

***Pantoea allii* sp. nov., a novel species isolated from onion and onion seed**

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Footnote: The GenBank/EMBL accession numbers for the sequences of *Pantoea allii* sp. nov. are AY530795 (16S rRNA gene); EF988782, EF988783, EF988778, FJ409060 – FJ409064 (*gyrB* gene); EF988950, EF988954, EF988955, GU458425 – GU458429 (*rpoB* gene), EF988864, EF988868, EF988869, GU458420 – GU458424 (*infB* gene), EF988691, EF988695, EF988696, GU458415 – GU458419 (*atpD* gene).

Summary

Eight yellow-pigmented, gram-negative, rod shaped, oxidase negative, motile, facultatively anaerobic bacteria were isolated from onion seed in South Africa and from an onion plant exhibiting centre rot symptoms in the U.S.A. The strains were placed in the genus *Pantoea* on the basis of phenotypic and biochemical tests. 16S rRNA gene sequencing and MLSA analysis, based on sequences of *gyrB*, *rpoB*, *atpD* and *infB*, confirmed their allocation to the genus *Pantoea*. MLSA further indicated that the strains constitute a novel *Pantoea* species, phylogenetically most closely related to *Pantoea ananatis* and *Pantoea stewartii*. AFLP analysis placed these strains into a cluster separate from the phylogenetically related *Pantoea* species. With only 11 – 55 % whole genome DNA relatedness to the type strains of *Pantoea* species showing > 97 % 16S rRNA gene sequence similarity, DNA-DNA hybridization confirmed the classification of these isolates as a novel species. The most useful phenotypic characteristics for differentiation of this species are its ability to produce acid from amygdalin, and utilize adonitol and sorbitol. The name *Pantoea allii* sp. nov. is proposed for this taxon with LMG 24248^T (= BD 390^T) as the type strain.

Introduction

Diseases of onion caused by *Pantoea ananatis* (Gitaitis & Gay, 1997) and *Pantoea agglomerans* (Edens *et al.*, 2006, Hattingh & Walters, 1981) are characterized by leaf blight, centre leaf rot, seed stalk necrosis and rot, and bulb decay. These disease symptoms can lead to significant economical losses (Hattingh & Walters, 1981, Walcott *et al.*, 2002). Centre rot of onion, induced by *P. ananatis*, has never been observed in South Africa, although this pathogen has been isolated from locally produced onion seed (Goszczyńska *et al.*, 2006). *Pantoea* species isolated from onion in the United States and South Africa are typically identified as *P. ananatis* based only on biochemical and physiological characteristics (Gitaitis & Gay, 1997, Walcott *et al.*, 2002) and 16S rRNA gene sequencing (Goszczyńska *et al.*, 2006). It has been noted that such methods are often insufficient for accurate species identification (Stackebrandt *et al.*, 2002, Wayne *et al.*, 1987), as is the case in the genus *Pantoea* (Brady *et al.*, 2008, 2009a, 2010).

In 2004 several yellow-pigmented bacterial strains were isolated from onion seed in South Africa. These strains were tentatively identified as *Pantoea* species following phenotypic testing and were used in pathogenicity tests, along with strains isolated from diseased onion plants in the United States. The strains were found to be pathogenic to the two onion cultivars tested, causing leaf and stalk necrosis (Goszczyńska *et al.*, 2006). Two representative strains from onion seed in South Africa and one strain from diseased onion in the USA were selected and included in a MLSA study of the genus *Pantoea* based on partial sequencing of four housekeeping genes, *gyrB*, *rpoB*, *atpD* and *infB* (Brady *et al.*, 2008). The MLSA study placed these strains from onion (referred to as

MLSA group G) in a separate strongly-supported cluster, phylogenetically most closely related to *Pantoea ananatis* and *Pantoea stewartii*, indicating they constitute a novel *Pantoea* species. In the present study, these three strains and five additional strains isolated from onion seed were investigated using a polyphasic taxonomic approach.

Strains

A list of strains used in this study is presented in Supplementary Table A on IJSEM Online. The strains from onion were maintained in milk-glycerol liquid medium at -20 °C and recovered on nutrient agar by incubation at 25 °C for 24 hours. Additional strains used in the study were recovered following instructions given by the provider (<http://www.belspo.be/bccm>).

