Erwinia toletana sp. nov., associated with *Pseudomonas savastanoi*-induced tree knots

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Gram-negative bacteria were isolated from knots induced by *Pseudomonas savastanoi* in olive trees (*Olea europaea* L.). A total of nine endophytic bacterial strains were isolated, each from inside a different tree knot. Biochemical characterization indicated that all the strains belong to the family *Enterobacteriaceae*. Phylogenetic analyses of the 16S rRNA genes of these novel isolates revealed that they formed a homogeneous cluster within *Erwinia* species. DNA signatures of these isolates were identical to those described for the genus *Erwinia*. The strains formed a homogeneous group as shown by DNA–DNA hybridization analysis and numerical analysis of phenotypic data, clearly differentiated from all species of *Erwinia* with validly published names. The data provide strong evidence of the differentiation of these strains from the most closely related species. Therefore, these isolates represent a novel species, for which the name *Erwinia toletana* sp. nov. is proposed. The isolates are available at CFBP, CECT and ATCC. The G+C content is 52±0·5 mol%. The type strain is CFBP 6631^T (=A37^T=ATCC 700880^T=CECT 5263^T).

Ewing & Fife (1971, 1972) concluded that strains isolated from clinical sources and strains belonging to the *Herbicola* group of Dye (1969) are the same species and referred to them as the '*Enterobacter agglomerans–Erwinia herbicola*' complex. Lelliott & Dickey (1984) defined *Erwinia* as associated with plants as pathogens, saprophytes or constituents of the epiphytic flora. They considered *Erwinia herbicola* as yellow and non-pigmented *Erwinia*-like organisms that exist either on plant surfaces or as secondary organisms in lesions caused by many plant pathogens, as described by Billing & Baker (1963). Gavini *et al.* (1989) described the new genus *Pantoea* and the species *Pantoea agglomerans*, which includes the type strains of *Enterobacter agglomerans*, *Erwinia herbicola* and *Erwinia milletiae*. Later, *Erwinia ananatis* (synonym *Erwinia uredovora*) and *Erwinia stewartii* were transferred to the genus *Pantoea* (Mergaert *et al.*, 1993). Based on 16S rRNA gene phylogenetic analyses, plant-associated bacteria were reclassified into four genera: *Erwinia*, *Pectobacterium*, *Brenneria* and *Pantoea* (Hauben *et al.*, 1998). Mergaert *et al.* (1999) reclassified non-pigmented *Erwinia herbicola* epiphytic strains isolated from trees as *Erwinia billingiae*, which clades within the first cluster of Hauben *et al.* (1998).

Samples from diseased olive trees were collected from the Navahermosa and Chozas areas in the Toledo region of central Spain. Strains from knots were isolated according to the method of García de los Ríos (1999) and were grown on King's medium B (KB) and nutrient agar for 48 h at 25 °C. Two colony types were easily distinguishable on both agar media. Pure cultures were established by single colony isolation onto fresh KB agar. The first type was identified as *Pseudomonas savastanoi*. The second type, which corresponded to large (3–5 mm), mucilaginous, pigmented and non-pigmented colonies, was identified as a member of

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences reported in this study are AF130885, AF130909, AF130910, AF130962–AF130966, AF130968 and AF130953.

A majority-rule consensus tree and a summary of discriminatory characters are available as supplementary material in IJSEM Online.

Erwinia sensu lato by Gram stain, catalase, oxidase and phenylalanine deaminase activities, Kligler iron agar fermentation, methyl red reaction and motility. No *Xanthomonas* strains were identified. In order to determine more precisely the taxonomic status of these novel isolates, a polyphasic taxonomic study was initiated.

