Pantoea allii sp. nov., isolated from onion plants and seed

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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 USA. The isolates were assigned to the genus *Pantoea* on the basis of phenotypic and biochemical tests. 16S rRNA gene sequence analysis and multilocus sequence analysis (MLSA), based on *gyrB*, *rpoB*, *infB* and *atpD* sequences, confirmed the allocation of the isolates to the genus *Pantoea*. MLSA further indicated that the isolates represented a novel species, which was phylogenetically most closely related to *Pantoea ananatis* and *Pantoea stewartii*. Amplified fragment length polymorphism analysis also placed the isolates into a cluster separate from *P. ananatis* and *P. stewartii*. Compared with type strains of species of the genus *Pantoea* that showed >97 % 16S rRNA gene sequence similarity with strain BD 390^T, the isolates exhibited 11–55 % whole-genome DNA–DNA relatedness, which confirmed the classification of the isolates in a novel species. The most useful phenotypic characteristics for the differentiation of the isolates from their closest phylogenetic neighbours are production of acid from amygdalin and utilization of adonitol and sorbitol. A novel species, *Pantoea allii* sp. nov., is proposed, with type strain BD 390^T (=LMG 24248^T).

Diseases of onion that are 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 diseases can lead to significant economic losses (Hattingh & Walters, 1981; Walcott *et al.*, 2002). Centre rot of onion, induced by *P. ananatis*, has never been

observed in South Africa, although the pathogen has been isolated from locally produced onion seed (Goszczynska *et al.*, 2006). Members of the genus *Pantoea* isolated from onion in the USA and South Africa are typically identified as *P. ananatis* on the basis of only biochemical and physiological characteristics (Gitaitis & Gay, 1997; Walcott *et al.*, 2002) and 16S rRNA gene sequencing (Goszczynska *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, 2009, 2010a).

In 2004, several yellow-pigmented bacterial strains were isolated from onion seed in South Africa. These isolates were tentatively identified as members of the genus *Pantoea* by phenotypic testing. Pathogenicity tests with the isolates and strains isolated from diseased onion plants in the USA showed them all to be pathogenic to the two onion cultivars tested, causing leaf and stalk necrosis (Goszczynska *et al.*,

Abbreviation: MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BD 390^T is AY530795 and those of the housekeeping gene sequences of strains BD 309, BD 377, BD 390^T, BD 380, BD 381, BD 383, BD 391 and BD 392 are EF988778, EF988782, EF988783, FJ409060–FJ409064 (*gyrB*), EF988950, EF988954, EF988955, GU458425–GU458429 (*rpoB*), EF988864, EF988868, EF988869, GU458420–GU458424 (*infB*) and EF988691, EF988695, EF988696, GU458415–GU458419 (*atpD*), respectively.

A supplementary figure and two supplementary tables are available with the online version of this paper.

2006). Two representative strains from South Africa and one strain from the USA were included in a multilocus sequence analysis (MLSA) of the genus *Pantoea* based on partial sequences of four housekeeping genes, *gyrB*, *rpoB*, *infB* and *atpD* (Brady *et al.*, 2008). MLSA placed the three isolates from onion (referred to as MLSA group G) in a separate and strongly supported cluster that was phylogenetically most closely related to *P. ananatis* and *Pantoea stewartii* and indicated that the isolates represented a novel species of the genus *Pantoea*. In the present study, these three isolates and five additional isolates from onion seed in South Africa were investigated.

Strains

A list of strains used in this study is presented in Supplementary Table S1 (available in IJSEM Online). The isolates from onion were maintained in milk-glycerol liquid medium at -20°C and recovered on nutrient agar by incubation at 25°C for 24 h. Reference strains were recovered following instructions given by the BCCM culture collection.