16S rRNA gene sequencing

The almost complete (1346 bp) 16S rRNA gene sequence was determined for LMG 24248^T using the primers (Weisburg *et al.*, 1991) and sequencing conditions as described by Goszczynska *et al.* (2006). The sequences were aligned using ClustalX (Thompson *et al.*, 1997) and the overhangs trimmed. The Modeltest 3.7 programme (Posada & Crandall, 1998) was then applied to the data sets to determine the best-fit evolutionary model. Maximum likelihood and neighbour joining analyses were performed using Phym1 (Guindon & Gascuel, 2003) and PAUP 4.0b10 (Swofford, 2000) respectively, by applying the models and parameters determined by Modeltest (only Maximum likelihood trees are shown). Bootstrap analysis with 1000 replicates was performed to assess the reliability of the clusters. In the phylogenetic trees based on 16S rRNA gene sequences

(Fig.1, ML tree), LMG 24248^T clustered with *Pantoea* species with high bootstrap support, but on a separate branch indicating that the strain probably represents a novel *Pantoea* species. Furthermore, LMG 24248^T shares the eight 16S rRNA gene signature nucleotides specific for *Pantoea* species differentiating them from the closely related *Tatumella* species (Brady *et al.*, 2009a). LMG 24248^T showed more than 97 % 16S rRNA gene sequence similarity to *P. agglomerans*, *P. ananatis*, *P. anthophila*, *P. brenneri*, *P. calida*, *P. conspicua*, *P. deleyi*, *P. dispersa*, *P. eucalypti*, *P. gaviniae*, *P. septica*, *P. stewartii* subsp. *stewartii* and subsp. *indologenes* and *P. vagans*. Although LMG 24248^T clusters closely with species of the genus *Pantoea* in the 16S rRNA gene phylogenetic tree with high support, partial housekeeping gene sequences have been shown to be more reliable genetic markers for identification and phylogenetic analyses of *Pantoea* species (Brady *et al.*, 2008).

***gyrB*, *rpoB*, *atpD*, and *infB* sequences**

MLSA, based on partial *gyrB*, *rpoB*, *atpD* and *infB* gene sequences, was previously carried out on three strains from onion (Brady *et al.*, 2008). In the present study, five additional strains from onion seed were included in the MLSA scheme, using the same primers and conditions. Sequence analysis and tree construction were performed on the concatenated sequences, as well as the single gene sequences, as described above. The closest phylogenetic relatives of *Pantoea*, namely *Erwinia* and *Tatumella*, were included in the analysis. The eight strains formed a distinct well-supported cluster closely related to *P. ananatis* and *P. stewartii*, not only in the phylogenetic tree based on the concatenated sequences (Fig.2), but also in each of the single gene-based trees (data not

shown), suggesting these strains form a novel species group. The *atpD* gene sequences of the strains were examined to determine if they share the signature nucleotides which can be used to characterize *Pantoea* species (Brady *et al.*, 2010). The strains were found to include all 23 *atpD* signature nucleotides.

AFLP analysis

Genomic DNA was extracted from six strains from onion using the GenElute Bacterial Genomic DNA Kit (Sigma). Fluorescent amplified fragment polymorphism analysis (FAFLP) was performed according to the method previously described (Brady *et al.*, 2007). AFLP band patterns were analyzed with BioNumerics 5.0 (Applied Maths) and compared to a database containing profiles of reference strains of *Pantoea* species. A UPGMA dendrogram was constructed using the Pearson correlation (see Supplementary Fig. A in IJSEM Online). The strains from onion constituted a cluster separate from the *Pantoea* species in the database with similarity values of 72 - 94 %, suggesting that these strains belong to a single novel species within the genus. The values are in keeping with those observed previously for species of *Pantoea* (Brady *et al.*, 2007).

