Erwinia oleae sp. nov., isolated from olive knots caused by Pseudomonas savastanoi pv. savastanoi

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Three endophytic bacterial isolates were obtained in Italy from olive knots caused by Pseudomonas savastanoi pv. savastanoi. Phenotypic tests in combination with 16S rRNA gene sequence analysis indicated a phylogenetic position for these isolates in the genera Erwinia or Pantoea, and revealed two other strains with highly similar 16S rRNA gene sequences (> 99%), CECT 5262 and CECT 5264, obtained in Spain from olive knots. Rep-PCR DNA fingerprinting of the five strains from olive knots with BOX, ERIC and REP primers revealed three groups of profiles that were highly similar to each other. Multilocus sequence analysis (MLSA) based on concatenated partial atpD, gyrB, infB and rpoB gene sequences indicated that the strains constituted a single novel species in the genus Erwinia. The strains showed general phenotypic characteristics typical of the genus Erwinia and whole genome DNA–DNA hybridization data confirmed that they represented a single novel species of the genus Erwinia. The strains showed DNA G+C contents ranging from 54.7 to 54.9 mol%. They could be discriminated from phylogenetically related species of the genus Erwinia by their ability to utilize potassium gluconate, L-rhamnose and D-arabitol, but not glycerol, inositol or D-sorbitol. The name Erwinia oleae sp. nov. (type strain DAPP-PG 531T=LMG 25322T=DSM 23398T) is proposed for this novel taxon.

Knot formation on olive trees (Olea europaea L.) is a serious disease found in many olive producing areas. It is caused by Pseudomonas savastanoi pv. savastanoi and characterized by outgrowth on trunks and branches, and less frequently leaves and fruits (Sisto et al., 2004). Olive knots are ideal niches for bacterial growth, not only of the causal agent of the disease, but also of a number of endophytic members of the class Gammaproteobacteria such as Erwinia toletana (Rojas et al., 2004), Pantoea agglomerans (Marchi et al., 2006; Quesada et al., 2007) and other bacteria from the genera Burkholderia, Hafnia, Pseudomonas and Stenotrophomonas (Ouzari et al., 2008). In the last few years, several studies have focussed on the effect of these endophytes in modulating olive knot disease severity (Marchi et al., 2006; Hosni, 2010). As such, it has been shown that Pantoea agglomerans, frequently isolated from olive knots when inoculated in olive plants together with Pseudomonas savastanoi pv. savastanoi, can either depress growth of the pathogen or produce an increase in knot size (Marchi et al., 2006).

In the present study, five endophytic strains from olive knots (DAPP-PG 531T, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264) were investigated using a polyphasic taxonomic approach.

In September 2003 and May 2007, young knots from branches of diseased olive trees located in orchards at Scanzano in the province of Perugia (Umbria, central Italy) and Valenzano in the province of Bari (Apulia, south Italy) were collected. Small portions of their internal water-soaked tissue were excised with a scalpel and crushed in a few drops of sterile distilled water. Subsequently, a loopful of the suspensions was streaked onto nutrient agar (NA; Oxoid) and the plates incubated at 27 ± 1 °C for 2 days. Along with circular (0.5–2.9 mm in diameter), white to pale yellow colonies resembling a ‘fried egg’, typical for Pseudomonas savastanoi pv. savastanoi, another bacterial colony type was frequently isolated. The latter type was...
selected for further investigation. Pure cultures of this type were obtained by picking up a single colony and streaking it onto NA plates amended with 5% sucrose. This way, strains DAPP-PG 531$^T$ and DAPP-PG 537 were obtained in 2003 and strain DAPP-PG 672 in 2007. Initial microbiological characterization of these strains revealed that their colonies were not fluorescent when cultivated on King’s medium B (20 g peptone l$^{-1}$, 1.5 g anhydrous K$_2$HPO$_4$ l$^{-1}$, 1.5 g MgSO$_4$ l$^{-1}$, 10 ml glycerol l$^{-1}$, 15 g agar l$^{-1}$). It also revealed that their cells were Gram-negative (as they lysed in 3% KOH; Suslow et al., 1982), oxidase-negative, catalase-positive and facultatively anaerobic, suggesting that they belonged to the family Enterobacteriaceae. Additional strains used in this study were obtained from various biological resource centres and cultivated following the instructions of the provider. All bacterial strains used in this study are listed in Supplementary Table S1 (available in IJSEM Online).