For sequence determination, total bacterial genomic DNA was isolated from the nine novel isolates by a CTAB miniprep procedure (Murray & Thompson, 1989). Bacteria were suspended in an extraction buffer containing 2 μl RNase A (1 mg ml^{-1}) and incubated at 37 °C for 1 h. The samples were then microcentrifuged at 15 000 r.p.m. for 5 min. The supernatant was collected and the DNA was precipitated, resuspended in TE and then quantified spectrophotometrically and adjusted to a concentration of approximately 100 ng μl^{-1}. An internal portion of the 16S rRNA gene sequence was obtained for each isolate using primer $P16S_{27F}$, which anneals at position 27 of the *Escherichia coli* 16S rRNA gene (5′-ATTGAACGCTGGCGGCAGGCCTAA-3′), and primer $P16S_{1455R}$ (5′-CCTTGTTACGACTTCACCCCAGTC-3′), derived in this study from alignments of *Erwinia* and other enterobacteria species available in databases.

PCR was performed in a final volume of 25 μl containing 0·5 μM of each primer, 200 μM dNTPs, 1·5 mM $MgCl_2$, 1·25 U *Taq* polymerase, 1× *Taq* polymerase reaction buffer and 25 ng chromosomal DNA. An initial denaturation step was performed in a thermocycler (Perkin Elmer, model 9600) at 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. Reactions were stored at 4 °C. A negative control contained sterile water instead of DNA template.

DNA was purified using a QIaexII gel extraction kit (Qiagen) and then ligated into a plasmid vector (TA cloning kit; Invitrogen) and transformed into cells of *Escherichia coli* INValpha-F′. The transformants were selected according to the blue-white screening procedure (Sambrook *et al.*, 1989). Transformants were screened for the presence of an insert by PCR amplification using M13 phage primers ($M13_{fwd}$, 5′-GGAAACAGCTATGACCAT-3′; $M13_{rev}$, 5′-CGTTGTAAAACGACGGCCAG-3′). Following confirmation of inserts, plasmid DNA was extracted using Qiaquick plasmid miniprep (Qiagen). At least 1 μg DNA was used for sequencing and at least two clones of each strain were sequenced in both directions using a Li-Cor Sequencer. Sequencing reactions were prepared using the Sequenase kit with 7-deaza-dGTP (Amersham Pharmacia) according to the manufacturer's instructions. The fluorescent-labelled sequencing primers correspond to $M13_{fwd}$ and $M13_{rev}$ (see above). The sequences from each strain were aligned using the SEQUENCHER 3.0 software for Macintosh (Gene Codes Corporation) in order to trim away the vector sequence and to establish a consensus sequence for the insert.

Prior to phylogenetic analysis, representative sequences of several members of the *Enterobacteriaceae* were extracted from NCBI via BLAST searches (Altschul *et al.*, 1990). Phylogenetic assays were conducted to identify the overall position of these strains in trees containing several sequences (data not shown). After this preliminary analysis, all species of *Erwinia* with validly published names (with the exception of *Erwinia aphidicola*, for which a 16S rRNA gene sequence is not available) and the closest phylogenetic neighbours from other genera were selected to perform more detailed phylogenies. All the sequences are derived from type strains with two exceptions (see Fig. 1 and Supplementary Fig. A, available in IJSEM Online). Sequence similarity comparisons were then conducted. Similarity values over 97 % were found for the following species: *Erwinia billingiae* (97·2 %), *Pantoea ananatis* (97·3 %), *Pantoea agglomerans* (98 %), *Erwinia persicina* (98 %), *Erwinia rhapontici* (98·1 %), *Erwinia amylovora* (97·4 %) and *Erwinia pyrifoliae* (97·1 %). As values of similarity higher than 97 % are generally not used to discriminate at the species level, DNA–DNA hybridization experiments were conducted for these strains. For other *Erwinia* species, the values were lower: *Erwinia tracheiphila* (95·6 %), *Erwinia mallotivora* (96·6 %) and *Erwinia psidii* (96·5 %). Nonetheless, we included in our analysis all species of *Erwinia* with validly published names, independently of the similarity value.