DNA-DNA hybridization

High quality DNA for DNA-DNA hybridization was prepared by the method of Wilson (1987), with minor modifications (Cleenwerck *et al.*, 2002). DNA-DNA hybridizations were performed using the microplate method (Ezaki *et al.*, 1989) with some modifications (Cleenwerck *et al.*, 2002). The hybridization temperature was $45\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$. Reciprocal reactions (A x B and B x A) were performed for every DNA pair from all

strains and their variation was within the limits of this method (Goris *et al.*, 1998). The values presented are based on a minimum of four replicates. Three representative strains from onion were selected and hybridized amongst each other. LMG 24248^T was hybridized to the type strains of all *Pantoea* species showing > 97 % 16S rRNA gene sequence similarity. These results are available in Supplementary Table B on IJSEM Online. The three strains from onion (LMG 24248^T, LMG 24202 and LMG 24203) exhibited high levels of DNA relatedness to each other, ranging from 90 to 99 % confirming they belong to the same species group. LMG 24248^T displayed low levels of DNA relatedness to the type strains of the *Pantoea* species, ranging from 11 to 55 %. The latter value is the DNA similarity between LMG 24248^T and the type strain of *Pantoea ananatis*, its closest phylogenetic neighbour. The DNA-DNA hybridization data prove that the strains from onion form a single distinct genetic group within the genus *Pantoea*.

DNA G + C content

The DNA G + C content, measured by HPLC (Mesbah *et al.*, 1989), for the representative strains LMG 24248^T, LMG 24202 and LMG 24203 was 53.6 mol %, 53.4 mol % and 53.5 mol % respectively. This is in keeping with the DNA G + C content of the genus *Pantoea*.

Phenotypic tests

Physiological and biochemical tests were performed on all strains from the novel *Pantoea* species using API 20E, API 50CHB/E and Biotype-100 strips (bioMérieux), according to the manufacturer's instructions and the conditions used for other novel *Pantoea* species

(Brady *et al.*, 2009a, b and c). Additional tests, using GN2 Microplates (Biolog) were performed on LMG 24248^T, LMG 24202 and LMG 24203 according to the manufacturer's instructions. Cell suspensions for inoculation were prepared from strains grown on tryptic soya agar for 12 h at 28 °C. The API and Biolog tests were read after 24 and 48 hours of incubation, whilst the Biotype-100 tests were observed for six days and read each day. A good correlation between the results from Biotype-100 and Biolog GN2 was observed, with consistent utilization of the major carbon sources by the strains tested. Results are presented in the species description below. *Pantoea allii* was found to share all the phenotypic traits identified as being characteristic of *Pantoea* species (Brady *et al.*, 2010). Furthermore, the strains from the novel species can be differentiated from their closest phylogenetic neighbours by the ability to produce acid from amygdalin and the ability to utilize adonitol and sorbitol. The most useful phenotypic characteristics for the differentiation of the novel species from its closest phylogenetic neighbours are listed in Table 1.

The genotypic and phenotypic data presented in this study demonstrate that the strains isolated from onion form a single novel species in the genus *Pantoea*. Therefore we propose to classify the strains as *Pantoea allii* sp. nov. with LMG 24248^T (= BD 390^T) as the type strain.

Description of *Pantoea allii* sp. nov.

Pantoea allii (al'li.i. N.L. gen. n. *allii* from *Allium*, isolated from *Allium cepa* L).

Cells are gram-negative, short rods, non-capsulated, motile and non-spore forming. Colonies are yellow, smooth, round and convex with entire margins on nutrient and tryptone glucose extract agar. Growth occurs at 30 °C, 37 °C and 40 °C, but not at 4 °C or 44 °C. Isolates are facultatively anaerobic, oxidase negative and catalase positive. Lysine and ornithine are not decarboxylated. Urease, gelatinase and H₂S are not produced. β -galactosidase, indole and acetoin are produced and citrate is utilized. Acid is produced from: glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, D-sorbitol, *N*-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, sucrose, D-trehalose, D-raffinose, gentiobiose and D-arabitol (API 50CHB/E). The following carbon sources are utilized at 30 °C: D-glucose, D-fructose, D-galactose, D-trehalose, D-mannose, D-melibiose, sucrose, D-raffinose, maltotriose, maltose, lactose, lactulose, β -galactopyranoside, α -galactopyranoside, D-cellobiose, gentiobiose, 1-*O*-methyl- β -D-glucopyranoside, esculin, D-ribose, L-arabinose, D-xylose, L-rhamnose, D-arabitol, glycerol, *myo*-inositol, D-mannitol, D-sorbitol, adonitol, D-saccharate, mucate, *meso*-tartrate, D-malate, L-malate, *cis*-aconitate, *trans*-aconitate, citrate, D-glucuronate, D-galacturonate, 2-keto-D-gluconate, 5-keto-D-gluconate, *N*-acetyl-D-glucosamine, D-gluconate, protocatechuate, quinate, DL-lactate, succinate, fumarate, DL-glycerate, D-glucosamine, L-aspartate, L-glutamate, L-proline, D-alanine, L-alanine and L-serine

