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New species of pathogenic *Pseudomonas* isolated from citrus in Tunisia: Proposal of *Pseudomonas kairouanensis* sp. nov. and *Pseudomonas nabeulensis* sp. nov.

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ABSTRACT

A collection of *Pseudomonas* strains was isolated in different regions of Tunisia in the period 2016–2017 from the fruits and leaves of *Citrus sinensis* cv. 'Valencia Late' and *Citrus limon* cv. 'Eureka' plants with symptoms of blast and black pit disease. A phylogenetic analysis of the housekeeping gene *rpoD* was used for strain identification at the species level. The results demonstrated the affiliation of these strains with the genus *Pseudomonas* and revealed the presence of 11 strains representing two putative new species in two monophyletic branches. These strains were analyzed morphologically and genotypically by multilocus sequence analyses of the *rpoD*, *gyrB* and 16S rRNA (*rrs*) gene sequences, and their phenotypic characteristics by API 20NE and Biolog GEN III. Plant pathogenic properties were confirmed on fruits and detached leaves of *C. limon* cv. 'Eureka'. Fatty acids and WC MALDI-TOF MS major protein profiles were determined. The genomes of both representatives were sequenced. The average nucleotide index and genome-to-genome distance from KC12^T and E10B^T are below the cut-off established for a described species. These results support the conclusion that the strains KC12^T, KC17, KC20, KC22, KC24A, KC25 and KC26 represent a novel species of *Pseudomonas*, for which the name of *Pseudomonas kairouanensis* is proposed. The type strain is KC12^T (=CECT9766 and CFBP 8662). The strains E10B^T, E10AB, E10CB1 and Iy3BA represent another novel species of *Pseudomonas* for which the name of *Pseudomonas nabeulensis* is proposed; the type strain is E10B^T (=CECT9765 and CFBP 8661).

Introduction

One of the most varied and complex groups of Gram-negative bacteria is the genus *Pseudomonas*, as it contains a very large number of species (more than 203 at the time of writing this manuscript) and can be isolated from many different abiotic (soil, water, air) and biotic (humans, animals, plant tissues) environments [26]. Their interactions with plants are extensive: some have roles as plant growth promoting rhizobacteria (PGPR) by stimulating the synthesis of phytohormones such as auxin and gibberellin and by improving the bioavailability of certain nutrients such as nitrogen and phosphate by solubilization [5]. Some species have antagonistic activity toward phytopathogens (fungi, nematodes, yeasts, bacteria) by producing antibiotics, by activating plant resistance, or through competitive exclusion, such as competition for carbon and iron [16]. The genus *Pseudomonas* also contains phytopathogenic species distributed within the *Pseudomonas fluorescens* lineage in several phylogenetic groups and subgroups as defined by Mulet et al. [20] and Gomila et al. [10]. Most plant pathogenic species are concentrated in the *Pseudomonas syringae* phylogenetic group (at least 14 species [11]), but others are located in the *Pseudomonas putida* group (*Pseudomonas monteilii*) or in the *P. fluorescens* group. In this last group, plant pathogens are

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found within the *Pseudomonas corrugata* subgroup (*P. corrugata* and *Pseudomonas mediterranea*), the *P. fluorescens* subgroup (*Pseudomonas constantinii*, *Pseudomonas lurida*, *Pseudomonas marginalis*, *Pseudomonas orientalis*, *Pseudomonas tolaasii*, *Pseudomonas panacis* and *Pseudomonas simiae*) and in the *Pseudomonas koreensis* subgroup (*Pseudomonas moraviensis*).

Significant economic losses due to the spread of black and blast pit disease, especially in lemon trees, have been reported in Tunisia. Recently, *P. syringae* pv. *syringae* has been reported as the causative agent of black pit in Tunisia, and strains have been characterized by REP-PCR and PCR melting profile (PCR MP) techniques to determine the potential genetic diversity among strains [2]. In this region, *P. syringae* is the only phytopathogenic bacterium on citrus as reported by farmers and researchers. However, studies in other geographical regions have shown that other citrus pathogenic *Pseudomonas* exist, such as *Pseudomonas caspiana*, *P. simiae, P. orientalis* and *P. lurida*, which induce blast disease symptoms on leaves and stems [4,6]. To identify and implement appropriate and adequate control methods and to minimize losses, it is necessary to identify the species of bacteria responsible for the disease. For this purpose, 462 strains were isolated in an initial screening from samples of citrus fruits and leaves of *Citrus sinensis* cv. 'Valencia Late' and *Citrus limon* cv. 'Eureka' with black pit symptoms collected from different regions of Tunisia (Nabeul, Ben Arous, Bizerte, Kairouan, Jendouba and Beja) in the period 2016–2017. The strains were analyzed by *rpoD* sequencing and by whole-cell MALDI-TOF mass spectrometry to assign them at the genus and species levels. Eleven strains were considered representatives of two putative new species because their *rpoD* sequence similarities to the currently known *Pseudomonas* species type strains were below the threshold values established for members of the same species in the genus; these strains were then deeply analyzed taxonomically. Their genotypic characteristics were studied by multilocus sequence analysis of the housekeeping genes *rpoD*, *gyrB* and 16S rRNA, which is considered one of the most effective and efficient molecular tools for species differentiation. Their phenotypic characteristics were studied by API 20NE and Biolog GEN III and chemotaxonomically by their MALDI-TOF MS profiles. The 11 strains were also tested for pathogenicity. The genomes of the two strains selected as representatives of these groups were sequenced and analyzed to obtain genomic insights into the two novel proposed species. The main objective of this study was to characterize and clarify the taxonomy of these putative new *Pseudomonas* species not previously described and present in citrus orchards in Tunisia.

Material and methods

Bacterial isolation

The characteristics of orchards where the eleven selected *Pseudomonas* strains were isolated are detailed in Table S1.

Sampling was performed on fruits showing the typical symptoms of black pit. Each fruit was placed separately in a sterile plastic bag before being transferred to the laboratory. For each sample, small pieces of the intermediate zone between the healthy and the necrotic parts were disinfected with 0.5% sodium hypochlorite and washed with sterile distilled water (SDW), macerated in phosphate buffer (K_2HPO_4: 8.75 g/l, KH_2PO_4: 6.75 g/l) and vortexed for 4 min. The liquid of the crushed material was taken, and decimal dilutions to 10^{-4} were made. One hundred microliters from the 10^{-4} dilution of each sample was inoculated in Trypticase Soy Agar (TSA, Biolife) and King B medium (KB, Biolife) supplemented with cycloheximide (Sigma, 200 mg/L), cephalexin (Sigma, 80 mg/L) and boric acid (BIOMATIQ, 1.5 g/100 mL) (KBC medium). After 2 days at 28 °C, colonies were subcultured on KB plates to ensure purity. The bacteria were stored in 20% glycerol at −80 °C and on KB at 4 °C for short-term use. The strains used in this study are listed in Tables 1 and S1.

Table 1
Strains used in this study and pathogenicity test on citrus fruits and leaves.

Isolate	Symptom on lemon fruit (cm) after injection of strain[a]	Symptom on lemon leaves (cm^2)[b]	Symptom on lemon fruit after spray (cm)[c]
KC12^T	2.5 ± 0.35	0.63	0.5 ± 0.30
KC17	1.0 ± 0.10	0.50	0.5 ± 0.12
KC20	1.5 ± 0.30	0.40	0.3 ± 0.14
KC22	0.9 ± 0.35	0.35	0.5 ± 0.06
KC24A	0.8 ± 0.17	0.70	0.3 ± 0.00
KC25	2.2 ± 0.70	0.63	1.2 ± 0.29
KC26	2.0 ± 0.30	0.64	1.2 ± 0.31
E10B^T	1.7 ± 029	0.36	0.6 ± 0.14
E10AB	3.4 ± .32	0.40	0.9 ± 0.31
E10CB1	2.8 ± 0.44	0.50	0.9 ± 0.14
ly3BA	2.1 ± 1.07	0.36	0.6 ± 0.14
Positive control	2.3 ± 0.36	0.5	1.4 ± 0.31
Negative control	0	0	0

The pathogenesis test was performed on lemon fruits and detached leaves of *Citrus lemon* cv. 'Eureka'. The strain *Pseudomonas syringae* pv. *syringae* KB49 (PG02) was used as positive control and sterile distilled water as negative control. All strains have negative result of HR on tobacco.