The sequences of all species of *Erwinia* with validly published names (accession numbers indicated in the phylogenetic trees) were aligned using the T-COFFEE tool (Notredame *et al.*, 2000) and further subjected to parsimony analysis using MEGA2.1 (http://www.megasoftware.net) as well as Bayesian probabilistic analysis using MrBayes (Huelsenbeck & Ronquist, 2001). The phylogenetic trees derived from these analyses included 1463 nucleotides and are shown in Fig. 1 (Bayesian tree) and in Supplementary Fig. A (parsimony tree). In the Bayesian analysis, 100 000 generations were run in four independent Markov chains. When convergence was reached, a total of 9000 trees were explored; Fig. 1 shows a consensus tree. The novel isolates form a very homogeneous group supported by a 99 % clade confidence value. The reliability of the parsimony tree (Supplementary Fig. A) was tested by a bootstrap analysis with 1000 replicates. Both trees showed the same topology with high confidence values.

Nucleotide signatures in the 16S rRNA genes are considered useful characters for assessing relatedness of bacteria. Hauben *et al.* (1998) assessed the 42 'signature' nucleotide positions in the 16S rRNA gene that can be used to classify different genera of the *Enterobacteriaceae*. Fifteen nucleotide positions comprise the genus *Erwinia* signature. These positions are indicated in the species description. All the novel isolates are identical in all 15 of the characteristic nucleotide signatures of *Erwinia* species.

Twenty-two conventional biochemical and physiological tests and assimilation of 99 carbon sources using Biotype 100 strips (bioMérieux) were performed, as described

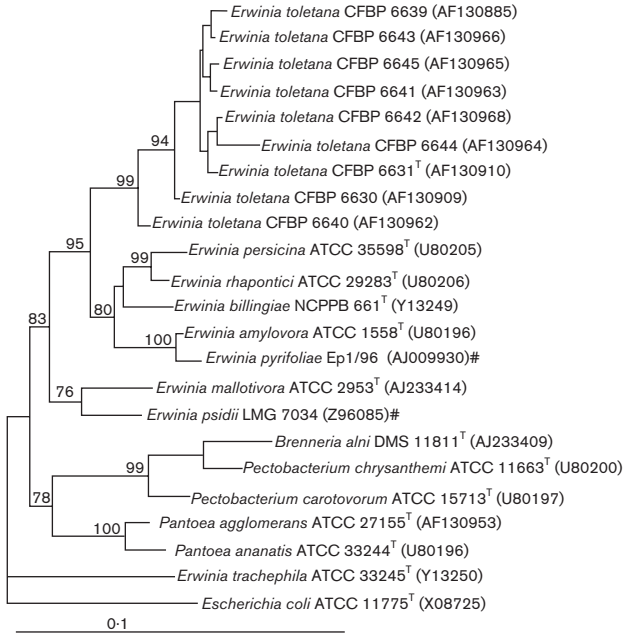

Fig. 1. Consensus (Bayesian) phylogenetic tree of 16S rRNA gene sequences. The likelihood model parameters are general time-reversible (GTR; number of states is 6) and gamma-distributed rate variation. The model was run for 100 000 generations in four independent Markov chains. When convergence was reached, 9000 trees were sampled and a consensus was generated. Numbers are clade confidence values in 9000 trees. Symbol # indicates unavailability of a 16S rRNA gene sequence for the corresponding type strain; therefore another strain was used. GenBank accession numbers are given in parentheses. *Escherichia coli* is the outgroup.

previously (Sutra *et al.*, 2001). A total of 121 tests were included in a numerical taxonomy analysis for 56 strains including the newly isolated strains and 43 reference or type strains representing the phylogenetic neighbours based on 16S rRNA gene sequence analysis. The distance matrix was calculated using the Jaccard coefficient (Sneath & Sokal, 1973). Cluster analysis was performed by using the unweighted pair group method with arithmetic averages (Sneath & Sokal, 1973). Discriminatory tests were selected using the diagnostic ability coefficient deduced from the numerical analysis (Descamps & Véron, 1981). The dendrogram of phenotypic distances among the 56 strains is shown in Fig. 2. At the distance level of 0·3277, 10 phena and 12 unclustered type strains were delineated. The phenotypic characteristics that differentiate the 10 phena and the unclustered type strains were deduced from the numerical taxonomy analysis (see Supplementary Table A in IJSEM Online). Phenon 1 gathered all nine endophytic strains shown in the phylogenetic tree (Fig. 1) at a distance of 0·06. Except *Erwinia amylovora* and *Erwinia pyrifoliae*, the closest species identified by 16S rRNA gene sequence comparisons (Fig. 1), i.e. *Erwinia billingiae*, *Erwinia rhapontici* and *Erwinia persicina*, as well as *Pantoea agglomerans*, *Pantoea ananatis* and *Pectobacterium cypripedii*, were found to be related to the endophytic strains on the basis of their phenotypic characteristics (Fig. 2). *Erwinia amylovora* and *Erwinia pyrifoliae* were phenotypically very distant and can be distinguished from the endophytic strains by their ability to assimilate sucrose and to produce reducing compounds from sucrose and their inability to grow at 36 °C.

