**Pseudomonas punonensis** sp. nov., isolated from straw

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During a study of the ‘tunta’ (frozen-dry potato) production process in Peru, a bacterial strain, LMT03T, was isolated from the straw grass in which the potatoes are dried. This strain was classified into the genus *Pseudomonas* on the basis of the 16S rRNA gene sequence analysis, and is most closely related to *Pseudomonas argentinensis* CH01T with 99.3 % identity in this gene and 96 %, 92 % and 86 % identities in *rpoB*, *rpoD* and *gyrB* genes, respectively. Strain LMT03T has a single polar flagellum, like other related yellow-pigment-producing pseudomonads. The major quinone is Q-9. The major fatty acids are C18:1ω7c in summed feature 8 (40.82 %), C16:1ω6c/C16:1ω6c in summed feature 3 (23.72 %) and C16:0 (15.20 %). The strain produces oxidase but it does not produce gelatinase, indole, urease, arginine dihydrolase or β-galactosidase. Catalase production was very weak after 28 and 48 h incubation on nutrient agar medium. Nitrate reduction is negative. It does not hydrolyse aesculin. The DNA G+C content is 57.8 mol%. DNA–DNA hybridization results showed lower than 52 % relatedness with respect to the type strain of *P. argentinensis*, CH01T. These results, together with other phenotypic characteristics, support the definition of a novel species within the genus *Pseudomonas*, for which the name *Pseudomonas punonensis* sp. nov. is proposed. The type strain is LMT03T (≡LMG 26839T ≡CECT 8089T).

Bitter potatoes (*Solanum juzepczukii* and *Solanum curtibulum*) play a definitive role in the balance of the fragile ecosystem of the Altiplano, because they can resist frost (down to −5 or −7 °C), drought and grow up to 4200 m. Since ancestral times, these potato varieties were domesticated by the ancient Andean people belonging to the Aymara culture, who also invented the process of dehydration and freezing potatoes for consumption and conservation, since bitter potatoes cannot be consumed fresh due to their high content of glycoalkaloids, a process named ‘tunta’. ‘Tunta’, elaborated in the southern highlands of Peru and northern Bolivia, is traditionally obtained from frozen potato tubers in the bitter cold frost, its immersion in river pools for periods between 15 and 20 days, drying, shelling and a final freeze. Strain LMT03T was isolated during a procedure for evaluating the microbiological quality control of this production process. It was found in certain grasses grown in moderate to strongly acidic soils (pH 5.0–5.5) from the Andean Churomaquera community in the province of El Collao (Puno, Peru) at 3860 m, used as bedding for the exposure of potatoes to the frost. For isolation, 10 g of these grasses were submerged in 90 ml peptone water 0.1 % and shaken thoroughly. One-millilitre aliquots were inoculated into asparagine broth tubes and incubated at 28 °C for 7 days, and tubes with positive growth were streaked on cetrimide agar and incubated at 28 °C for 48 h. Strain LMT03T was classified into the genus *Pseudomonas* after 16S rRNA gene analysis and the phylogenetic, chemotaxonomic and
phenotypic analysis showed that it represents a novel species for which we propose the name *Pseudomonas punonensis* sp. nov.

Cells were stained according to the Gram procedure described by Doetsch (1981). Motility was checked by phase-contrast microscopy after growth in nutrient agar at 22 °C for 48 h. The flagellation type was determined by electron microscopy after 48 h incubation in TSA at 22 °C as was previously described (Rivas et al., 2007). Strain LMT03T is Gram-negative, rod-shaped (0.4–0.5 × 1.2–1.3 μm) and motile by a single polar flagellum (see Fig. S1 in IJSEM online). Cells grew as round translucent yellow-coloured colonies on nutrient agar.

For 16S rRNA gene sequencing and comparison analysis, DNA extraction, amplification and sequencing were performed as reported by Rivas et al. (2007). The amplification and partial sequencing of *gyrB*, *rpoB* and *rpoD* housekeeping genes was performed as described by Mulet et al. (2010), using the primers PsEG30F/PsEG790R for *rpoD* gene (Mulet et al. 2009), LAPS5F/LAPS27R for *rpoB* gene (Tayeb et al., 2005) and GyrBPUN1F (5′-AAGGAGCTGGTGYTGACC-3′) and GyrBPUN1R (5′-GCCTCAGTCATCTTGGC-3′) designed in this study for amplification of the *gyrB* gene.