[a] Necrotic diameters were measured in cm ± standard deviation (average of three measurements).

[b] The lesion area of one assay was expressed in cm^2.

[c] Necrotic diameters of sprayed suspension of three replicates in cm ± standard deviation.

Morphology and, biochemical and physiological tests

Cell size, morphology and flagellar insertion were determined by transmission electron microscopy of cells from the exponential growth phase in Luria Broth medium (LB, Difco). A Hitachi model H600 electron microscope was used at 75 kV. The samples were negatively stained with phosphotungstic acid (1%, pH 7.0) as previously described [13].

The production of fluorescent pigments was tested on King B medium, and pyocyanin production was tested on King A medium (*Pseudomonas* agar P, Biolife). The strains were characterized phenotypically using API 20 NE strips (bioMérieux) and Biolog GEN III MicroPlates (Biolog, Hayward, CA). Growth temperatures (4, 6, 10, 15, 18, 25, 30, 37 and 42 °C) were determined in LB, and growth in the presence of NaCl (0–15% w/v) and pH ranges (4–11) were tested in Nutrient Broth (Merck).

Chemotaxonomic analysis

Whole cell fatty acid methyl esters (FAME) analysis was performed at the Spanish Type Culture Collection, CECT, Valencia, Spain (http://cect.org/identificaciones) under high standardized conditions. Fatty acids were extracted, prepared and analysed as previously described [25].

The chemotaxonomic data obtained with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of whole cells (WC MALDI-TOF MS) of the 11 selected strains, together with their closely related species type strains, were obtained at the Scientific-Technical Services (University of Balearic Islands, Spain) and were analyzed as previously described [25].

Tests on plants

Tests of pathogenicity were performed on lemon fruits and detached leaves of *C. limon* cv. 'Eureka'. Fruits and leaves were dipped in a solution of sodium hypochlorite (1% active hypochlo-

rite) for 5 min, rinsed three times in SDW and gently dried with filter paper [27]. The pathogenic strain *P. syringae* pv. *syringae* KB49 (a member of phylogroup PG02) isolated from a citrus orchard in Tunisia with black pit symptoms was used as a positive control. Strains were grown on LB medium at 28 °C for 48 h. One or two colonies were suspended in sterile distilled water to an absorbance at 580 nm between 0.06 and 0.12, which corresponds to approximately 10^8 CFU/mL. The suspensions (10 μL) were inoculated either by injection with a tuberculin needle at the fruit wall or by depositing on the surface of the fruit. Negative controls were treated similarly with SDW. Three fruits were inoculated for each strain as well as for the controls. For leaves, 10 μL of the bacterial suspension was injected into the central veins of the abaxial side, the leaves were incubated for 5 days at 20 °C, and the areas of necrosis were measured.

The capacity of the strains KC12^T and E10B^T to induce a hypersensitive response (HR) in tobacco was assessed as described previously [18]. Bacterial suspensions (10^8 CFU/mL) were infiltrated into *Nicotiana tabacum* L. cv. Samsun plant leaves. Strain CC94 was used as a positive control, and SDW was used as a negative control. HR was checked after 24 h.

Antibiotic resistance

The *Pseudomonas* isolates were evaluated against antibiotics of different families using the disc diffusion method of Bauer et al. [3]. The antibiotics used in the resistance test were penicillins (piperacillin and oxacillin), cephalosporin (ceftazidime, cephalothin, cefazolin, ceftriaxone, cefuroxime), carbapenem (imipenem), aminopenicillin (amoxicillin), aminosides (gentamicin, streptomycin, neomycin, tobramycin, kanamycin), fluoroquinolone (ciprofloxacin, pefloxacin, ofloxacin), quinolones (nalidixic acid), sulfamides-trimethoprim (sulfamethoxazole), polypeptide (colistin), carboxypenicillin (carbenicillin), monobactam (aztreonam), tetracyclines (tetracycline), phenicolates (chloramphenicol and rifampicin). Antibiotic discs were placed on nutrient agar medium previously inoculated with a pure culture of the strains grown for 48 h and adjusted to 10^6 colony-forming units (CFU) mL^{-1}.

After incubation at 30 °C for 24 h, inhibition zones were measured in mm, comparing them with critical values of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org) to evaluate whether a strain was sensitive or resistant. Multiresistance classification was performed according to Magiorakos et al. [14]. Multidrug resistance (MDR) is defined as nonsusceptible to at least one agent in three or more antimicrobial categories. Extensive drug resistance (XDR) is defined as nonsusceptible to at least one agent in all but two or fewer antimicrobial categories. Pandrug resistance (PDR) is defined as nonsusceptible to all agents in all microbial categories.

DNA extraction, PCR amplification and DNA sequencing conditions

The DNA extraction, PCR amplification, primers used, purification of the amplified products and DNA sequencing conditions, as well as the sequence analysis procedures, were previously described [19].

Phylogenetic analysis

Partial sequences of the 16S rRNA (1285 nucleotides), *gyrB* (801 nucleotides) and *rpoD* (665 nucleotides) genes were analyzed individually. An analysis of these three concatenated gene sequences was also performed, comprising a total of 2751 nt [20]. The Jukes–Cantor (JC) [12], maximum likelihood (ML) [8] and maximum parsimony (MP) [22] algorithms were used for the comparisons.

Genome sequencing and analysis

Genomic DNA was isolated from the strains KC12^T and E10B^T using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. The obtained Illumina HiSeq 2000 paired-end library reads were assembled *de novo* using the Newbler Assembler v2.7 program (Roche). The draft genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The whole genome shotgun projects for strains KC12^T and E10B^T have been deposited in DDBJ/ENA/GenBank under the accession numbers QUZU00000000 and QUZT00000000. The version described in this paper is the first version. Analysis and comparison of the functional annotation were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG Automatic Annotation Server [KAAS]) [17].

The genomic relatedness of the strains KC12^T and E10B^T to the whole genome shotgun sequences of all species type strains in the *P. fluorescens* phylogenetic subgroup available in public databases was determined based on the tetranucleotide frequency correlation coefficients (TETRA), average nucleotide identity (ANI) and the BLASTN algorithm (ANIb), using the MUMMER ultrarapid aligning tool (ANIm) as well as genome-to-genome distance (GGDC) methods. ANIb and ANIm were calculated using the JSpecies software tool available at the webpage http://www.imedea.uib.es/jspecies. The recommended species cut-off is 95–96% for the ANIb and ANIm indices [24]. GGDC was calculated using the web service http://ggdc.dsmz.de [15] and using the recommended BLAST method. The GGDC results shown are based on the recommended formula 2, which is independent of genome length and is thus robust against the use of incomplete draft genomes. The recommended species cut-off is 70% for the GGDC index.

Results

Origin of bacterial strains and initial identification

Several orchards in Tunisia with traditional types of culture, using drip or traditional irrigation and fertilization were selected for the initial screening of *Pseudomonas* strains. All strains were isolated from different samples from trees characterized by moderate symptoms of citrus black pit. The varieties of citrus trees studied were *C. sinensis* cv. 'Valencia Late', cultivated since 1966, and *C. limon* cv. 'Eureka'. As depicted in Tables 1 and S1, the KC strains were isolated from 3 different trees of *C. sinensis* cv. 'Valencia Late' in the Chaabani2 orchard in the Kairouan/Sbikha region of north central Tunisia. Strains E10B^T, E10AB, and E10CB1 were isolated in 2017 from one tree of the *C. limon* 'Eureka' cultivar in the Chakib orchard in the Nabeul/Bnikhalled region, situated in northeastern Tunisia on the south coast near the Cap Bon peninsula. Strain Iy3BA was isolated from *C. sinensis* cv. 'Valencia Late' in the Ayari orchard in the region of Ben Arous/Naasen, which is a city in northeastern Tunisia.

