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

Carrie L. Brady,1 Teresa Goszczynska,2 Stephanus N. Venter,1 Ilse Cleenwerck,3 Paul De Vos,3 Ronald D. Gitaitis4 and Teresa A. Coutinho1

1Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
2Agricultural Research Council, Plant Protection Research Institute, Department of Microbiology and Plant Pathology, Private Bag X134, Queenswood 0121, Pretoria, South Africa
3BCCM/LMG Bacteria Collection, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium
4Department of Plant Pathology, Coastal Plain Experiment Station, University of Georgia, Tifton, GA 31793, USA

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 390T, 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 390T (=LMG 24248T).

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., 2006).
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 390T 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 390T 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 390T 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 390T showed >97 % 16S rRNA gene sequence similarity to *Pantoea agglomerans* NCTC 9381T, *P. ananatis* LMG 2665T, *P. anthophila* LMG 2558T, *P. brenneri* LMG 5343T, *P. calida* 1400/07T, *P. conspicua* LMG 24534T, *P. deleyi* LMG 24200T, *P. dispersa* LMG 2603T, *P. eucalypti* LMG 24197T, *P. gaviniae* A18/07T, *P. septica* LMG 5345T, *P. stewartii* subsp. *stewartii* LMG 2715T, *P. stewartii* subsp. *indologenes* LMG 2632T and *P. vagans* LMG 24199T.

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

Although strain BD 390T 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 ± 1 °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 390T 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 11775T 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 Et1/99T and S. dysenteriae Sd197 were obtained from genome sequence databases (http://www.ncbi.nlm.nih.gov, http://www.sanger.ac.uk, http://asap.ahabs.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 390T, 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 390T 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 390T and P. ananatis LMG 2665T, 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 390T, 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 390T, 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 characterisation 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>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole production (API 20E)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acid from (API 50 CHB/E):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amygdalin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Fucose</td>
<td>-</td>
<td>d</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>d</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>(d)</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>-</td>
<td>+</td>
<td>d</td>
<td>-</td>
<td>d</td>
<td>-</td>
</tr>
<tr>
<td>Utilization of (Biotype 100):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adonitol</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quinate</td>
<td>+</td>
<td>-</td>
<td>(d)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Sorbitol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Tartrate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**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. anthropila* (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.

**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 ol’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-xylene, 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-galactosyranoside, cellobiose, gentiobiose, 1-O-methyl β-D-glucopyranoside, ascinul, D-ribose,
L-arabinose, D-xylene, 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-gluconurate, D-galacturonate, 2- and 5-keto-D-gluconate, N-acetyl-D-glucomamine, D-gluconate, protocatechuic acid, quinate, DL-lactate, succinate, fumarate, DL-glyceraldehyde, D-glucosamine, L-aspartate, L-glutamate, L-proline, D- and L-alanine and L-serine (Biotype 100), xanthine, L-theanine, D-glucose 6-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, tricarballylate, L-trytophan, phenylacetate, 4-hydroxybenzoate, gentisate, 3-hydroxybenzoate, benzoate, 3-phenylpropionate, m-coumarate, trigoneline, betaine, putrescine, 4-aminoxybutirate, histamine, caprate, caprylate, L-histidine, glutarate, 5-aminovalerate, ethanolamine, tryptamine, itaconate, 3-hydroxybutyrate, propionate and L-tyrosine (Biotype 100), D-galactonate acid lactone, oxaloacetate and D-β-hydroxybutyric acid, z-ketobutyric acid, z-ketoglutric acid, z-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 390T (=LMG 24248T), 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.

Acknowledgements

This study was supported by the South African–Flemish Bilateral Agreement, the Agricultural Research Council (ARC), the National Research Foundation (NRF) and the THRIP support programme of the Department of Trade and Industry, South Africa. The BCCM/ LMG Bacteria Collection is supported by the Federal Public Planning Service – Science Policy, Belgium. The authors thank Katrien Vandemeulebroecke for technical assistance.

References