The sequences obtained were compared with those from the GenBank using the BLASTN (Altschul et al., 1990) and EzTaxon (Kim et al., 2012) programs. For phylogenetic analysis, sequences were aligned using CLUSTAL_X software (Thompson et al., 1997). Distances were calculated according to Kimura’s two-parameter model (Kimura, 1980). Phylogenetic trees of 16S rRNA were inferred using neighbour-joining (NJ; Saitou & Nei, 1987), and maximum-likelihood (ML; Rogers & Swofford, 1998) analyses. MEGA5 software (Tamura et al., 2011) was used for all analyses.

The comparison of the 16S rRNA gene sequence of strain LMT03T against the type strains of bacterial species recorded in the EzTaxon database showed that the new strain belonged to the genus *Pseudomonas*, its closest relative being *Pseudomonas argentinensis* CH01T with 99.3% identity (11 different nucleotides). Other closely related species are *Pseudomonas straminea* IAM 1598T and *Pseudomonas flavescens* B62T, with 98.8% (17 different nucleotides) and 98.5% (22 different nucleotides) identity, respectively. The remaining species of the genus *Pseudomonas* had identities lower than 98.5%. All species having more than 97% identity in the 16S rRNA gene, as well as the type species of the genus, *Pseudomonas aeruginosa* LMG 1242T, were included in the phylogenetic analysis. The NJ phylogenetic tree (Fig. 1) showed that strain LMT03T occupied a branch related to a cluster formed by *P. argentinensis* CH01T, *P. straminea* IAM 1598T and *P. flavescens* B62T. Similar results were obtained after ML phylogenetic analysis (data not shown).

In addition to the 16S rRNA gene, three housekeeping genes widely used in the phylogenetic analysis of *Pseudomonas* species were studied in this work (Tayeb et al., 2005; Mulet et al., 2009, 2010). In agreement with the results of the 16S rRNA gene analysis, the phylogenies obtained with these housekeeping genes also show the affiliation of LMT03T as a separate species within the *P. straminea* group. The concatenated *rpoD*, *rpoB* and *gyrB* genes phylogenetic tree (Fig. 2) showed that LMT03T cluster together with the type strains of *P. argentinensis*, *P. straminea* and *P. flavescens*, being *P. argentinensis* the closest related species (Fig. 2). The identities of *rpoD* gene calculated from pairwise distances matrix done by MEGA 5.0 program were 91.6% with respect to *P. argentinensis* and *P. straminea* and 86% with respect to *P. flavescens*. For the *rpoB* gene, identities were 95.8%, 90.3% and 92.7%, respectively, and for the *gyrB* gene 87%, 89.6% and 89%, respectively. These values are similar or lower than those found among several species of the genus *Pseudomonas*. For example, in the case of *rpoD* gene, *Pseudomonas jessenii* showed about 92% identity with respect to *Pseudomonas vancouverensis*, *Pseudomonas moorei* and *Pseudomonas mohnii*, *Pseudomonas reinekii* showed 94% identity with respect to *P. moorei* and *P. mohnii*, *P. moorei* and *P. mohnii* showed 96% identity and *P. koreensis* and *P. moraviensis* 93.7% identity. In the *rpoB* gene, *P. vancouverensis* and *P. mohnii* have 95.6% identity, and *P. moorei* and *P. mohnii*, *P. jessenii* and *P. reinekii*, *P. koreensis* and *P. moraviensis* and *P. vancouverensis*, *P. jessenii* and *P. reinekii* showed about 97% identity. All these species showed values ranging from 89% to 97% in the *gyrB* gene among them. Therefore the results of the *rpoD*, *rpoB* and *gyrB* gene analysis also suggested that strain LMT03T belongs to an undescribed species of *Pseudomonas*.