Genomic DNA was extracted from strain DAPP-PG 531$^T$ according to the protocol of Niemann et al. (1997). Amplification of the 16S rRNA gene was performed with the conserved primers 16F27 (5‘-AGAGTTTGTATCCTGGCTCAG-3‘) and 16R1522 (5‘-AAGGAGGTGATCCAGCCGCA-3‘). Purification of the amplification product was conducted with the NucleoFast 96 PCR Clean-up kit (Macherey-Nagel). Sequencing reactions were performed with the internal primers listed by Coenye et al. (1999) using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Purification of the sequencing reaction products was conducted using the BigDye XTerminator$^R$ Purification kit (Applied Biosystems). Sequencing was performed using an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). Sequence assembly was done using the software package BioNumerics (Applied Maths). A nearly complete 16S rRNA gene sequence (1494 nt) was obtained for strain DAPP-PG 531$^T$ and compared with 16S rRNA gene sequences deposited at NCBI, using BLAST. This analysis indicated that strain DAPP-PG 531$^T$ belonged to the genera Erwinia or Pantoea, and it revealed two strains with very similar 16S rRNA gene sequences (>99% pairwise similarity), ‘Pantoea oleae’ CECT 5262 and CECT 5264, that were obtained in Spain from olive knots. Using the software package BioNumerics (Applied Maths), nearly complete 16S rRNA gene sequences of strains DAPP-PG 531$^T$, CECT 5262 and CECT 5264 were compared with those of reference strains of the species of the genera Erwinia, Pantoea and related taxa collected from GenBank. Pairwise similarities were calculated using an open gap penalty of 100% and a unit gap penalty of 0%. A neighbour-joining phylogenetic tree (Fig. 1) was constructed using BioNumerics, and the robustness of the branches was evaluated by bootstrap analysis (Felsenstein, 1985). A maximum-likelihood phylogenetic tree was constructed (see Supplementary Fig. S1 in IJSEM Online) as described previously (Brady et al., 2008). The three strains from olive knots showed more than 99% 16S rRNA gene sequence similarity with each other and less than 97% with recognized species of the genera Erwinia and Pantoea. As strains showing less than 97% 16S rRNA gene sequence similarity are not likely to have more than 60–70% DNA–DNA relatedness (Stackebrandt & Goebel, 1994), these similarity values strongly suggested that the strains from olive knots represented at least one novel species in the family Enterobacteriaceae.

Genomic DNA was extracted from strains DAPP-PG 531$^T$, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 and from Erwinia toletana CFBP 6631$^T$ with the GenElute Bacterial Genomic DNA kit (Sigma Aldrich). Rep-PCR fingerprinting was performed with the BOX (Versalovic et al., 1994), ERIC (Hulton et al., 1991) and REP (Higgins et al., 1982; Versalovic et al., 1991) primers, according to the method described by Rademaker & de Bruijn (1997). Repeat experiments were performed and identical results were obtained. The rep-PCR profiles are shown in Supplementary Fig. S2. Irrespective of the primers used, strains DAPP-PG 531$^T$ and DAPP-PG 537 generated the same fingerprints as well as strains CECT 5262 and CECT 5264. The Dice coefficient between the two groups of strains was 0.88. Strain DAPP-PG 672 had a similarity index of 0.88 with strains DAPP-PG 531$^T$ and DAPP-PG 537 and 0.94 with strains CECT 5262 and CECT 5264. E. toletana CFBP 6631$^T$ generated different fingerprints and with low similarity (0.36) in comparison with the other tested strains. Based on previous studies (Gevers et al., 2001; De Vuyst et al., 2008), the rep-PCR data suggested that strains DAPP-PG 531$^T$, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 probably constituted a single species.