An initial screening for *Pseudomonas* was performed by MALDI-TOF MS analysis. The 11 selected strains were identified as members of the *Pseudomonas* genus, but they could not be identified at the species level because of the low score values given by the Biotyper identification program.

Pathogenicity tests on citrus plants

All strains were positive for lemon fruit and leaf necrosis tests when compared with *P. syringae* pv. *syringae* KB49 as a positive control. Disease severity values on lemon fruit ranged from 1.0 to

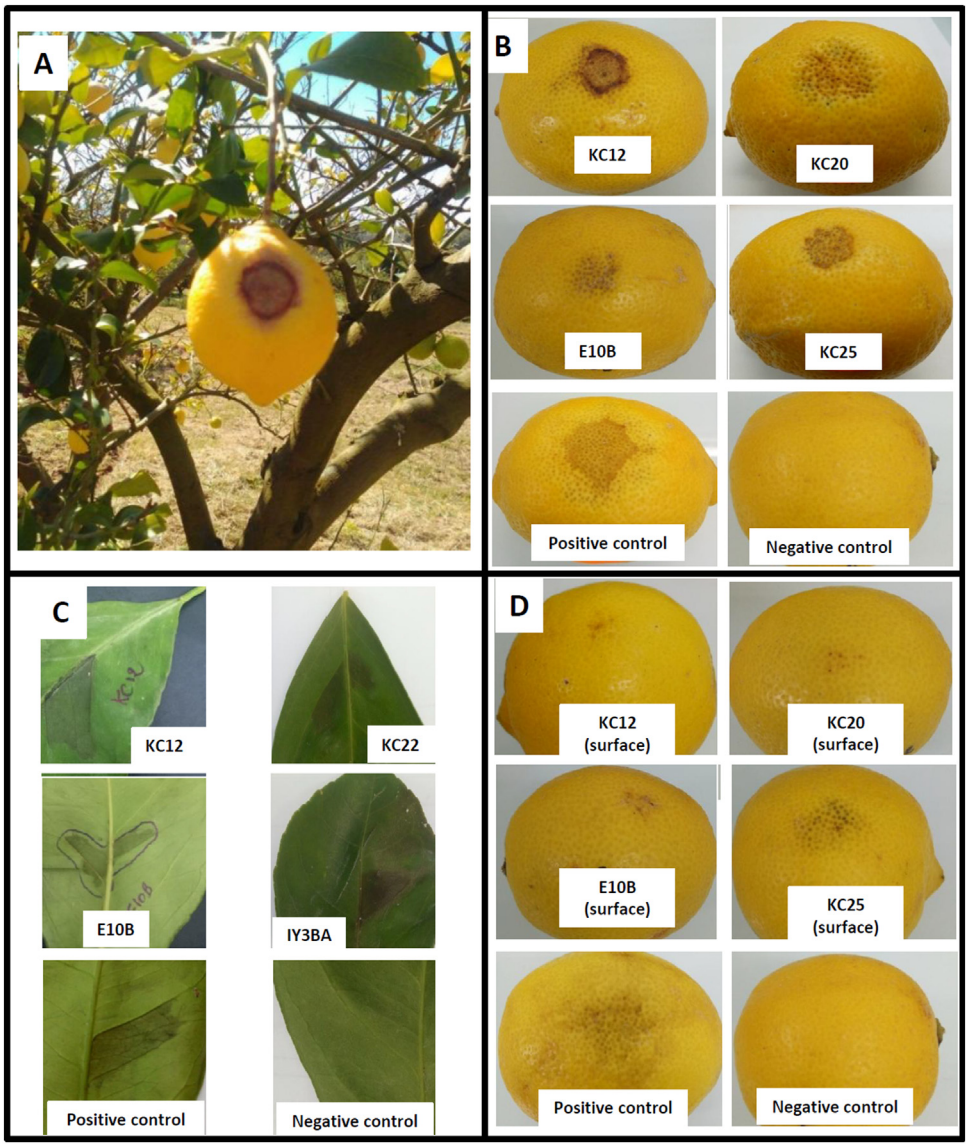

Fig. 1. Pathogenicity tests in *Citrus limon* cv 'Eureka' lemons fruits and leaves for the strains of study. A) Symptom of bacterial black pit in *Citrus limon* orchard in Nabeul region, B) Pathogenity test with injection on the lemon fruit, C) In surface on lemon leaves and D) Spray inoculation on the lemon fruit with the strains of the study. The strain *Pseudomonas syringae* pv. *syringae* KB49 (PG02b) was used for positive control. The sterile distilled water was used for negative control.

3.4 cm (Table 1). In lemon leaves, the necrotic lesion area varied from 0.3 to 0.7 cm^2. In both tests, 54% of the values obtained were equal or superior to those of the control (*P. syringae* pv. *syringae* KB49). Table 1 indicates the diameter of the necrotic lesion and area for each strain. Fig. 1 depicts the pathogenicity test results, where black necrotic spots with irregular shapes surrounded by chlorotic halos were formed.

Although strains $KC12^T$ and $E10B^T$ produced symptoms on citrus, they did not produce HR in tobacco. It seems that these new species have a different means of attacking plants than that of the citrus pathogenic *P. syringae* strains.

Morphology and phenotypic characterization

The cells from all strains are Gram-negative, short rods, 2.1–3.5 μm long and 0.9–1.0 μm wide, and motile with one or multiple monopolar flagella, as shown in Figs. S2 and S3. The colonies of the strains KC12, KC17, KC20, KC22, KC24A, KC25 and KC26 were round, flat, translucent and beige-colored, with regular margins (2–3 mm in diameter) on LB plates after incubation for 48 h at 30 °C. Strains $E10B^T$ and Iy3BA showed similar colonial morphologies, but smaller, with sizes between 1–2 mm in diameter. All strains grew in KB and KBC media and produced fluorescent pigments, but no pyocyanin was produced on King A (*Pseudomonas* agar P, Difco). All were catalase- and oxidase-positive and strictly aerobic. Growth occurred at temperatures between 4 and 37 °C (optimum at 25 °C), with pH 5.0–9.0 (optimum at pH 6–7) and NaCl concentrations from 0 to 6% (w/v) (optimum at 0–2%). Biolog GN III and API 20NE results are given in Tables 2 and S2. The type strains of *Pseudomonas canadensis* and *Pseudomonas grimontii* were included because they were the strains with the highest similarity in the phylogenetic analyses based on the concatenated partial sequences of the 16S rRNA, *gyrB* and *rpoD* genes (see below). Both groups of strains were positive in the API 20NE tests by the presence of arginine dihydrolase and assimilation of glucose, arabinose, mannose, mannitol, *N*-acetyl-D-glucosamine, potassium gluconate, capric acid, adipic acid, malate, and trisodium citrate. In the Biolog GN III plates, all strains were positive for the utilization

Table 2
Differential phenotypic characteristics of *Pseudomonas kairouanensis* and *Pseudomonas nabeulensis* from related *Pseudomonas* type strains. Strains: *P. kairouanensis* (1. KC12^T, 2. KC20) and *P. nabeulensis* (3. E10B^T, 4. Iy3BA), 5. *P. grimontii* ATCC BAA-140^T, 6. *P. canadensis* 2-92^T, 7. *P. fluorescens* ATCC 13525^T.

