**Pantoea intestinalis** sp. nov., isolated from the human gut

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A novel bacterial strain, 29Y89B\(^\mathrm{T}\), was isolated from a faecal sample of a healthy human subject. Cells were Gram-stain-negative, motile, non-spore-forming and rod-shaped. Strain 29Y89B\(^\mathrm{T}\) formed cream-coloured colonies 2 mm in diameter on trypticase soy agar and showed optimum growth at 35 °C. Strain 29Y89B\(^\mathrm{T}\) showed highest 16S rRNA gene sequence similarity to *Pantoea gaviniae* A18/07\(^\mathrm{T}\) (98.4 %) followed by *Pantoea calida* 1400/07\(^\mathrm{T}\) (97.2 %). Multi-locus sequence analysis using *atpD* (ATP synthase \(\beta\) subunit), *gyrB* (DNA gyrase), *infB* (initiation translation factor 2) and *rpoB* (RNA polymerase \(\beta\) subunit) genes also supported the result of 16S rRNA gene sequence based phylogeny. Strain 29Y89B\(^\mathrm{T}\) showed 62 and 40.7 % DNA–DNA relatedness with *P. calida* DSM 22759\(^\mathrm{T}\) and *P. gaviniae* DSM 22758\(^\mathrm{T}\). Strain 29Y89B\(^\mathrm{T}\) contained C\(_{17} : 0\) cyclo, C\(_{19} : 0\) cyclo \(\omega 8\)c, C\(_{16} : 0\), C\(_{14} : 0\) and C\(_{12} : 0\) as predominant fatty acids. In addition, strain 29Y89B\(^\mathrm{T}\) showed physiological and phenotypic differences from its closest relatives *P. gaviniae* DSM 22758\(^\mathrm{T}\) and *P. calida* DSM 22759\(^\mathrm{T}\). The polar lipid profile mainly comprised phospholipids. The DNA G + C content was 59.1 mol%. Thus, based on the findings of the current study, strain 29Y89B\(^\mathrm{T}\) showed clear delineations from its closest relatives *P. gaviniae* DSM 22758\(^\mathrm{T}\) and *P. calida* DSM 22759\(^\mathrm{T}\), and is thus considered to represent a novel species of the genus *Pantoea*, for which the name *Pantoea intestinalis* sp. nov. is proposed. The type strain is 29Y89B\(^\mathrm{T}\) (=DSM 28113\(^\mathrm{T}\)=MCC 2554\(^\mathrm{a}\)).

The human gut is a complex reservoir of trillions of microbes (De Filippo et al., 2010; Gill et al., 2006; Kallus & Brandt, 2012; Kau et al., 2011). These microbial cells outnumber the human body cells by about 100 times (Fujimura et al., 2010). Gut bacteria perform important functions and protect the host against pathogen invasion, improve the host’s metabolic activities, enhance the host’s immunity (De Filippo et al., 2010), and play an important role in the host’s pathogenesis and obesity (Zhang et al., 2009). They help the host in extracting energy from fermentation of undigested carbohydrates and subsequent absorption of short chain fatty acids (Cummings & Macfarlane, 1997). Most reports on the human microbiome have been mainly based on culture-independent molecular studies. Culture-independent approaches have provided deeper insight into the unexplored diversity of micro-organisms, which could not be attained through culture-dependent studies (Hugenholtz et al., 1998). However, culture-independent methodologies fail to clarify the functionality of micro-organisms (Prakash et al., 2013b). Thus, it is essential to cultivate the bacteria for a better understanding of their physiology and functions regarding host health and ecology.

The family *Enterobacteriaceae* contains members of the genera *Escherichia*, *Klebsiella*, *Citrobacter*, *Vernini*, *Salmonella*, *Shigella*, *Enterobacter*, *Pantoea* and many others. The genus *Pantoea* was first reported by Gavini et al. (1989). Members of the genus *Pantoea* are Gram-stain-negative, coccoid to rod-shaped bacteria. They inhabit various habitats such as plants, soil, water, animals, and infant formula milk and its production environment (Gavini et al., 1989; Popp et al., 2010; Liu et al., 2013). Different species of the genus *Pantoea* have been reported as causative agents of diseases in plants and humans. For example, *Pantoea ananatis* causes maize white spot disease (Bomfeti et al., 2008) and *Pantoea agglomerans* causes nosocomial infections regarding host health and ecology.
infections in immunocompromised patients (Cruz et al., 2007). Fritz et al. (2014) recently demonstrated that some strains of Pantoea calida were the causal agent of post-surgical meningitis in humans. Pantoea species serve as plant epiphytes or endophytes; they are also used as biocontrol agents (De Maayer et al., 2012a, b; Swart, 2009).