Multilocus sequence analysis (MLSA) of concatenated partial atpD, gyrB, infB and rpoB gene sequences enables the phylogenetically related genera Erwinia, Pantoea and Tatumella to be differentiated from each other (Brady et al., 2008, 2009, 2010a, b, c). To refine the taxonomic position of the five strains from olive knots, partial sequences of the above-mentioned housekeeping genes were determined for three representative strains, DAPP-PG 531$^T$, DAPP-PG 672 and CECT 5264, and 12 reference strains from recognized species of the genus Erwinia (see Fig. 2 and Supplementary Fig. S3; gene sequences from GenBank accession nos HM439612–HM439619, GU991653–GU991656, HQ393588–HQ393635 were determined in this study). Partial fragments of the atpD, gyrB, infB and rpoB genes for strains DAPP-PG 531$^T$, DAPP-PG 672 and CECT 5264 were amplified and sequenced using the protocol of Brady et al. (2008) with the following modifications: (i) primer atpD 08-R (5‘-CCGAGCCGGAGATCCTC-3‘) was used instead of atpD 04-R; (ii) primer infB 05-F (5‘-ACGGBATGRTBACSTTCCTK-3‘) was used instead of infB 03-F. The modifications were needed because good technical sequences could not be obtained with primers atpD 04-R and infB 03-F, probably because they could not bind efficiently. Primers atpD 08-R and infB 05-F were designed based on sequences obtained with the primers atpD 03-F and infB 04-R, respectively. Sequence assembly was performed using the BioNumerics software package (Applied Maths), and partial nucleotide atpD, gyrB, infB and rpoB gene sequences were concatenated and aligned with concatenated partial atpD,
gyrB, infB and rpoB gene sequences of reference strains of species of the genera Erwinia, Pantoaea and Tatumella taken from GenBank. The BioNumerics software package (Applied Maths) was used for this analysis and neighbour-joining and maximum-likelihood phylogenetic trees (Fig. 2 and Supplementary Fig. S3) were constructed as described for the 16S rRNA gene. MLSA revealed that strains DAPP-PG 531\textsuperscript{T}, DAPP-PG 672 and CECT 5264 belonged to the genus Erwinia oleae sp. nov. and related taxa within the family Enterobacteriaceae. Brenneria alni ICMP 12481\textsuperscript{T} was used as the outgroup. Bar, 1% nucleotide substitutions. Numbers at branching points are bootstrap percentage values based on 1000 replications. Only values >50% are shown.

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the phylogenetic relationship between Erwinia oleae sp. nov. and related taxa within the family Enterobacteriaceae. Brenneria alni ICMP 12481\textsuperscript{T} was used as the outgroup. Bar, 1% nucleotide substitutions. Numbers at branching points are bootstrap percentage values based on 1000 replications. Only values >50% are shown.
Erwinia and also suggested that they probably constituted a single novel species.

Strains DAPP-PG 531\textsuperscript{T}, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 were subjected to API 20E and API 50CHE systems (bioMérieux), according to the manufacturer’s instructions. The results are presented in the species description below. API 50CHE tests were also carried out on type and reference strains of the 12 recognized species of the genus Erwinia. The strains studied are presented in Supplementary Fig. S4, and the data obtained were analysed numerically to reveal the phenotypic relationship between the five strains from olive knots and the recognized species of the genus Erwinia. A distance matrix was calculated from similarity matrices generated using the Dice coefficient (Dice, 1945) and subjected to the unweighted pair-group method with arithmetic mean (UPGMA) clustering algorithm using NTSYSpc software (Exeter Software) version 2.1. A cophenetic value of 0.86 was determined for this matrix, which indicated a high goodness-of-fit. The dendrogram in Fig. 2. Neighbour-joining tree based on concatenated partial atpD, gyrB, infB and rpOB gene sequences showing the phylogenetic relationship between Erwinia oleae sp. nov. and related taxa of the genera Erwinia, Pantoea and Tatumella. Cronobacter sakazakii ATCC BAA-894 was included as the outgroup. Bar, 10\% nucleotide substitutions. Numbers at branching points are bootstrap percentage values based on 1000 replications. Only values >50\% are shown.