Characteristics	1	2	3	4	5	6	7
NaCl (%) (w/v)	0–6	0–6	0–6	0–8	0–6	0–6	0–8
Reduction of nitrates to nitrites (API 20NE)	–	–	–	–	+	–	–
Hydrolysis (API 20NE):							
Esculin	–	–	+	+	+	–	–
Assimilation (API 20NE):							
Adipic acid	+	+	+	+	+	+	–
Phenyl acetic acid	+	+	–	–	+	–	–
Substrates utilization in Biolog GENIII							
Sucrose	–	–	–	–	+	+	+
Pectin	–	–	–	–	–	+	–
p-Hydroxy-phenylacetic acid	–	+	–	–	+	+	–
α-Keto-butyric acid	+	+	–	–	+	+	+

of D-trehalose, *N*-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycogen, D-serine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, methyl pyruvate, L-lactic acid, citric acid, α-keto glutaric acid, D-malic acid, L-malic acid, bromo-succinic acid, γ-amino-butyric acid, α-hydroxy butyric acid, β-hydroxy-D,L-butyric acid, propionic acid, acetic acid, and formic acid; they were resistant to the following in the chemical sensitivity assays: 1% sodium lactate, fusidic acid, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, guanidine HCl, Niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue, nalidixic acid, lithium chloride, potassium tellurite, aztreonam, sodium butyrate, and sodium bromate. The KC group of strains showed three traits that differentiated them from the E10 group of strains: they were esculin negative and alpha keto-butyric acid and phenyl acetic acid positive. The *p*-hydroxy-phenylacetic acid test was variable. The other tests were negative or weak. Moreover, the KC group of strains can be differentiated from *P. grimontii* (the species with the highest similarity in the concatenated three-gene sequence analysis) by their inability to reduce nitrate to nitrite, their inability to hydrolyze aesculin and their inability to oxidize sucrose. The E10 group of strains can be differentiated from *P. canadensis* (the species with the highest similarity in the concatenated three-gene sequence analysis) by their ability to hydrolyze aesculin and their inability to utilize sucrose, pectin, *p*-hydroxy-phenylacetic acid and alfa-keto-butyric acid. All phenotypic data are shown in Table S2.

Chemotaxonomic analysis

Fatty acid profiles of strains KC12^T and E10B^T were compared with those of closely related species of the *P. fluorescens* subgroup generated under the same standardized conditions (Table S3). The major cellular fatty acid of strains KC12^T and E10B^T were, $C_{16:1}$ ω6c/$C_{16:1}$ ω7*c* (33.75–31.23%; summed feature 3), $C_{16:0}$ (31.05–33.49%); $C_{18:1}$ ω7c/$C_{18:1}$ ω6c (14.84–13.28%; summed feature 8), $C_{12:0}$ 3-OH (5.32–5.21%), $C_{12:0}$ 2-OH (4.06–4.63%), $C_{10:0}$ 3-OH (3.14–3.79%), $C_{12:00}$ (4.33–2.98%) and $C_{17:0}$ *cyclo* (2.65–3.69%), respectively. The presence of $C_{10:0}$ 3-OH, $C_{12:0}$ 2-OH and $C_{12:0}$ 3-OH fatty acids in the profile of strains KC12^T and E10B^T are consistent with the classification as *sensu stricto* pseudomonad [23]. No significant differences among the analyzed strains were found (Table S3).

The WC MALDI-TOF MS protein m/z profile differentiated the strains of group KC from group E10 by nine differential peaks. Table S3 shows the m/z values of the differential proteins. The protein profiles are shown in Fig. S1.

Antibiogram analysis

All strains in the KC and E10-Iy3BA groups are resistant to oxacillin, cephalothin, cefazolin, cefuroxime, amoxicillin, and tobramycin. Moreover, strains of both groups, KC and E10-Iy3BA, are multidrug resistant to 3 (MDR3) or 4 (MDR4) different antibiotic classes (Table S4).

Phylogenetic analysis

A dendrogram of the 16S rRNA gene sequences of the strains of this study was constructed and is shown in Fig. 2. It also includes 35 species type strains in the *P. fluorescens* phylogenetic subgroup, as well as *P. syringae* and *Pseudomonas aeruginosa* as outgroups. The corresponding similarity matrix is given in Table S5. The 11 strains are located in two different branches but with very short distances between them. Strains in the KC group were 99.6% similar to the strains in group E10-Iy3BA.

The similarities of the partial *rpoD* gene sequences of the strains in the KC group (KC12^T, 17, 20, 22, 24A, 25 and 26) were identical and were compared with the corresponding sequences of all *Pseudomonas* species type strains available in our in-house database (200 *Pseudomonas* sp. type strains). The results indicated that all were 94.1% similar to the closest type strain, *P. grimontii*, in the *P. fluorescens* subgroup. The strains in group E10-Iy3BA were 93.4% similar to the closest type strain, *P. canadensis*, which is also a member of the *P. fluorescens* phylogenetic subgroup. The strains in the KC and E10-Iy3BA groups were 94.4% similar (Table 3). The *rpoD* nucleotide sequence threshold for species differentiation in the *Pseudomonas* genus is 95% [20,21]. The values were lower than 95% and indicated that the nucleotide sequences corresponded to putative new species . The corresponding similarity matrix is given in Table S5. When the *rpoD* nucleotide sequences of the strains of this study were compared with all data available in NCBI, some *rpoD* sequences, HF932179.1 and HF932186.1, had similarity values of 93.5% to the strains in the KC group and 98.5% to the E10-Iy3BA group. These sequences corresponded to uncultured *Pseudomonas* clone 633 and clone 640 from the bulk cultivable fraction from the potato field of the Central Andean Highlands of Ecuador [9]. Twenty-four clones from the same study, together with two *Pseudomonas* sp. Irchel 2F7 and Irchel 3B17 strains [7], had similarity values between 95–96%.

The multilocus sequence analysis of the concatenated partial sequences of the housekeeping genes *rpoD*, *gyrB* and 16S rRNA had a similarity value of 95.3% between the KC strains and the closest species type strain *P. grimontii*. These sequences were identical for all strains in this group. The strains E10B^T, E10AB, E10CB1, Iy3BA and the closest species type strain *P. canadensis* had a similarity of 95.9% in the 3-gene partial sequence analysis (Table 3). Matrix distances are shown in Table S5. The MLSA threshold established