The nine strains formed a very homogeneous phenon, which can be clearly distinguished from the other species by numerous phenotypic characteristics (Supplementary Table A). The major discriminating character of phenon 1 is the lack of assimilation of sucrose. Among the 56 strains studied, this character is only shared by two other strains, the type strains of *Erwinia billingiae* and *Enterobacter cancerogenus*.

For DNA–DNA hybridization studies, DNA extractions were performed with the Qiagen Plasmid Mega kit according to an adapted protocol for genomic DNA extraction. For each strain, bacteria were grown overnight at 25 °C on four trypticase soy agar plates. Cultures were scraped off in TES buffer (50 mM EDTA, 50 mM Tris/HCl, 100 mM NaCl, pH 8·0) and centrifuged at 5000 ***g*** for 30 min at 4 °C. Bacterial pellets were washed twice in TES buffer and resuspended in 26 ml B1 buffer (50 mM Tris/HCl, 50 mM EDTA, 0·5 % Tween 20, 0·5 % Triton X-100; pH 8·0). Cell suspensions were freeze–thawed from −20 to 37 °C, diluted fourfold in B1 buffer and distributed into four tubes. Each suspension was thoroughly mixed with 1 ml of a lysozyme solution (100 mg ml^{-1}), 1·25 ml of a 5 mg Pronase ml^{-1} solution (Sigma) and 900 µl of a 25 % SDS solution and incubated overnight at 37 °C. Fourteen microlitres of a 100 mg RNase ml^{-1} solution [98 Kunitz units (mg protein)$^{-1}$] were added to each tube, which were then incubated for 1 h at 60 °C. After addition of 9·5 ml B2 buffer (3 M guanidine hydrochloride, 20 % Tween 20) to the clear lysate, the tubes were incubated for 30 min at 50 °C. The following steps of the DNA extraction are described in the Qiagen genomic DNA handbook, but volumes are adapted. After the flow of the sample through the equilibrated tip, the tip was washed twice with 100 ml Qiagen QC buffer. Genomic DNA was eluted with 35 ml Qiagen QF buffer and precipitated with 0·7 vols room-temperature 2-propanol. The DNA was then dissolved in 1 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7·5); the concentration of the extracted DNA was determined by measuring the A_{260} and the purity was checked by determination of the value A_{260}/A_{280}. Native DNA of strain CFBP 6631^T was labelled *in vitro* with tritium-labelled nucleotides (Amersham) by random-priming (Feinberg & Vogelstein, 1983) using a Megaprime kit (Amersham). The S1 nuclease-trichloroacetic acid method was used for DNA–DNA hybridizations (Crosa *et al.*, 1973; Grimont *et al.*, 1980). The reassociation was performed at 60 °C in 0·42 M NaCl.