(Biotype 100), dextrin, tween 40, tween 80, pyruvic acid methyl ester, acetic acid, bromosuccinic acid, D-alanine, L-alanine, L-asparagine, urocanic acid, thymidine, D-glucose-1-phosphate and D-glucose-6-phosphate (Biolog GN). The following carbon substrates are not utilized at 30 °C: L-sorbose, palatinose, L-fucose, D-melezitose, L-arabitol, xylitol, dulcitol, D-tagatose, maltitol, D-turanose, hydroxyquinoline- β -glucuronide, erythritol, L-tartrate, D-tartrate, tricarballylate, L-tryptophan, phenylacetate, 4-hydroxybenzoate, gentisate, 3-hydroxybenzoate, benzoate, 3-phenylpropionate, *m*-coumarate, trigonelline, betain, putrescine, 4-aminobutyrate, histamine, caprate, caprylate, L-histidine, glutarate, 5-aminovalerate, ethanolamine, tryptamine, itaconate, 3-hydroxybutyrate, propionate and L-tyrosine (Biotype 100), D-galactonic acid lactone, α -hydroxybutyric acid, β -hydroxybutyric acid, α -keto butyric acid, α -keto glutaric acid, α -keto valeric acid, malonic acid, sebacic acid, L-alaninamide, L-leucine, L-phenylalanine, D-serine, L-threonine, 2-aminoethanol and 2,3-butanediol (Biolog GN). The DNA G + C content of the type strain is 53.6 mol %.

Strains belonging to this species were isolated from onion seed and onion plants exhibiting symptoms of leaf blight and bulb decay. The type strain is LMG 24248^T (= BD 390^T) and was isolated from onion seed in South Africa.

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Table 1. Phenotypic characteristics distinguishing *Pantoea allii* sp. nov. from the closest phylogenetic *Pantoea* species

1 = *P. allii* sp. nov. (8 strains), 2 = *P. agglomerans* (3), 3 = *P. ananatis* (4), 4 = *P. anthophila* (2), 5 = *P. brenneri* (1), 6 = *P. conspicua*

(1), 7 = *P. deleyi* (1), 8 = *P. eucalypti* (2), 9 = *P. stewartii* ssp. *stewartii* (1), 10 = *P. stewartii* ssp. *indologenes* (1), 11 = *P. vagans* (7)

+, 90-100 % strains positive in 1-2 days; (+), 90-100 % strains positive in 1-4 days; -, negative; d, 11-89 % strains positive in 1-4

days; (d), 11-89 % strains positive in 3-4 days; ND, not determined

Data were taken from the following sources: taxon 1 (this study), taxa 2, 3, 9 and 10 (Brady *et al.*, 2009a; Grimont & Grimont, 2005),

taxa 4, 7, 8, and 9 (Brady *et al.*, 2009a), taxa 5 and 6 (Brady *et al.*, 2009b). Only data generated under the same conditions have been

selected. Data generated using ^a API 20E, ^b API 50CHB/E and ^c Biotype 100 strips according to the manufacturer's instructions.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Indole production ^a	+	-	+	-	-	-	-	-	-	+	-
Acid from ^b :											
Amygdalin	+	-	-	-	-	-	-	-	-	-	-
D-Fucose	-	d	d	-	+	-	+	d	-	+	+
Gentiobiose	+	+	d	-	-	-	+	-	-	+	-
Glycerol	+	(d)	+	+	d	-	+	d	-	d	d
Utilization of ^c :											
Adonitol	+	-	-	-	-	-	-	-	-	-	-
Gentiobiose	+	-	+	-	-	+	+	-	-	d	d
Quinate	+	-	(+)	d	-	-	-	-	-	+	-
L-Rhamnose	+	+	d	+	+	+	+	+	+	d	+
D-Sorbitol	+	-	d	-	-	-	-	-	-	-	-
L-Tartrate	-	-	-	-	d	+	+	+	-	-	+