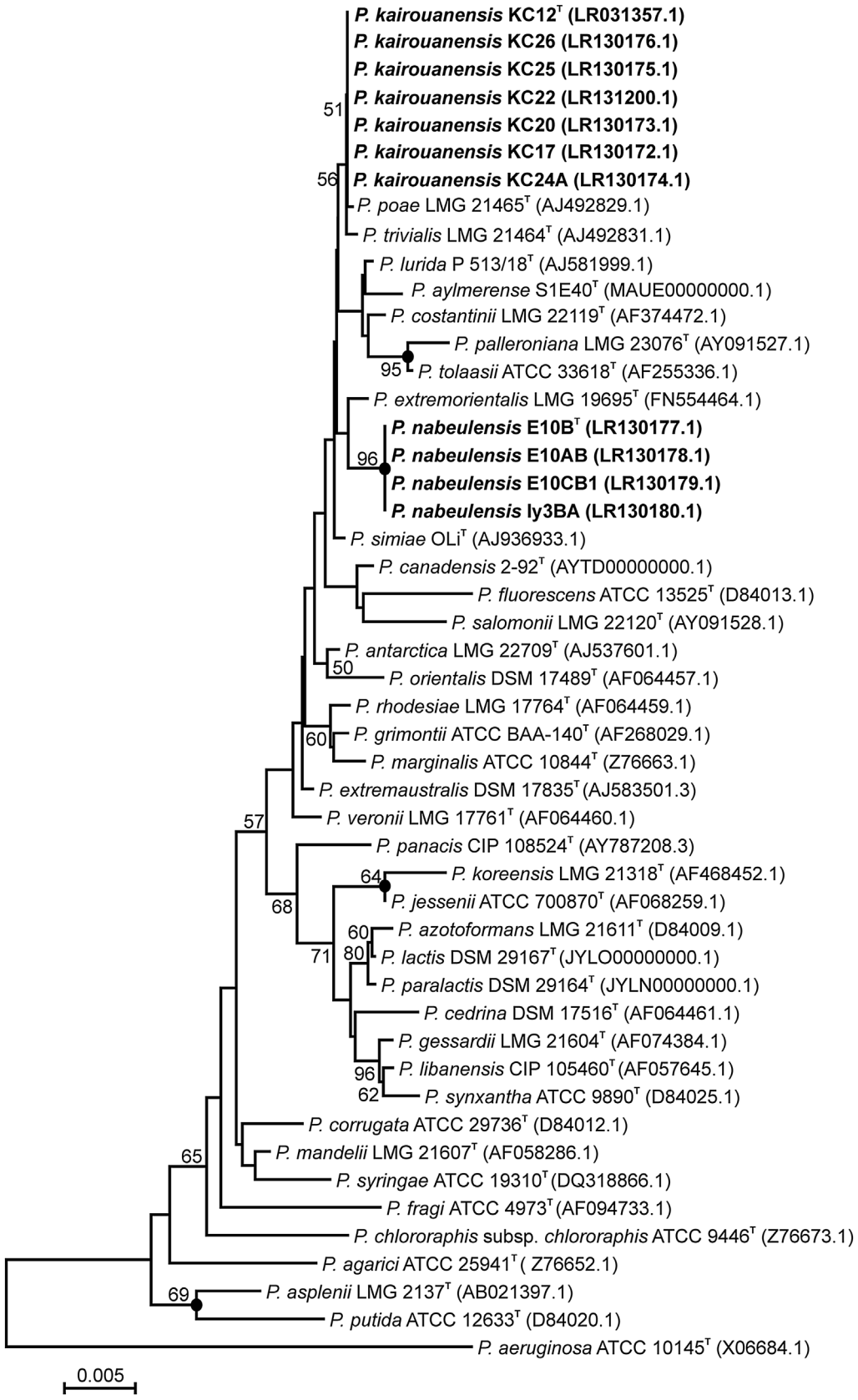

Fig. 2. Phylogenetic tree based on 16S rRNA gene of *P. kairouanensis*, *P. nabeulensis* and phylogenetically close members of *Pseudomonas*. Distance matrices were calculated by the Jukes–Cantor method. Dendrograms were generated by the neighbour-joining method. *P. aeruginosa* ATCC 10145^T was used as the outgroup. The bar indicates sequence divergence. Percentage bootstrap values of more than 50% (from 1000 replicates) are indicated at the nodes. Filled circles indicate that the corresponding nodes were also obtained in the trees generated with the maximum-likelihood and maximum parsimony. GenBank accession numbers are given in parentheses. Accession numbers indicated in bold are for sequences determined in this study.

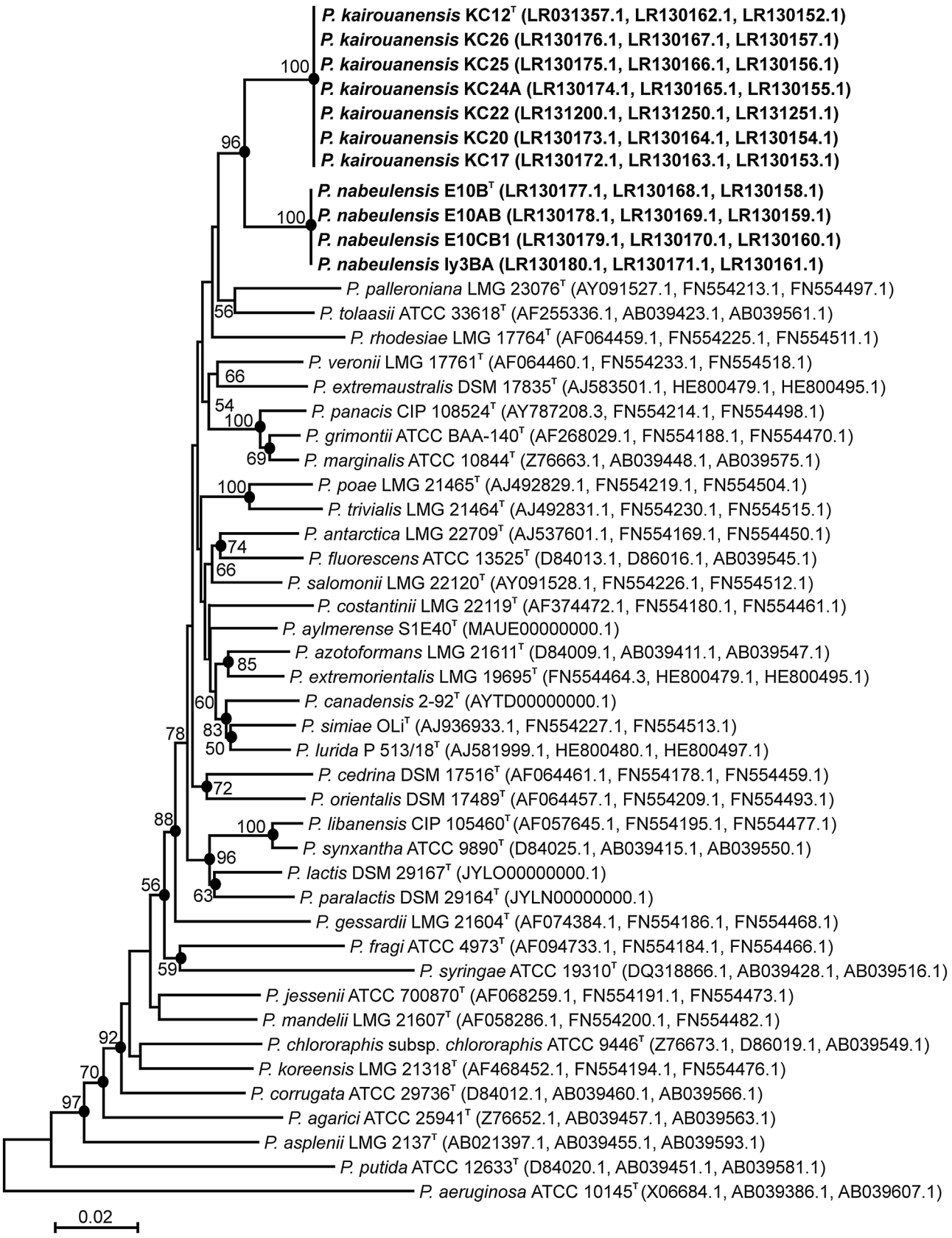

Fig. 3. Phylogenetic tree based on concatenated sequences of the 16S rRNA, *gyrB* and *rpoD* genes of *P. kairouanensis*, *P. nabeulensis* and phylogenetically close members of *Pseudomonas*. Distance matrices were calculated by the Jukes-Cantor method. Dendrograms were generated by the neighbour-joining method. *P. aeruginosa* ATCC 10145^T was used as the outgroup. The bar indicates sequence divergence. Percentage bootstrap values of more than 50% (from 1000 replicates) are indicated at the nodes. Filled circles indicate that the corresponding nodes were also obtained in the trees generated with the maximum-likelihood and maximum parsimony. GenBank accession numbers are given in parentheses in the following order: 16S rRNA, *gyrB* and *rpoD* genes. Accession numbers indicated in bold are for sequences determined in this study.

Table 3
Phylogenetic assignation of strains studied based on *rpoD* gene and the concatenated sequences of the 16S rRNA, *gyrB* and *rpoD* genes.

Isolate	Similarity to the closest type strain				Similarity to the closest strain		Group or Subgroup
	rpoD gene (in %)		Concatenated (in %)		Concatenated (in %)		
KC12^T	94.1	*P. grimontii*	95.3	*P. grimontii*	96.7	E10B	*P. fluorescens* SG
KC17	94.1	*P. grimontii*	95.3	*P. grimontii*	96.7	E10B	*P. fluorescens* SG
KC18	94.1	*P. grimontii*	95.3	*P. grimontii*	96.7	E10B	*P. fluorescens* SG
KC20	94.1	*P. grimontii*	95.3	*P. grimontii*	96.7	E10B	*P. fluorescens* SG
KC22	94.1	*P. grimontii*	95.3	*P. grimontii*	96.7	E10B	*P. fluorescens* SG
KC24A	94.1	*P. grimontii*	95.3	*P. grimontii*	96.7	E10B	*P. fluorescens* SG
KC25	94.1	*P. grimontii*	95.3	*P. grimontii*	96.7	E10B	*P. fluorescens* SG
KC26	94.1	*P. grimontii*	95.3	*P. grimontii*	96.7	E10B	*P. fluorescens* SG
E10B^T	93.8	*P. canadensis*	95.9	*P. canadensis*	96.7	KC12	*P. fluorescens* SG
E10AB	93.5	*P. canadensis*	95.9	*P. canadensis*	96.7	KC12	*P. fluorescens* SG
E10CB1	93.8	*P. canadensis*	95.9	*P. canadensis*	96.7	KC12	*P. fluorescens* SG
Iy3BA	93.8	*P. canadensis*	95.9	*P. canadensis*	96.7	KC12	*P. fluorescens* SG

Table 4
Genomic characteristics of the new pathogenic *Pseudomonas* strains: *P. kairouanensis* KC12^T and *P. nabeulensis* E10B^T isolated from citrus in Tunisia.