16S rRNA gene sequence analysis

The almost-complete (1346 bp) 16S rRNA gene sequence was determined for strain BD 390^T using the primers described by Weisburg *et al.* (1991) and sequencing conditions as described by Goszczynska *et al.* (2006). Sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997) and overhangs were trimmed. MODELTEST version 3.7 (Posada & Crandall, 1998) was used to determine the best-fit evolutionary model. Maximum-likelihood and neighbour-joining analyses were performed using PhyML (Guindon & Gascuel, 2003) and PAUP version 4.0b10 (Swofford, 2000), respectively, by applying the models and parameters determined by MODELTEST. Bootstrap analysis with 1000 replicates was performed to assess the reliability of the clusters.

In the maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences (Fig. 1), strain BD 390^T clustered with members of the genus *Pantoea* with high bootstrap support, but on a separate branch, which indicated that the isolate probably represented a novel *Pantoea* species. Furthermore, strain BD 390^T had the eight 16S rRNA gene signature nucleotides that are specific for the genus *Pantoea* and that differentiate the genus from the closely related genus *Tatumella* (Brady *et al.*, 2010a). Strain BD 390^T showed >97% 16S rRNA gene sequence similarity to *Pantoea agglomerans* NCTC 9381^T, *P. ananatis* LMG 2665^T, *P. anthophila* LMG 2558^T, *P. brenneri* LMG 5343^T, *P. calida* 1400/07^T, *P. conspicua* LMG 24534^T, *P. deleyi* LMG 24200^T, *P. dispersa* LMG 2603^T, *P. eucalypti* LMG 24197^T, *P. gaviniae* A18/07^T, *P. septica* LMG 5345^T, *P. stewartii* subsp. *stewartii* LMG 2715^T, *P. stewartii* subsp. *indologenes* LMG 2632^T and *P. vagans* LMG 24199^T.

gyrB, *rpoB*, *infB* and *atpD* sequence analysis

Although strain BD 390^T clustered closely with members of the genus *Pantoea* in the 16S rRNA gene phylogenetic tree with high support, this study also used partial sequences of housekeeping genes, which have been shown to be more reliable genetic markers for identification and phylogenetic analysis (Brady *et al.*, 2008). MLSA based on partial *gyrB*, *rpoB*, *infB* and *atpD* sequences had been carried out previously on three of the isolates included in this study (Brady *et al.*, 2008). In the present study, five additional isolates from onion seed were included in the MLSA scheme using the same primers and conditions. Sequence analysis and tree construction were performed on concatenated sequences as well as single gene sequences, as described above. Members of the closest phylogenetic relatives of the genus *Pantoea*, the genera *Erwinia* and *Tatumella*, were included in the analysis.

The eight isolates formed a distinct well-supported cluster that was 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 (not shown), which suggested that the isolates represented a novel species. The *atpD* sequences of the isolates were examined to determine whether they contained the signature nucleotides that can be used to characterize members of the genus *Pantoea* (Brady *et al.*, 2010a); the isolates were found to include all 23 *atpD* signature nucleotides.

Amplified fragment length polymorphism analysis

Genomic DNA was extracted from six isolates using the GenElute Bacterial Genomic DNA kit (Sigma). Fluorescent amplified fragment polymorphism analysis was performed according to the method described previously (Brady *et al.*, 2007). Band patterns were analysed with BioNumerics 5.0 (Applied Maths) and compared to a database containing profiles of reference strains of species of the genus *Pantoea*. An unweighted pair group method with averages dendrogram was constructed using Pearson's correlation (Supplementary Fig. S1). The isolates from onion formed a cluster separate from members of the genus *Pantoea*, with similarity values of 72–94%, which suggested that the isolates belonged to a single novel species within the genus. The similarity values are in keeping with those observed previously for species of the genus *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 hybridization was performed using the microplate method (Ezaki *et al.*, 1989) with some modifications (Cleenwerck *et al.*, 2002). The hybridization temperature was $45 \pm 1^{\circ}\text{C}$. Reciprocal reactions were performed for every hybridization pair and variation was within the limits of this method