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Supplementary Fig. S4 revealed that strains DAPP-PG 531<sup>T</sup>, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 formed a very homogeneous cluster with an overall similarity of about 93% that was well discriminated from the 12 recognized species of the genus Erwinia that each formed a separate cluster. It also showed that the five strains from olive knots had phenotypic features common to the genus Erwinia. Table 1 lists a selected number of phenotypic features that enable strains DAPP-PG 531<sup>T</sup>, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 to be differentiated from recognized species of the genus Erwinia, including Erwinia toletana, the phylogenetically most closely related species of the genus also isolated from olive knots. Table 1 also reveals that the strains can be discriminated from each recognized species of the genus Erwinia, including Erwinia piriflorinigrans (López et al., 2011), by at least two characteristics.

To confirm whether or not strains DAPP-PG 531<sup>T</sup>, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 truly constituted a single novel species of the genus Erwinia, DNA–DNA hybridizations were performed. High-molecular mass DNA for DNA–DNA hybridization studies and DNA base composition determination was extracted using the method of Wilson (1987), with minor modifications (Cleenwerck et al., 2002). DNA quantity and quality were determined by measuring the absorptions at 260, 280 and 234 nm and only high quality DNA with A<sub>260</sub>/A<sub>280</sub> and A<sub>235</sub>/A<sub>260</sub> ratios of 1.8–2.0 and 0.40–0.60, respectively, was selected for further use. The size of the DNA was estimated by agarose gel electrophoresis. DNA–DNA hybridizations were performed with strains DAPP-PG 531<sup>T</sup>, DAPP-PG 672 and CECT 5264 and E. toletana LMG 24162<sup>T</sup> using a modification (Goris et al., 1998; Cleenwerck et al., 2002) of the microplate method described by Ezaki et al. (1989). The hybridization temperature was 44°C. Reciprocal reactions (A × B and B × A) were performed and their variation was generally taken to be within the limits of this method (Goris et al., 1998). The DNA–DNA relatedness values reported are the mean of minimum six hybridizations. Strains DAPP-PG 531<sup>T</sup>, DAPP-PG 672 and CECT 5264 exhibited high levels of DNA–DNA relatedness (>80%) and showed low levels (<25%) with E. toletana LMG 24162<sup>T</sup> (Table 2). As levels of 60–70% DNA–DNA relatedness are generally accepted as the limit for species delineation (Wayne et al., 1987), the DNA–DNA hybridization results confirmed that the strains from olive knots represented a single novel species of the genus Erwinia.

The DNA G + C content of strains DAPP-PG 531<sup>T</sup>, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 was determined by HPLC according to the method of Mesbah et al. (1989), and varied from 54.7 to 54.9 mol%, which was within the range reported previously for members of the genus Erwinia (Hauben et al., 1998; Mergaert et al., 1999; Kim et al., 1999; Gardan et al., 2004; Rojas et al., 2004; Geider et al., 2006). The DNA G + C content range was also less than 2%, the generally accepted range within a species.