Given the importance of culturable diversity we isolated different bacterial groups from faecal samples of healthy individuals and identified them using 16S rRNA gene sequencing. In the current study, we selected a Pantoea-like strain, 29Y89B^T, isolated from the human gut for extensive characterization using a polyphasic taxonomic approach (Prakash et al., 2007).

Strain 29Y89B^T was isolated from a faecal sample of a healthy human subject. Consent from the subject and institutional ethical committee approval was given before sampling. The faecal sample was collected and processed as discussed by Prakash et al. (2014a). For isolation of bacteria, the sample was diluted in normal saline (0.9 % NaCl) and plated on different medium plates. Morphologically different colonies were picked and purified by restreaking several times on fresh medium plates. Strain 29Y89B^T was picked from a trypticase soy agar (TSA) plate. After isolation and purification, the strain was preserved in liquid nitrogen for future study as discussed by Prakash et al. (2013a).

For 16S rRNA gene sequencing, genomic DNA was extracted from freshly grown cultures using a PureLink Pro 96 Genomic DNA Purification kit (Invitrogen). PCR amplification of the 16S rRNA gene was carried out using bacterial-specific primers (27F: 5'-GAGTTTGATCMTGGCTCAG-3'; 1492R: 5'-TACGGYTACCTTGTTA-3') as described previously (Prakash et al., 2007, 2014a, b). Sequencing was carried out using Big Dye Terminator v 3.1 and 3730XL DNA Analyser (Applied BioSystems). Raw sequence files were manually edited and the contigs were generated using ChromasPro software (http://www.technelysium.com.au/ChromasPro.html). The nearly full-length 16S rRNA gene sequence of strain 29Y89B^T was obtained. Similarity searches were conducted using the EzTaxon database (http://www.eztaxon.org, Kim et al., 2012) with validated type strains. Results of these searches indicated that strain 29Y89B^T shared 98.4 % 16S rRNA gene sequence similarity with P. gaviniae A18/07^T, followed by 97.2 % with P. calida 1400/07^T.

For 16S rRNA gene sequence based phylogenetic reconstruction, validly published sequences of all species of the genus Pantoea were used and aligned using CLUSTAL X2 (Thompson et al., 1997). Aligned sequences were used to reconstruct phylogenetic trees using MEGA 5 (Tamura et al., 2011). The neighbour-joining method was employed to reconstruct the phylogenetic tree, and the robustness was evaluated using bootstrap analysis based on 1000 replicates (Saitou & Néi, 1987). The tree topologies (branching pattern and clustering) were similar to those presented in recent reports of Pantoea species (Gueule et al., 2015; Popp et al., 2010). Evaluation of the phylogenetic tree (Fig. 1) revealed that strain 29Y89B^T formed a separate clade with P. gaviniae A18/07^T and P. calida 1400/07^T. Furthermore, strain 29Y89B^T was clearly distinct from the subclade containing P. calida and P. gaviniae (Fig. 1). Thus, our phylogenetic analysis indicated that strain 29Y89B^T represents a novel species of the genus Pantoea and is most closely related to P. calida and P. gaviniae. Additionally, we carried out multi-locus sequence analysis (MLSA) for the bacterial strain under study. As partial sequences of protein-coding genes are useful for species identification in the family Enterobacteriaceae, the atpD (coding for ATP synthase β subunit), gyrB (encoding DNA gyrase), infB (encoding initiation translation factor 2) and rpoB (encoding RNA polymerase β subunit) genes were investigated. We used the primers reported by Brady et al. (2008) to amplify and sequence the rpoB, gyrB, atpD and infB genes from strain 29Y89B^T. Sequencing was carried as described for the 16S rRNA gene. Concatenated datasets for all four genes were prepared as described by Brady et al. (2008) and a phylogenetic tree was reconstructed using the neighbour-joining method. The MLSA-based phylogenetic data suggested that strain 29Y89B^T was most closely related to P. gaviniae A18/07^T and P. calida 1400/07^T, but simultaneously formed a separate subclade (Fig. 2). Thus, our MLSA data also substantiate the phylogenetic analysis carried out in this study using 16S rRNA gene sequences and confirmed that strain 29Y89B^T is a member of the genus Pantoea and closely related to P. calida and P. gaviniae.