Supplementary Table A. Strains used in this study

LMG = BCCM/LMG Bacteria Collection, Ghent University, Belgium, BD = Plant Pathogenic and Plant Protecting Bacteria (PPPPB)

Culture Collection, ARC-PPRI, Pretoria, South Africa

Species name	Strain No	Source	Location
<i>Pantoea agglomerans</i>	LMG 1286 ^T	Human	Zimbabwe
<i>Pantoea ananatis</i>	LMG 2665 ^T	Pineapple	Brazil
<i>Pantoea anthophila</i>	LMG 2558 ^T	<i>Impatiens balsamina</i>	India
<i>Pantoea brenneri</i>	LMG 5343 ^T	Clinical	USA
<i>Pantoea calida</i>	LMG 25383 ^T	Powdered infant formula	Switzerland
<i>Pantoea conspicua</i>	LMG 24534 ^T	Clinical	USA
<i>Pantoea cypripedii</i>	LMG 2657 ^T	Orchid	USA
<i>Pantoea deleyi</i>	LMG 24200 ^T	Eucalyptus	Uganda
<i>Pantoea dispersa</i>	LMG 2603 ^T	Soil	Japan
<i>Pantoea eucalypti</i>	LMG 24197 ^T	Eucalyptus	Uruguay
<i>Pantoea gaviniae</i>	LMG 25382 ^T	Powdered infant formula	Switzerland
<i>Pantoea septica</i>	LMG 5345 ^T	Clinical	USA
<i>Pantoea stewartii</i> subsp. <i>stewartii</i>	LMG 2715 ^T	Maize	USA
<i>Pantoea stewartii</i> subsp. <i>indologenes</i>	LMG 2632 ^T	Fox millet	India
<i>Pantoea vagans</i>	LMG 24199 ^T	Eucalyptus	Uganda
<i>Pantoea allii</i>	LMG 24248 ^T = BD 390 ^T	Onion seed	South Africa
	LMG 24202 = BD 309	Onion plant	USA
	LMG 24203 = BD 377	Onion seed	South Africa
	BD 380	Onion seed	South Africa
	BD 381	Onion seed	South Africa
	BD 383	Onion seed	South Africa
	BD 391	Onion seed	South Africa
	BD 392	Onion seed	South Africa