**Table 1.** Phenotypic characteristics differentiating strains of *Erwinia oleae* sp. nov. from other species of the genus *Erwinia*

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*The strains tested for each *Erwinia* species are given in Supplementary Table S1.
‡Data from López et al. (2011).
§Tests performed according to Schaad et al. (2001).
In conclusion, based on the genotypic data (from 16S rRNA gene sequence analysis, rep-PCR DNA fingerprinting, MLSA and DNA–DNA hybridizations) and phenotypic data obtained in this study, the five endophytic bacterial strains DAPP-PG 531<sup>T</sup>, DAPP-PG 537, DAPP-PG 672, CECT 5262 and CECT 5264 from olive knots, caused by *Pseudomonas savastanoi* pv. *savastanoi*, represent a novel species for which the name *Erwinia oleae* sp. nov. is proposed. Strain DAPP-PG 531<sup>T</sup> (=LMG 25322<sup>T</sup> = DSM 23398<sup>T</sup>) is the type strain.

**Description of *Erwinia oleae* sp. nov.**

*Erwinia oleae* [o’leae. L. gen. fem. n. oleae of olive (*Olea europaea*), the plant from which the bacterium was isolated]. Strains have all the characteristics of members of the family *Enterobacteriaceae*. Cells are Gram-negative rods, measuring 0.9 × 1.5–3.0 μm, and occur as single and pairs. Cells are motile and non-spore-forming. After growth for 24–48 h on nutrient agar at 27 ± 1 °C, colonies are light beige, circular (1–1.2 mm in diameter) and convex and with entire margins. Does not produce fluorescent pigment on King’s medium B. Growth in yeast salt and lipid 523 medium (Schaad *et al.*, 2001) occurs at 36 °C, but not at 39 °C. Able to grow in 5% (w/v) NaCl. Facultatively anaerobic. Oxidase is not produced. Results obtained with API20E tests (bioMérieux) give a positive result for β-galactosidase activity, but a negative result for activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase, phenylalanine deaminase and gelatinase (except strain DAPP-PG 672). Citrate is not utilized; hydrogen sulfide, indole and acetoin (except strains CECT 5262 and CECT 5264) are not produced. Nitrate is reduced to nitrite. Results obtained with API 50CHE tests (bioMérieux) indicate that strains utilize the following substrates as sole carbon sources at 27 ± 1 °C within 2 days: L-arabinose, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, N-acetylgalcosamine, ascorul, trehalose, D-arabitol, potassium glucanate and potassium 2-ketogluconate, and arbutin and salicin (except strains DAPP-PG 531<sup>T</sup> and DAPP-PG 537). The following carbon sources are not utilized at 27 ± 1 °C within 2 days: glycerol, erythritol, D-arabinose, D-xylene, L-xylene, D-adenitol, methyl β-D-xylopyranoside, L-sorbose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, cellobiose, maltose, lactose, melibiose, sucrose, inulin, melezitose, raffinose, starch, glycogen, xyitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol and potassium 5-ketogluconate. The DNA G + C content of the five strains ranges from 54.7 to 54.9 mol% as determined by the method of Mesbah *et al.* (1989).

The type strain, DAPP-PG 531<sup>T</sup> (=LMG 25322<sup>T</sup> = DSM 23398<sup>T</sup>) and DAPP-PG 537 (=LMG 25323 = DSM 23412), were isolated in Umbria (Italy) from olive knots caused by *Pseudomonas savastanoi* pv. *savastanoi*. Additional strains were isolated in Apulia (Italy) (DAPP-PG 672 = LMG 25321 = DSM 23411) and Spain (CECT 5262 = LMG 25327, CECT 5264 = LMG 25328) also from olive knots caused by *Pseudomonas savastanoi* pv. *savastanoi*.

**Acknowledgements**

This research was supported by ‘Fondazione Cassa di Risparmio di Perugia’, Italy, project ‘Indagini sui batteri endofiti associati a piante di olivo affette da roagna, volte all’individuazione di nuove strategie di lotta alla malattia’ to R. B. The BCCM/LMG Bacteria Collection is supported by the Federal Public Planning Service – Science Policy, Belgium. C. B. is the beneficiary of a fellowship granted by the Federal Science Policy Office, Belgium. The authors wish to acknowledge Katrien Engelbeen and Luca Bonciarelli for their technical support.

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