For DNA–DNA hybridization experiments, genomic DNA was extracted and purified as discussed by Marmur (1961). The analysis was done by fluorimetry using a Step One Plus Real-Time PCR system (Applied Biosystems) fitted with 96-well thermal cycling block in a 96-well plate as described by Prakash et al. (2014a). Data were obtained in triplicate and are presented as the mean of these three values. DNA–DNA reassociation was carried out at optimum reassociation temperatures of 76.0–78 °C (Tm = 0.51 × (% G+C) + 47.0) according to the procedure described by De Ley et al. (1970) and Gillis et al. (1970). DNA G+C content was calculated in triplicate as discussed by Gonzalez & Saiz-Jimenez (2002) using a StepOnePlus Real-Time PCR system (Applied Biosystems). DNA–DNA hybridization experiments indicated that strain 29Y78BT^ shared 62 and 40.7 % relatedness with P. calida DSM 22759^T and P. gaviniae DSM 22758^T, respectively, which is less than the threshold value described by Wayne et al. (1987) for species delineation in bacterial taxonomy. Thus, based on the result of DNA–DNA hybridization we concluded that strain 29Y78BT^ represents a novel species of the genus Pantoea. The DNA G+C content of strain 29Y78BT^ was 59.1 mol%.

Fatty acid methyl ester (FAME) analysis was carried out as described by Prakash et al. (2014a). In brief, strain 29Y89B^T and its closest relatives, P. calida DSM 22759^T and P. gaviniae DSM 22758^T, were cultivated under a similar set of conditions on TSA plates. An equal amount of cell biomass was harvested from the same physiological stage
FAMEs were extracted and analysed using the method described by Sasser (1990) and comparison of peak profiles was achieved using the TSBA-6 database of MIDI (Microbial ID). A comparison of the fatty acid profiles of strain 29Y89BT and P. calida DSM 22759T and P. gaviniae DSM 22758T is given in Table S1 (available in the online Supplementary Material). Polar lipids and isoprenoid quinones were extracted and analysed by one- and two-dimensional TLC, as discussed by Tindall (1990a, b) and DiSpirito et al. (1983), respectively.

Fatty acid comparisons showed that along with C17:0 cyclo and C19:0 cyclo ω8c all three strains contained C16:0, C14:0, and C12:0 as predominant fatty acids in their FAME profiles (Table S1). Unsaturated fatty acids were present in lower proportions and were unequally distributed among the selected strains. Although there were high levels of similarity in the FAME profiles, there were also qualitative as well as quantitative differences. Comparative study indicated that the FAME data are in agreement with profiles of recognized Pantoea species (Brady et al., 2012). Thus, the FAME data support the findings of phylogenetic analysis that strain 29Y89BT belongs to the genus Pantoea. Based on the similarity and differences in the FAME profile of strain 29Y89BT with its closest relatives P. calida DSM 22759T and P. gaviniae DSM 22758T, we conclude that strain 29Y89BT belongs to the genus Pantoea and represents a novel species. The polar lipid profile of strain 29Y89BT is presented in Fig. S1. It mainly contained phospholipids while amino- and choline-containing lipids were not detected (Fig. S1). Results of isoprenoid quinone analysis indicated that P. calida DSM 22759T and P. gaviniae DSM 22758T contained only ubiquinone while strain 29Y89BT contained both ubiquinone and menaquinone (Fig. S2).

For phenotypic characterization, the type strains of phylogenetically most closely related species of the genus Pantoea, i.e. P. calida DSM 22759T and P. gaviniae DSM 22758T, were
purchased from DSMZ and all the tests were conducted using a similar set of conditions as recommended by Tindall et al. (2010). Growth response was evaluated using nutrient agar, TSA, Luria agar and R2A agar. Colony morphologies and pigmentation were studied on TSA agar after 48 h of incubation at 37 °C. Tolerance to NaCl, optimum pH and temperature were evaluated as discussed by Prakash et al. (2014a, b). The Gram-reaction was studied using a Gram-stain kit (HiMedia) and was confirmed by an aminopeptidase assay and KOH lysis method (Smibert & Krieg 1994). Strain 29Y89BT was a Gram-stain-negative, non-spore-forming, motile, rod-shaped bacterium and formed 2 mm diameter cream-coloured colonies on TSA after 24 h of incubation at 35 °C. It tolerated up to 10 % NaCl, but grew optimally with 0.5 % NaCl. It grew at 15–45 °C with optimum growth at 35 °C. It showed several variable features in terms of colonial morphology and in physiological and biochemical tests with its closest relatives P. gaviniae and P. calida DSM 22759T and P. gaviniae DSM 22758T (Table 1). Cells showed positive test results for catalase, nitrate reductase, cellulase and Voges–Proskauer reaction but negative results for oxidase, urease, gelatinase and amylase. 16S rRNA gene sequence phylogeny as well as MLSA indicated that strain 29Y89BT belongs to the genus Pantoea but is separate from recognized species of the genus. FAME data of strain 29Y89BT (Table S1) generated using a similar set of conditions showed agreement with previous data generated for other species of the genus Pantoea (Brady et al., 2012; Gueule et al., 2015) and confirmed that strain 29Y89BT is a recognized species of the genus Pantoea. Furthermore, morphological and physiological variations along with DNA–DNA hybridization value lower than the prescribed threshold for species delineation with P. calida DSM 22759T and P. gaviniae DSM