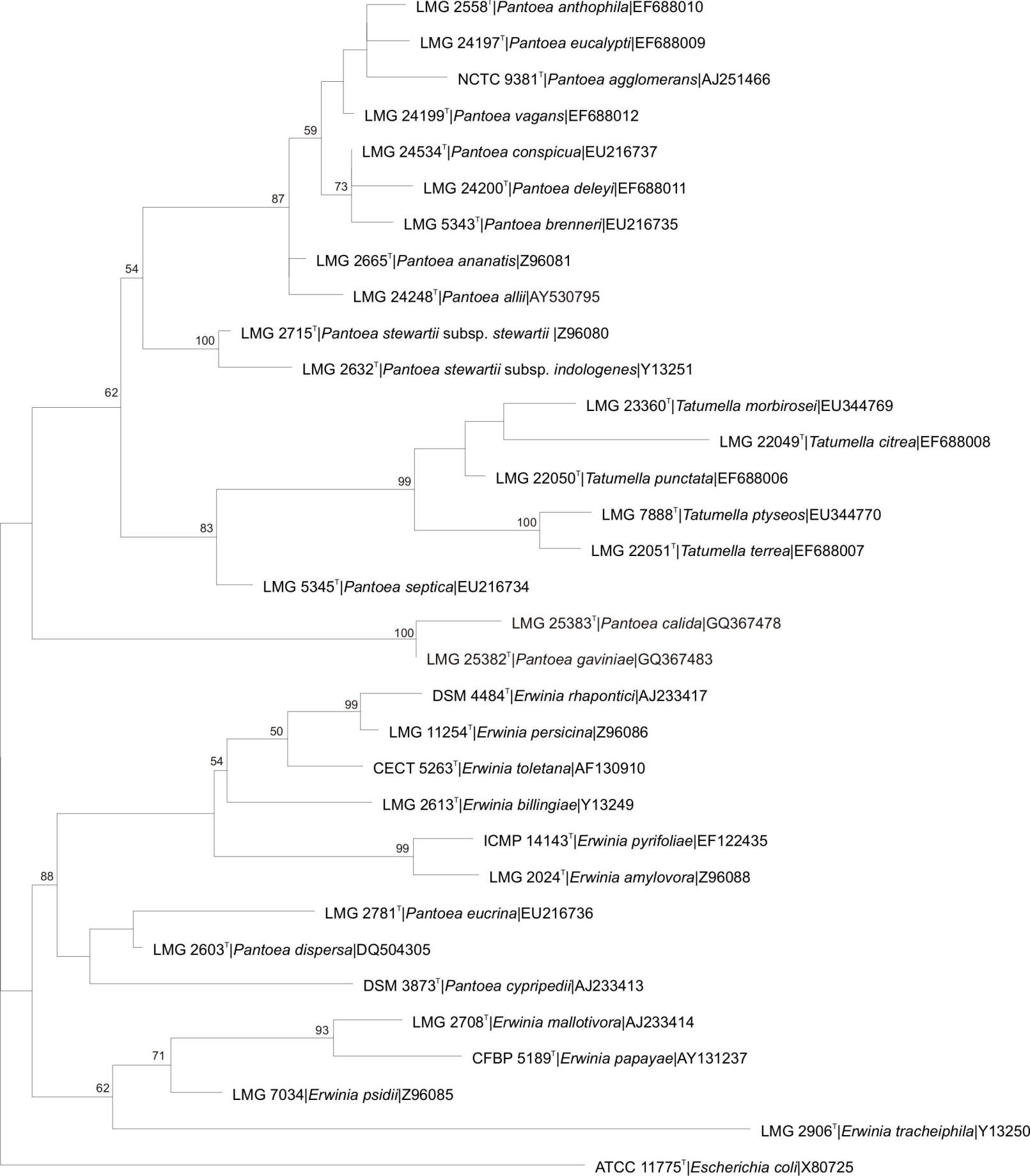
Supplementary Table B. DNA-DNA hybridization values amongst strains belonging to the novel species *Pantoea allii* sp. nov. and type strains of the species of the genus *Pantoea* showing more than 97 % 16S rRNA gene sequence similarity to *P. allii* sp. nov.

	1	2	3
<i>Pantoea allii</i> :			
1. LMG 24248 ^T	100		
2. LMG 24202	99	100	
3. LMG 24203	99	90	100
<i>Pantoea agglomerans</i> LMG 1286 ^T	26		
<i>Pantoea ananatis</i> LMG 2665 ^T	55	57	44
<i>Pantoea anthophila</i> LMG 2558 ^T	23		
<i>Pantoea brenneri</i> LMG 5343 ^T	19		
<i>Pantoea calida</i> LMG 25383 ^T	11		
<i>Pantoea conspicua</i> LMG 24534 ^T	21		
<i>Pantoea cypripedii</i> LMG 2657 ^T	12		
<i>Pantoea deleyi</i> LMG 24200 ^T	27		
<i>Pantoea dispersa</i> LMG 2603 ^T	22		
<i>Pantoea eucalypti</i> LMG 24197 ^T	21		
<i>Pantoea gaviniae</i> LMG 25382 ^T	15		
<i>Pantoea septica</i> LMG 5345 ^T	18		
<i>Pantoea stewartii</i> ssp. <i>stewartii</i> LMG 2715 ^T	18		
<i>Pantoea vagans</i> LMG 24199 ^T	24		