Characteristics	KC12^T	E10B^T
GeneBank ID	QUZU00000000	QUZT00000000
Genome size (bp)	6,702,324	6,763,512
No. contigs	107	150
N50 contig size	112,454	77,761
Largest contig size	503,235	294,970
Q40 Plus Bases (%)	6,702,242	6,763,396
Q39 Minus Bases (%)	82	116
GC-content (%)	60.4%	60.3%
Total genes	6261	6241
Protein-coding genes (CDS)	6077	6049
No. hypotethical proteins	771	753
RNA genes (clusters)	1	1
tRNAs	59	52
Pseudogenes[a]	116	131
Mobilome:		
Integrases	9	12
Transposases	27	21

[a] The number of total Pseudogenes indicated includes genes with ambiguous residues, frameshifted genes, incomplete genes, genes with internal stops or other multiple problems.

to differentiate species is 97% [20]. The Jukes–Cantor/neighbor-joining tree of the concatenated gene sequences revealed the presence of an independent branch supported by high bootstrap values (96%) that can be differentiated from the other *Pseudomonas* species type strains as depicted in Fig. 3.

General taxonomic genome features

The average nucleotide index and genome-to-genome distance from KC12^T to the closest type strain, *P. canadensis*, were 86.96 and 35.1%, respectively; for strain E10B^T, these values were 87.2 to *P. canadensis* and 35.1% to *P. extremorientalis*. These data clearly indicate that are below the established cut-off for each (ANIb: 95%, ANIm: 95%, GGDC: 70%), confirming that these two groups of strains represents a novel genomic species in the group. The results are shown in Table S6.

Genomic insights into the KC12^T and E10B^T strains

One strain of the KC group, KC12^T, was chosen as a representative of the group. The representative strain selected for the group including E10B^T, E10AB, E10CB1 and Iy3BA was E10B^T.

A total of 6261 genes were detected in strain KC12^T and 6,241 in strain E10B^T; 6077 and 6049 were predicted as protein coding genes (CDS), respectively (Table 4).

Both strains share many genes characteristic of the genus, such as genes related to motility, flagellation, twitching motility, chemotaxis, swarming activity, biofilm formation and alginate biosynthesis. Several metabolic traits can be inferred from the genome analyses. As examples, for catabolism, all genes necessary for starch hydrolysis to maltose or the degradation of the natural acyclic monoterpenoid citronellol were detected. Aromatic ring cleavage has been used in taxonomy for the characterization of *Pseudomonas*. In both genomes, 2 complete sets of genes were found for the degradation of 4-hydroxybenzoate through the protocatechuate 3,4-dioxygenase pathway and another for the catechol ring cleavage producing *cis*,*cis*-muconate (ortho pathway). These pathways converge in 3-oxoadipate enol-lactone and finally produce succinyl-CoA and acetyl-CoA. In the E10B^T genome, 10 additional genes were annotated, some for the degradation of *p*-cymene to pyruvate and acetaldehyde, and others for the degradation of 2,3-dihydroxybenzoate, finally producing acetyl-CoA and pyruvate. Other traits considered relevant in *Pseudomonas* taxonomy are those related to iron acquisition. Both genomes contained genes for Fe^{3+} siderophores and many genes for TonB dependent siderophore receptors. Other assimilatory genes are related to sulfate reduction and to a pathway for nitrate reduction to ammonia.

Secretion systems are also relevant for plant pathogenic bacteria. Genes for 4 types of secretion systems (TSS) were present in both the genomes analyzed: TSSI, TSSII, TSSIII and TSSVI. TSSIII belongs to the EscC/YscC/HrcC family secretion system export apparatus, which has an essential role in virulence. The exotoxin ExoU, an important cytotoxin, and the effector HopJ, which are both transported by TSSIII, were detected in the genome of strain E10B^T but absent in strain KC12^T. Three models of TSSVI are possibly encoded. The presence of genes coding for *TssA*, *TssJ*, *hcp* and *VgrG* indicates the possibility of a TSSVI-i model, and the presence of *DotU* and a second copy of *VgrG* could indicate the TSSVI-ii model. The presence of *tssK*, *tssG*, *tssF* and *tssE* are genes common to the TSSVIi and iii models. Three different VgrG genes are present in strain KC12 with percentage identity values ranging from 82, 83 and 97 with the corresponding genes of several type strains in the *P. fluorescens* group of species. Similarly, in strain E10B^T, three VgrG genes are present, and these are related to the corresponding genes of the *Pseudomonas poae* type strain (94, 96 and 99% identities for each gene) [1].

Several toxin-antitoxin systems of type II, typically phospholipases or pore-forming cytotoxins, are predicted in strains KC12^T and E10B^T: RelE/ParE family toxin, HicA/HicB, MqsR/Mqsa. Multidrug efflux pumps are one of the major mechanisms of antimicrobial resistance, and AdeC/AdeK/OprM genes have been annotated in both genomes.

Phage-related genes are mostly considered strain-specific. However, they can also be shared by strains in closely related species. A set of 25 correlative phage proteins are present in the genome of strain E10B^T, and these proteins are similar to those detected in the genomes of *P. grimontii* strains (96–94% identities), but these proteins were not found in the KC12^T genome.

Description of Pseudomonas kairouanensis *sp. nov.*

P. kairouanensis (N.L. fem. adj. kairouanensis, kai.rou.an.en'sis) pertaining to Kairouan, the geographical location of first isolation.

Cells are Gram-negative straight rods (2.1–3.5 μm long and 0.9–1.0 μm wide), motile with one or multiple monopolar flagella, as shown in Figs. S2 and S3. The colonies were round, flat, translucent and beige-colored, with regular margins (2–3 mm in diameter) on LB plates after incubation for 48 h at 30 °C. The strains grew in KB and KBC media and produced fluorescent pigments, but no pyocyanin was produced on King A (*Pseudomonas* agar P, Difco). All were catalase- and oxidase-positive and strictly aerobic. Growth occurred at temperatures between 4 and 37 °C (optimum at 25 °C), with pH 5.0–9.0 (optimum at pH 6–7) and NaCl concentrations from 0 to 6% (w/v) (optimum at 0–2%). Biolog GN III and API 20NE results are given in Tables 2 and S2. The strains were positive in the API 20NE tests by the presence of arginine dihydrolase and assimilation of glucose, arabinose, mannose, mannitol, *n*-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate and phenylacetate. In the Biolog GN III plates, the strains were positive for the utilization of D-trehalose, *N*-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycogen, D-serine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, methyl pyruvate, L-lactic acid, citric acid, α-keto glutaric acid, D-malic acid, L-malic acid, bromo-succinic acid, γ-amino-butyric acid, α-hydroxy butyric acid, β-hydroxy-D,L-butyric acid, alfa-keto-butyric acid, propionic acid, acetic acid, and formic acid; they were resistant to the following in the chemical sensitivity assays: 1% sodium lactate, fusidic acid, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, guanidine HCl, Niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue, nalidixic acid, lithium chloride, potassium tellurite, aztreonam, sodium butyrate and sodium bromate. The other tests were negative or weak. All phenotypic data are shown in Table S2. The predominant fatty acids are summed feature 3 ($C_{16:1}$ ω6c/$C_{16:1}$ ω7*c*), $C_{16:0}$, summed feature 8 ($C_{18:1}$ 8ω7c/$C_{18:1}$ ω6c), $C_{12:0}$ 3-OH, $C_{12:0}$ 2-OH, $C_{10:0}$ 3-OH, $C_{12:00}$ and $C_{17:0}$ *cyclo*.