Fig. 2. Neighbour-joining tree based on concatenated sequences of the gyrB, rpoB, atpD and infB genes of recognized species of the genus Pantoea as described by Gueule et al. (2015). Similar gene sequences of Tatumella morbirosei DSM 23960T were used as an outgroup. Bootstrap values based on 1000 replicates are expressed as percentages. Bar, 0.02 substitutions per site.
Table 1. Differential features between strain 29Y89B<sup>T</sup> and its closest relatives in the genus <em>Pantoea</em>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony size (mm)</td>
<td>2–3</td>
<td>3</td>
<td>5–6</td>
</tr>
<tr>
<td>Colony colour</td>
<td>Cream</td>
<td>Cream</td>
<td>Cream mucoid</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>1.2 × 0.6</td>
<td>1.5 × 0.7</td>
<td>1.4 × 0.5</td>
</tr>
<tr>
<td>Malic acid</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Valine arylamidase</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Trypsin</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Phosphatase</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Simons citrate</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Protease</td>
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<td>+</td>
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<tr>
<td>DNase test</td>
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<td>−</td>
<td>+</td>
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<tr>
<td>Naphthol-AS-BI-phosphohydrolase</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>α-Aesculin</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Leucine arylamidase</td>
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<td>+ +</td>
<td>+</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>w</td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>59.1</td>
<td>57.4*</td>
<td>58.4*</td>
</tr>
</tbody>
</table>

*Data taken from Popp et al. (2010). Number of “+” signs increased with increasing the intensity of the reaction.

22758<sup>T</sup> suggested that strain 29Y89B<sup>T</sup> represents a novel species of the genus <em>Pantoea</em>, and the first one isolated from a human faecal sample. Thus, based on phenotypic, phylogenetic, MLSA and genotypic data we conclude that strain 29Y89B<sup>T</sup> is a novel member of the genus <em>Pantoea</em> for which the name <em>Pantoea intestinalis</em> sp. nov. is proposed.

**Description of <em>Pantoea intestinalis</em> sp. nov.**

<em>Pantoea intestinalis</em> (in.tes.ti.na’lis. N.L. fem. adj. intestina-lis pertaining to the intestines, from where the type strain was isolated).

Gram-stain-negative, non-spore-forming, motile, rod-shaped bacterium with a cell size of 1.2 × 0.6 μm. Good growth occurs on universal laboratory media such as Luria agar, nutrient agar, R2A agar and TSA with optimum growth on TSA plates. Forms smooth cream-coloured colonies 2 mm in diameter after 24 h of incubation on TSA. Grows at 15–45 °C with optimal growth at 35 °C. Range of pH for growth is 4–9 with optimum growth at pH 7. Tolerates up to 10 % NaCl but grows optimally with 0.5 % NaCl. Positive for catalase, nitrate reductase, cellulose and acid phosphatase but negative for esterase, esterase lipase, lipase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, urease, amylase and oxidase. Assimilates D-glucose, L-arabinose, d-mannose, D-mannitol, N-acetylglucosamine and maltose but shows no growth on trisodium citrate, gelatin, capric acid, adipic acid and phenylacetic acid. Differential features in comparison with <em>P. calida</em> DSM 22759<sup>T</sup> and <em>P. gaviniae</em> DSM 22758<sup>T</sup> are presented in Table 1. Contains C<sub>17</sub>:0 cyclo, C<sub>19</sub>:0 cyclo ω8c, C<sub>16</sub>:0, C<sub>14</sub>:0 and C<sub>12</sub>:0 as predominant fatty acids. Phospholipids are the main constituents of the polar lipid profile while other lipids are not detected.

The type strain, 29Y89B<sup>T</sup> (=DSM 28113<sup>T</sup>=MCC 2554<sup>T</sup>), was isolated from a stool sample of a healthy human subject residing in Pune, Maharashtra, India. The DNA G+C content of the type strain is 59.1 mol%.

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**References**

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