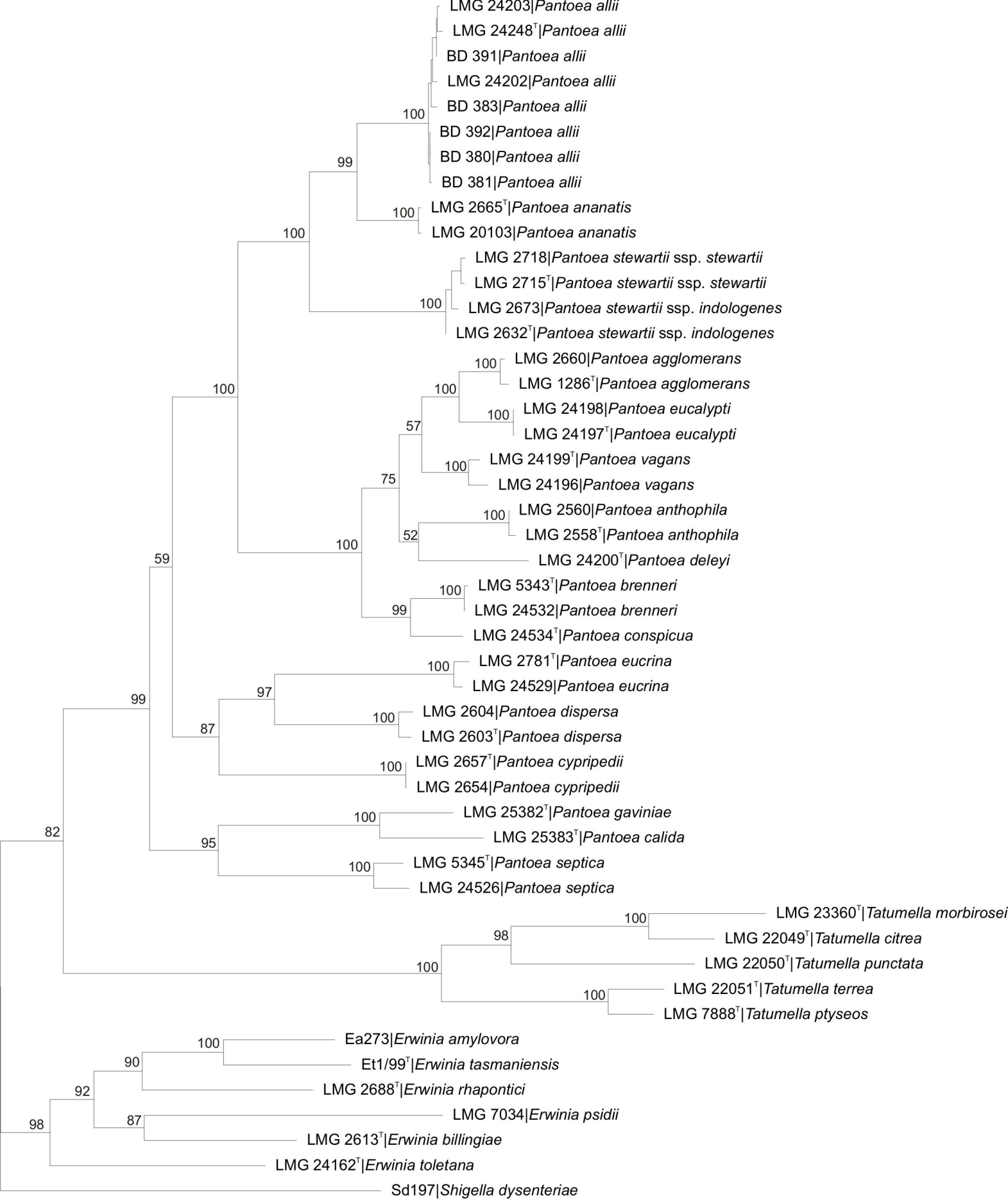
Figure 1: Maximum likelihood tree based on almost complete 16S rRNA gene sequences of species of *Pantoea* and phylogenetically related species. Bootstrap values after 1000 replicates are expressed as percentages. *Escherichia coli* is included as an outgroup. The scale bar indicates the fraction of substitutions per site.

Figure 2: Maximum likelihood tree based on concatenated housekeeping gene sequences of *Pantoea* strains. Bootstrap values after 1000 replicates are expressed as percentages. *Shigella dysenteriae* was included as an outgroup. Gene sequences for *Erwinia amylovora*, *Erwinia tasmaniensis* and *Shigella dysenteriae* were obtained from genome sequencing databases (<http://www.ncbi.nlm.nih.gov>, <http://www.sanger.ac.uk>, <http://asap.ahabs.wisc.edu/asap>). The scale bar indicates the fraction of substitutions per site.

Supplementary Figure A: UPGMA dendrogram based on FAFLP analysis of selected *Pantoea* species using the selective primer combination Eco-C/Mse-GC. The levels of similarity representing the Pearson similarity coefficient, are expressed as percentages. The banding patterns adjacent to each branch are normalised and background-subtracted digitised gel strips processed using BioNumerics.



0.01



0.1