´-94ºC, 45´´-62ºC, 30´´-72ºC (X 30 cycles); 5´-72ºC	This study
hrpJ specific-PCR	hrpJPaF: 5´-GACCTTCGCGGAACAGGTTG-3´ hrpJPaR: 5´-CGGCAGATGGTAGACCAG-3´	2´-94ºC; 45´´-94ºC, 45´´-60ºC, 45´´-72ºC (X 30 cycles); 5´-72ºC	This study
BOX-PCR	BOXA1R: 5´-CTACGGCAAGGCGACGCTGACG-3´	7´-94ºC; 1´-94ºC, 1´-53ºC, 8´-65ºC (X 30 Cycles); 15´-65ºC	Rademaker et al. 1998
gyrB-PCR	gyrB3i: 5´-AACGCWATCGACGAAGC-3´ gyrB4i: 5´-TGGAAVCCRTCRTTCCAC-3´	2´-94°C; 1´-94°C, 1´-60°C, 1´- 72°C (X 30 cycles); 5´-72°C	Modified from Delétoile et al. 2009
rpoB-PCR	Vic3: 5´-GGCGAA ATGGCWGAGAACCA-3´ Vic2: 5´-GAGTCTTCGAAGTTGTAACC-3´	4´-94°C; 45´´-94°C, 45´´-50°C, 45´´-72°C (X30 cycles); 5´-72°C	Modified from Delétoile et al. 2009