Fig. 1. Maximum-likelihood tree based on almost-complete 16S rRNA gene sequences showing the relationships of strain BD 390^T and type strains of species of the genera *Pantoea*, *Erwinia* and *Tatumella*. Bootstrap values (>50%) based on 1000 replicates are shown at branch nodes. *Escherichia coli* ATCC 11775^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Fig. 2. Maximum-likelihood tree based on concatenated housekeeping gene sequences showing the relationships of members of strains of *Pantoea allii* sp. nov. and the genera *Pantoea*, *Erwinia* and *Tatumella*. Bootstrap values (>50%) based on 1000 replicates are shown at branch nodes. *Shigella dysenteriae* Sd197 was used as an outgroup. Sequences for *Erwinia amylovora* Ea273, *Erwinia tasmaniensis* Et1/99^T and *S. dysenteriae* Sd197 were obtained from genome sequence databases (<http://www.ncbi.nlm.nih.gov>, <http://www.sanger.ac.uk>, <http://asap.hahbs.wisc.edu/asap/home.php>). Bar, 0.1 substitutions per nucleotide position.

(Goris *et al.*, 1998). The values presented are based on a minimum of four replicates (Supplementary Table S2). Three representative isolates from onion (BD 390^T, BD 309 and BD 377) exhibited high levels of DNA–DNA relatedness to each other (90–99 %), which confirmed that they belonged to the same species. Strain BD 390^T was tested against the members of the genus *Pantoea* with which it showed >97 % 16S rRNA gene sequence similarity. The isolate displayed low levels of DNA–DNA relatedness (11–55 %) with these type strains. DNA–DNA relatedness between strain BD 390^T and *P. ananatis* LMG 2665^T, its closest phylogenetic neighbour, was 55 %. The DNA–DNA hybridization data confirmed that the isolates from onion belonged to 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 isolates BD 390^T, BD 309 and BD 377 was 53.6, 53.4 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 of the isolates from onion and representative strains of the genus *Pantoea* using the API 20 E, API 50 CHB/E and Biotype-100 systems (bioMérieux), according to the manufacturer's instructions and conditions used for other novel *Pantoea* species (Brady *et al.*, 2009, 2010a, b). Additional tests using GN2 MicroPlates (Biolog) were performed on strains BD 390^T, BD 309 and BD 377, according to the manufacturer's instructions. Cell suspensions for inoculation were prepared from cells prepared on tryptic soy agar for 12 h at 28 °C. The API and Biolog tests were read after 24 and 48 h and the Biotype-100 tests were read each day for 6 days.

A good correlation between the Biotype-100 and Biolog GN2 results was observed, with consistent utilization of the major carbon sources by the strains tested. The results of the phenotypic characterization are presented in the species description. The isolates from onion were found to share all of the phenotypic traits that have been identified as characteristic of the genus *Pantoea* (Brady *et al.*, 2010a). Furthermore, the isolates could be differentiated from their closest phylogenetic neighbours by their ability to produce acid from amygdalin and to utilize adonitol and sorbitol. The most useful phenotypic characteristics for the differentiation of the isolates from their closest phylogenetic neighbours are listed in Table 1.

In conclusion, the genotypic and phenotypic data presented in this study demonstrate that the strains isolated from onion represent a single novel species in the genus *Pantoea*. We therefore propose to classify the isolates in a novel species, for which the name *Pantoea allii* sp. nov. is proposed.