The DNA G + C base composition is 60.4 mol% and the genome size is 6.70 Mb. The type strain KC12^T (=CECT 9766 = CFBP 8662) was isolated from citrus tree of *C. sinensis* cv. 'Valencia late' in Kairouan, Tunisia. Digital Protologue Taxonumber: TA000738.

Description of Pseudomonas nabeulensis *sp. nov.*

P. nabeulensis (N.L. fem. adj. nabeulensis, na.beul.en'sis) pertaining to Nabeul, the geographical location of first isolation.

Cells are Gram-negative straight rods (2.1–3.5 μm long and 0.9–1.0 μm wide), motile with one or multiple monopolar flagella, as shown in Figs. S2 and S3. The colonies were round, flat, translucent and beige-colored, with regular margins (1–2 mm in diameter) on LB plates after incubation for 48 h at 30 °C. The strains grew in KB and KBC media and produced fluorescent pigments, but no pyocyanin was produced on King A (*Pseudomonas* agar P, Difco). All were catalase- and oxidase-positive and strictly aerobic. Growth occurred at temperatures between 4 and 37 °C (optimum at 25 °C), with pH 5.0–9.0 (optimum at pH 6–7) and NaCl concentrations from 0 to 6% (w/v) (optimum at 0–2%). Biolog GN III and API 20NE results are given in Tables 2 and S2. The strains were positive in the API 20NE tests by the presence of arginine dihydrolase, hydrolysis of aesculin and assimilation of glucose, arabinose, mannose, mannitol, *n*-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, malate and trisodium citrate. In the Biolog GN III plates, the strains were positive for the utilization of D-trehalose, N-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycogen, D-serine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, methyl pyruvate, L-lactic acid, citric acid, α-keto glutaric acid, D-malic acid, L-malic acid, bromo-succinic acid, γ-amino-butyric acid, α-hydroxy butyric acid, β-hydroxy-D,L-butyric acid, propionic acid, acetic acid and formic acid; they were resistant to the following in the chemical sensitivity assays: 1% sodium lactate, fusidic acid, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, guanidine HCl, Niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue, nalidixic acid, lithium chloride, potassium tellurite, aztreonam, sodium butyrate and sodium bromate. The other tests were negative or weak. All phenotypic data are shown in Table S2. The predominant fatty acids are summed feature 3 ($C_{16:1}$ ω6c/$C_{16:1}$ ω7*c*), $C_{16:0}$, summed feature 8 ($C_{18:1}$ 8ω7c/$C_{18:1}$ ω6c), $C_{12:0}$ 3-OH, $C_{12:0}$ 2-OH, $C_{10:0}$ 3-OH, $C_{12:00}$ and $C_{17:0}$ *cyclo*.

The DNA G + C base composition is 60.3 mol% and the genome size is 6.76 Mb. The type strain E10B^T (=CECT 9765 = CFBP 8661) was isolated from citrus tree of *C. lemon* cv. 'Eureka' in Nabeul, Tunisia. Digital Protologue Taxonumber: TA00744.

Discussion

Most species of plant pathogenic *Pseudomonas* belong to the *P. fluorescens* phylogenetic group of species. This includes the *P. syringae* phylogenetic subgroup of species, which is the most abundant. However, deep taxonomic studies based on the isolation of a high number of strains demonstrate that plant pathogenic *Pseudomonas* strains classified in other phylogenetic branches have also been isolated: *P. lurida*, *P. orientalis* and *P. simiae* of the *P. fluorescens* subgroup, *P. moraviensis* of the *P. koreensis* subgroup (both subgroups belong to the *P. fluorescens* group), and *P. monteilii* of the *P. putida* group [4]. In Tunisia, *P. syringae* has been associated previously with symptoms resembling bacterial blast and black pit epidemics [2] [Oueslati, manuscript in preparation]. A deep knowledge of the presence of pathogenic species and their diversity in citrus in the Mediterranean areas is crucial due to its economic implications, but knowledge of the microbiota present in these trees and their environment is also important to elucidate their role. In this study, we characterized 2 groups of strains that could not be ascribed to any known *Pseudomonas* species. Strains in the KC group were isolated from *C. sinensis* cv. 'Valencia Late', while the E10B-Iy3BA group was isolated from *C. limon* cv. 'Eureka', indicating that this species can be present in both *Citrus* species. These strains have a wide distribution, ranging from the central region of Kairouan to Nabeul and Ben Arous, septentrional regions not previously studied.

The pathogenic tests showed that these strains probably have different mechanisms of plant attack than does *P. syringae*, as demonstrated by the hypersensitive response in tobacco. As predicted by the genome analysis, virulence determinants such as the 4 types of secretion systems and toxins can be involved in phytopathogenic strategies. Necrotic lesions were consistently observed on both leaves and fruits for both groups of strains. The lesions induced by the KC strains were similar in severity to those of the *P. syringae* positive control, whereas the E10 strains were as severe or more so.

Table 5
Protologue *Pseudomonas kairouanensis* sp. nov.

Taxonumber	TA00738
Species name	*Pseudomonas kairouanensis*
Genus name	*Pseudomonas*
Specific epithet	*kairouanensis*
Species status	sp. nov.
Species etymology	N.L. fem. adj. kairouanensis, kai.rou.an.en'sis pertaining to Kairouan, the geographical location of first isolation.
Designation of the type strain	KC12
Strain collection numbers	CECT 9766, CFBP 8662
16S rRNA gene accession number	LR031357
Alternative housekeeping genes	*rpoD* [LR130152], *gyrB* [LR130162]
Genome accession number	QUZU00000000
Genome status	Draft
Genome size	6,702,324
GC mol%	60.4
Country of origin	Tunisia
Region of origin	Kairouan
Other	Sbikha
Date of isolation	2017
Source of isolation	Citrus tree of *Citrus sinensis* 'Valencia late'
Sampling date	2017
Geographic location	Sbikha, orchard Chaabani 2
Latitude	35°51′02.7″N
Longitude	10°03′15.6″E
Depth	0
Altitude (alti)	0
Number of strains in study	7
Source of isolation of non-type strains	Citrus tree of *Citrus sinensis* cv. 'Valencia late'
Growth medium, incubation conditions used for standard cultivation	28 °C in KBC medium (King B media supplemented with cycloheximide (200 mg/L), cephalexin (80 mg/L) and boric acid (1,5 g/100 mL)
Gram stain	NEGATIVE
Cell shape	rod
Cell size (length or diameter)	2.1–3.5 μm long 0.9–1.0 μm wide
Motility	Motile
If motile	flagellar
If flagellated	one or multiple monopolar flagella
Sporulation (resting cells)	none
Colony morphology	Round, flat, translucent and beige coloured, with regular margins (2–3 mm in diameter) on LB plates after incubation for 48 h at 30 °C
Temperature range	4–37
Lowest temperature for growth	4
Higuest temperature for growth	37
Temperature optimum	25
Lowest pH for growth	5
Highest pH for growth	9
Lowest NaCl concentration for growth	0
Highest NaCl concentration for growth	6
Salinity category	Moderate halophile (optimum 7-15% NaCl)
Positive tests with BIOLOG	Biolog GENIII plates: utilization of D-trehalose, *N*-acetyl-D-glucosamine, alfa-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycogen, D-serine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, methyl pyruvate, L-lactic acid, citric acid, alfa-keto glutaric acid, D-malic acid, L-malic acid, bromo-succinic acid, gamma-amino-butyric acid, alfa-hydroxy butyric acid, beta-hydroxy-D,L-butyric acid, alfa-keto-butyric acid, propionic acid, acetic acid, formic acid and resistant in the chemical sensitivity assays: 1% sodium lactate, fusidic acid, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, guanidine HCl, niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue, nalidixic acid, lithium chloride, potassium tellurite, aztreonam, sodium butyrate, sodium bromate
Negative tests with BIOLOG	Biolog GENIII plates: dextrin, D-maltose, D-cellobiose, gentiobiose, sucrose, turanose, stachyose, D-raffinose, alfa-D-lactose, D-melibiose, beta-methyl-D-glucoside, D-salicin, *N*-acetyl-beta-D-mannosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-neuraminic acid, 3-methyl glucose, L-fucose, D-glucose-6-PO4, D-aspartic acid, gelatin, pectin
Variable tests with BIOLOG	Biolog GENIII plates: *p*-hydroxy-phenylacetic acid
Positive tests with API	Presence of arginine dihydrolase, glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, gluconate, caprate, adipate, malate, citrate, phenylacetate
Negative tests with API (APIN)	Reduction of nitrate to nitrite, indole production, glucose fermentation, urease, hydrolysis of aesculin, hydrolysis of gelatin, beta-galactosidase, maltose
Variable tests with API (APIV)	
Commercial kits used?	BIOLOG GENIII, API 20NE
Energy metabolism	Chemoorganotroph
Oxidase	Positive
Catalase	Positive
Positive tests	Fluorescence on King B agar
Negative tests	Pigment on King A
Biosafety level	1
Habitat	Citrus family (food source) (http://purl.obolibrary.org/obo/FOODON_03411139)
Biotic relationship	Free-living
Known pathogenicity	Plant pathogen

Table 6
Protologue *Pseudomonas nabeulensis* sp. nov.