DNA–DNA hybridizations experiments with the labelled DNA of strain CFBP 6631^T showed very high levels of

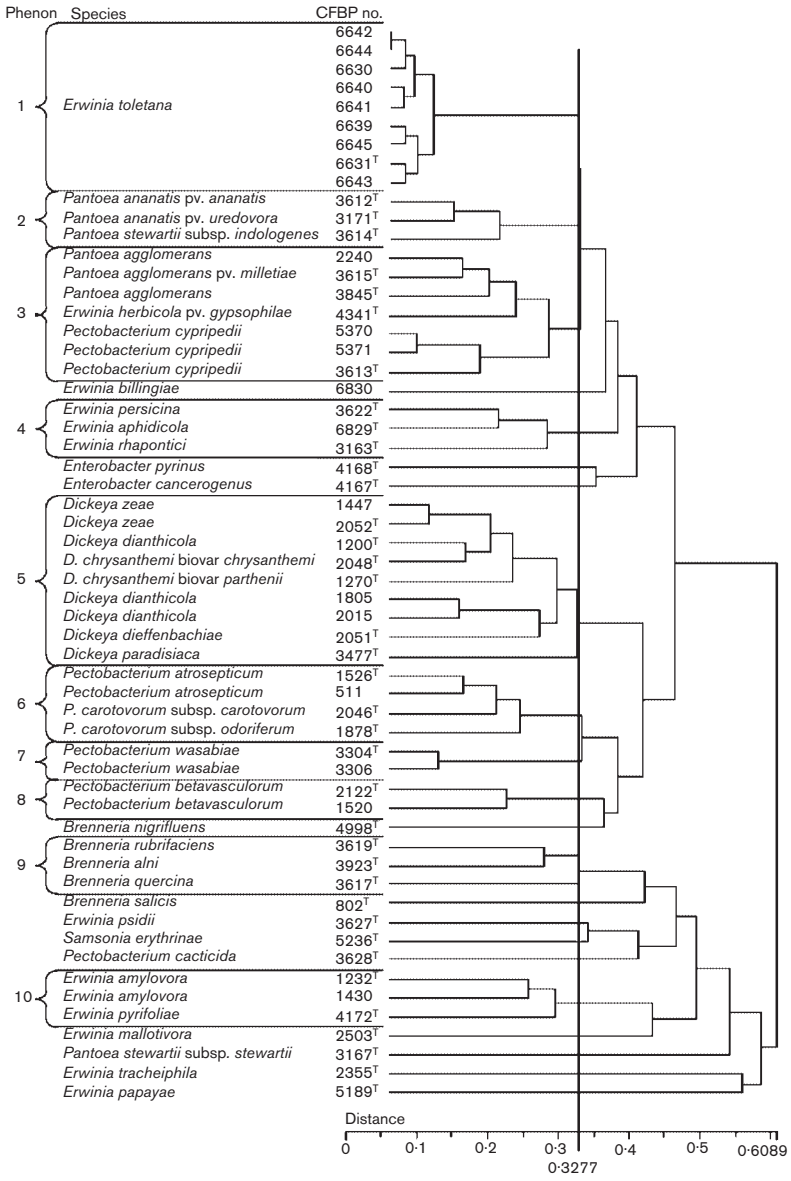

Fig. 2. Dendrogram of phenotypic characteristics of the 56 strains based on the unweighted pair group method with averages. Distance is shown as 1−Jaccard coefficient.

reassociation (93–100 % hybridization) within the endophytic strains. In contrast, low levels of reassociation (9–22 % hybridization) were observed with DNAs from type strains of the closest phylogenetic neighbours: *Erwinia rhapontici*, *Erwinia persicina*, *Erwinia billingiae*, *Erwinia pyrifoliae* and *Erwinia amylovora* (Fig. 1). From the DNA–DNA hybridization results, we can conclude that the nine strains of phenon 1 belong to the same genomospecies (Table 1).

The G+C content of the DNA of strains CFBP 6631^T and CFBP 6630, measured using the denaturation method of Marmur & Doty (1962), was 52±0·5 mol%. This value is in the range of the G+C contents of other *Erwinia* species (Hauben *et al.*, 1998).

To summarize, phenotypic tests, 16S rRNA gene sequence analysis, nucleotide signature identification and DNA–DNA hybridization procedures were performed on nine endophytic bacterial strains associated with olive trees, all of which were members of the *Enterobacteriaceae* with at least a superficial resemblance to the genus *Erwinia*. Phylogenetic analyses of 16S rRNA gene sequences, using two different methods, and nucleotide signature identification confirmed their taxonomic position in the genus *Erwinia*. DNA–DNA hybridizations experiments supported their status as a novel species. Numerical analysis of 121 phenotypic tests allowed determination of discriminatory characters.