Table 1. Phenotypic characteristics that distinguish *Pantoea allii* sp. nov. from its closest phylogenetic neighbours

Species/subspecies: 1, *Pantoea allii* sp. nov. (*n*=8); 2, *P. agglomerans* (*n*=3); 3, *P. ananatis* (*n*=4); 4, *P. anthophila* (*n*=2); 5, *P. brenneri* (*n*=1); 6, *P. conspicua* (*n*=1); 7, *P. deleyi* (*n*=1); 8, *P. eucalypti* (*n*=2); 9, *P. stewartii* subsp. *stewartii* (*n*=1); 10, *P. stewartii* subsp. *indologenes* (*n*=1); 11, *P. vagans* (*n*=7). Data for reference taxa were taken from Brady *et al.* (2009) and Grimont & Grimont (2005) (columns 2, 3, 9 and 10), Brady *et al.* (2009) (columns 4, 7, 8 and 11) and Brady *et al.* (2010b) (columns 5 and 6). All data were generated under the same test conditions. +, 90–100 % strains positive in 1–2 days; (+), 90–100 % strains positive in 1–4 days; d, 11–89 % strains positive in 1–4 days; (d), 11–89 % strains positive in 3–4 days; –, negative.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Indole production (API 20E)	+	–	+	–	–	–	–	–	–	+	–
Acid from (API 50 CHB/E):											
Amygdalin	+	–	–	–	–	–	–	–	–	–	–
D-Fucose	–	d	d	–	+	–	+	d	–	+	+
Gentiobiose	+	+	d	–	–	–	+	–	–	+	–
Glycerol	+	(d)	+	+	d	–	+	d	–	d	d
Utilization of (Biotype 100):											
Adonitol	+	–	–	–	–	–	–	–	–	–	–
Gentiobiose	+	–	+	–	–	+	+	–	–	d	d
Quinate	+	–	(+)	d	–	–	–	–	–	+	–
L-Rhamnose	+	+	d	+	+	+	+	+	+	d	+
D-Sorbitol	+	–	d	–	–	–	–	–	–	–	–
L-Tartrate	–	–	–	–	d	+	+	+	–	–	+

Description of *Pantoea allii* sp. nov.

Pantoea allii [al'li.i. N.L. n. *Allium* (from L. n. *allium* garlic) the scientific generic name of the onion (*Allium* sp.); N.L. gen. n. *allii* of/from *Allium*, referring to the isolation of the first strains 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 30–40 °C, but not at 4 or 44 °C. Facultatively anaerobic. Oxidase-negative and catalase-positive. Lysine and ornithine are not decarboxylated. Produces β-galactosidase, indole and acetoin, but not urease, gelatinase or H₂S. Utilizes citrate. 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, cellobiose, maltose, lactose, sucrose, trehalose, raffinose, gentiobiose and D-arabitol (API 50CHB/E). The following carbon sources are utilized at 30 °C: D-glucose, D-fructose, D-galactose, trehalose, D-mannose, melibiose, sucrose, raffinose, maltotriose, maltose, lactose, lactulose, α- and β-galactopyranoside, cellobiose, gentiobiose, 1-O-methyl β-D-glucopyranoside, aesculin, 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- and 5-keto-D-gluconate, *N*-acetyl-D-glucosamine, D-glucuronate, protocatechuate, quinate, DL-lactate, succinate, fumarate, DL-glycerate, D-glucosamine, L-aspartate, L-glutaminate, L-proline, D- and L-alanine and L-serine (Biotype 100), dextrin, Tweens 40 and 80, pyruvic acid methyl ester, acetic acid, bromosuccinic acid, D- and L-alanine, L-asparagine, urocanic acid, thymidine, α -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, melezitose, L-arabitol, xylitol, dulcitol, D-tagatose, maltitol, turanose, hydroxyquinoline β -glucuronide, erythritol, L- and D-tartrate, tricarballoylate, L-tryptophan, phenylacetate, 4-hydroxybenzoate, gentisate, 3-hydroxybenzoate, benzoate, 3-phenylpropionate, *m*-coumarate, trigonelline, betaine, 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 - and β -hydroxybutyric acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric 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%.

The type strain is BD 390^T (=LMG 24248^T), isolated from onion seed in South Africa. Strains have been isolated from onion seed and onion plants exhibiting symptoms of leaf blight and bulb decay.

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