Taxonumber	TA00744
Species name	*Pseudomonas nabeulensis*
Genus name	*Pseudomonas*
Specific epithet	*nabeulensis*
Species status	sp. nov.
Species etymology	N.L. fem. adj. nabeulensis, na.beul.en'sis pertaining to Nabeul, the geographical location of first isolation.
Designation of the type strain	E10B
Strain collection numbers	CECT 9765, CFBP 8661
16S rRNA gene accession number	LR130177
Alternative housekeeping genes	*rpoD* [LR130158], *gyrB* [LR130168]
Genome accession number	QUZT00000000
Genome status	Draft
Genome size (pb)	6,763,512
GC mol%	60.3
Country of origin	Tunisia
Region of origin	Nabeul
Other	Bni khalled
Date of isolation	2016
Source of isolation	Citrus tree of *C. lemon* cv.'Eureka'
Sampling date	2016
Geographic location	Bni khalled, orchard Chakib
Latitude	36°38′59.2″N
Longitude	10°35′13.8″E
Depth	0
Altitude (alti)	0
Number of strains in study	4
Source of isolation of non-type strains	Citrus tree of *C. lemon* cv. 'Eureka' and *C. sinensis* cv.' Valencia late'
Growth medium, incubation conditions used for standard cultivation	28 °C in KBC médium (King B media supplemented with cycloheximide (200 mg/L), cephalexin (80 mg/L) and boric acid (1,5 g/100 mL)
Gram stain	NEGATIVE
Cell shape	rod
Cell size (length or diameter)	2.1–3.5 μm long 0.9–1.0 μm wide
Motility	Motile
If motile	flagellar
If flagellated	one or multiple monopolar flagella
Sporulation (resting cells)	none
Colony morphology	Round, flat, translucent and beige coloured, with regular margins (1–2 mm in diameter) on LB plates after incubation for 48 h at 30 °C
Temperature range	4–37
Lowest temperature for growth	4
Higuest temperature for growth	37
Temperature optimum	25
Lowest pH for growth	5
Highest pH for growth	9
Lowest NaCl concentration for growth	0
Highest NaCl concentration for growth	6
Salinity category	Moderate halophile (optimum 7–15% NaCl)
Positive tests with BIOLOG	Biolog GENIII plates: utilization of D-trehalose, *N*-acetyl-D-glucosamine, alfa-D-glucose, D-mannose, D-fructose, D-galactose, D-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycogen, D-serine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, methyl pyruvate, L-lactic acid, citric acid, alfa-keto glutaric acid, D-malic acid, L-malic acid, bromo-succinic acid, gamma-amino-butyric acid, alfa-hydroxy butyric acid, beta-hydroxy-D,L-butyric acid, propionic acid, acetic acid, formic acid and resistant in the chemical sensitivity assays: 1% sodium lactate, fusidic acid, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, guanidine HCl, niaproof 4, vancomycin, tetrazolium violet, tetrazolium blue, nalidixic acid, lithium chloride, potassium tellurite, aztreonam, sodium butyrate, sodium bromate
Negative tests with BIOLOG	Biolog GENIII plates: dextrin, D-maltose, D-cellobiose, gentiobiose, sucrose, turanose, stachyose, D-raffinose, alfa-D-lactose, D-melibiose, β-methyl-D-glucoside, D-salicin, *N*-acetyl-beta-D-mannosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-neuraminic acid, 3-methyl glucose, L-fucose, D-glucose-6-PO4, D-aspartic acid, gelatin, pectin, *p*-hydroxy-phenylacetic acid, alfa-keto-butyric acid
Variable tests with BIOLOG	
Positive tests with API	Presence of arginine dihydrolase, hydrolysis of aesculin, glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, gluconate, caprate, adipate, malate, citrate
Negative tests with API (APIN)	Reduction of nitrate to nitrite, indole production, glucose fermentation, urease, hydrolysis of gelatin, beta-galactosidase, maltose, phenylacetate
Variable tests with API (APIV)	
Commercial kits used?	BIOLOG GENIII, API 20NE
Energy metabolism	Chemoorganotroph
Oxidase	Positive
Catalase	Positive
Positive tests	Fluorescence on King B agar
Negative tests	Pigment on King A
Biosafety level	1
Habitat	Citrus family (food source) (http://purl.obolibrary.org/obo/FOODON_03411139)
Biotic relationship	Free-living
Known pathogenicity	Plant pathogen

Both groups of strains were characterized by their morphology and physiological and biochemical traits, as well as through a phylogenomic analysis. These characteristics clearly classified the studied strains in the genus *Pseudomonas*, but they could not be assigned to any known species. Therefore, we conclude that the strains in the KC group are representatives of a new species, for which we propose the name *P. kairouanensis*, with strain KC12^T as the type strain. The E10 strain group is representative of a second new species, for which we propose the name *P. nabeulensis*, with strain E10B^T as the type strain. Both species could be differentiated by some phenotypic traits in the MALDI-TOF MS protein profile. The phylogenomic analyses of these two species, although related, indicate that they belong to different species, as indicated by the threshold values of *rpoD* sequences, the three gene sequence analyses and the ANIb and GGDC values.

Conclusion

The protein profiles of the two groups of strains studied, KC and E10, which were isolated from citrus, were analyzed by MALDI-TOF, confirming the relationships among these strains. The concatenated nucleotide sequence analysis confirms this status and locates these strains in the *P. fluorescens* group and the *P. fluorescens* subgroup, adjacent to *P. grimontii* and *P. canadensis*. Considering the phylogenetic, chemotaxonomic, genotypic and phenotypic characteristics presented, we propose two new species, *P. kairouanensis* sp. nov., with *P. kairuanensis* KC12^T as the type strain, and *P. nabeulensis* sp. nov., with *P. nabeulensis* E10B^T as the type strain. The full descriptions of the new taxa are shown in Tables 5 and 6 as obtained from the Digital Protologue website (http://imedea.uib-csic.es/dprotologue/), in which the new species was registered under references TA000738 and TA000744.

Note

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences reported in this study are as follows: LR031357, LR131200 and LR130172-LR130180 for the 16S rRNA of *P. kairouanensis* (strains KC12^T, KC17, KC20, KC22, KC24A, KC25, KC26) and *P. nabeulensis* (strains E10B^T, E10AB, E10CB1 and Iy3BA); LR131250 and LR130162-LR130171 for the *gyrB* gene of *P. karouanensis* (strains KC12^T, KC17, KC18, KC20, KC22, KC24A, KC25, KC26) and *P. nabeulensis* (strains E10B^T, E10AB, E10CB1 and Iy3BA); and LR131251 and LR130152-LR130161 for the *rpoD* gene of *P. karouanensis* (strains KC12^T, KC17, KC18, KC20, KC22, KC24A, KC25, KC26) and *P. nabeulensis* (strains E10B^T, E10AB, E10CB1 and Iy3BA).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2019.03.002.

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