Description of *Erwinia toletana* sp. nov.

Erwinia toletana (to.le.ta′na. L. fem. adj. *toletana* from *Toletum*, the Roman name for Toledo, the location from which the organisms were isolated).

Table 1. Levels of DNA–DNA reassociation among *Erwinia toletana* sp. nov. strains, type strains of *Erwinia* and *Pantoea agglomerans* and *Pectobacterium cypripedii* strains

Strain	Binding with labelled DNA from CFBP 6631^T (%)
Erwinia toletana sp. nov.	
CFBP 6631^T (=A37^T)	100
CFBP 6630 (=A33)	100
CFBP 6639 (=A4)	100
CFBP 6640 (=A43)	100
CFBP 6641 (=A64)	100
CFBP 6642 (=A75)	100
CFBP 6643 (=A78)	100
CFBP 6644 (=A82)	93
CFBP 6645 (=A89)	100
Erwinia persicina CFBP 3622^T	22
Erwinia rhapontici CFBP 3163^T	18
Erwinia billingiae CFBP 6830^T	19
Erwinia amylovora CFBP 1232^T	15
Erwinia pyrifoliae CFBP 4172^T	9
Erwinia papayae CFBP 5189^T	16
Erwinia psidii CFBP 3627^T	4
Erwinia tracheiphila CFBP 2355^T	3
Erwinia mallotivora CFBP 2503^T	11
Erwinia aphidicola CFBP 6829^T	10
Pantoea agglomerans CFBP 4898*	11
Pectobacterium cypripedii CFBP 3613^T	10

*The type strain of *Erwinia herbicola* was used.

Strains show the general characteristics of the *Enterobacteriaceae* and the specific characteristics of the genus *Erwinia*, as described by Hauben *et al.* (1998). Cultures are Gram-negative, oxidase-negative, catalase-positive, motile and ferment glucose without gas formation. The optimal growth temperature is 28 °C. Growth occurs at 36 °C but not at 39 °C. Colonies grown on nutrient agar are circular, slightly convex with entire margins, translucent and non-pigmented. In KB medium, colonies are circular, convex, highly mucoid, translucent and non-pigmented. Gelatin is not liquefied; indole, acetoin and hydrogen sulfide are not produced. The methyl red reaction is positive and nitrate is not reduced to nitrite. Strains exhibit β-galactosidase, α-galactosidase and β-glucosidase activity, but no urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, phenylalanine deaminase, *N*-acetyl-β-glucosaminidase, α-maltosidase, lipase or L-aspartic acid arylamidase activities. Acid is produced when growth medium contains mannitol, glucose, trehalose, *meso*-inositol, melibiose, amygdalin, L-arabinose, galacturonate, maltose, D-arabitol or cellobiose. Strains grow on the following substrates as sole carbon sources: D-glucose, L-arabinose, D-ribose, mannitol, salicin, melibiose, citrate, acetate, propionate, 2-ketogluconate, *N*-acetylglucosamine, malonate, L-alanine, L-proline and L-serine. Strains possess signature nucleotides identical to the signatures described by Hauben *et al.* (1998) for the genus *Erwinia*: A408, A594, C598, G639, G646, C839, G847, G987, G988, C989, G1216, C1217, C1218, C1308 and G1329, using the *Escherichia coli* 16S rRNA gene sequence numbering (Brosius *et al.*, 1981). The DNA G+C content of strains CFBP 6631^T and CFBP 6630 is 52$\pm$0·5 mol%.

The type strain is strain A37^T (=CFBP 6631^T=ATCC 700880^T=CECT 5263^T). Several other isolates from this species have been deposited in the CFBP (see Fig. 1) and CECT. Strains have been isolated from olive knots in association with *Pseudomonas savastanoi* pv. *savastanoi* as secondary invaders on diseased plants.

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