DNA–DNA hybridization was carried out by the method of Ezaki et al. (1989), following the recommendations of Willems et al. (2001). LMT03T was hybridized with *P. argentinensis* CH01T and *P. argentinensis* PA01, and after four replicates less than 52% hybridization was obtained in both cases. LMT03T showed a mean value of 51% (47/56 reciprocal values) with respect to CH01T and 46% (42/50 reciprocal values) with respect to PA01. Therefore, strain LMT03T represents a different species within the genus *Pseudomonas* when the recommendation of a threshold value of 70% DNA–DNA similarity for definition of a bacterial species is considered (Wayne et al., 1987).

For base composition analysis, DNA was prepared according to Chun & Goodfellow (1995), and the DNA G+C content of strain LMT03T was determined by thermal denaturation (Mandel & Marmur, 1968) to be 57.8 mol%. This value is similar to that obtained for *P. argentinensis* and related species (Peix et al., 2005).

Cellular fatty acids were analysed by using the Microbial Identification System (MIDI; Microbial ID) Sherlock 6.1 and the library RTSBA6 according to the technical instructions provided by this system (Sasser, 1990). Strain LMT03T was grown on TSA plates (Becton Dickinson, BBL) for 24 h at 28 °C as previously described for *P.
argentinensis CH01T, P. straminea IAM 1598T and P. flavescens DSM 12071T. The major fatty acids of strain LMT03T were C18:1ω7c in summed feature 8 (40.82 %), C16:1ω6c/C16:1ω6c in summed feature 3 (23.72 %) and C16:0 (15.20 %). As expected, all the relatives clustering in the same phylogenetic group as strain LMT03T shared similar fatty acid profiles (Table 1), although slight differences were found in the amounts of C10:03-OH, C12:03-OH and C16:0. Therefore LMT03T has the three fatty acids typically present in the genus Pseudomonas according to Palleroni (2005), which are C10:0 3-OH, C12:0 3-OH and C16:0.

Strain LMT03T was cultivated for 24 h in TSA plates (Becton Dickinson, BBL) at 28 °C to obtain the cell mass required for quinone analysis that was carried out by the Identification Service of the DSMZ (Braunschweig, Germany) from freeze-dried cells using the methods described by Tindall (1990a, 1990b). Strain LMT03T contained Q-9 as major quinone (96 %) and low levels of Q-8 (4 %). The presence of Q9 as the major quinone is in agreement with the results obtained for other species of the genus Pseudomonas (Palleroni, 2005).

For pigment analysis, cells were grown in King B agar and nutrient agar, and testing for pigment production and spectral characteristics was performed by extraction with methanol according to Hildebrand et al. (1994), using a visible-UV Kontron Uvikon 860 spectrophotometer. The spectral analysis of the methanol-extracted yellow pigment of strain LMT03T revealed a major peak at 446 nm, the same absorbance position of the yellow pigment of P. flavescens (Hildebrand et al. 1994) and slightly different to that of the closest relative, P. argentinensis, whose major peak was at 442 nm (Peix et al., 2005), revealing high similarity of yellow-insoluble pigments in this phylogenetic subcluster of the genus Pseudomonas. As for the fluorescent pigment analysis, the spectral study of supernatants from King’s B broth cultures revealed a peak at 334 nm, which is also in the range found for absorption peaks of other Pseudomonas cremoricolorata NRIC 0181T (AB060136)
Pseudomonas fulva IAM 1529T (D84015)
Pseudomonas parafulva AJ 2129T (AB060132)
Pseudomonas putida DSM 291T (Z76667)
Pseudomonas taiwanensis BCRC 17751T (EU103629)
Pseudomonas mossellii CIP 105259T (AF072688)
Pseudomonas montelii CIP 104883T (AF064458)
Pseudomonas plecoglossicida FPC951T (AB009457)
Pseudomonas vancouverensis ATCC 700688T (AJ011507)
Pseudomonas moorei RW10T (AM293566)
Pseudomonas mohii ipA-2 (AM293567)
Pseudomonas koreensis Ps 9-14T (AF468452)
Pseudomonas reinekei MT1T (AM293565)
Pseudomonas jessenii CIP 105274T (AF068259)
Pseudomonas moraviensis CCM 7280T (AY970952)
Pseudomonas baetica a390T (FM201274)
Pseudomonas benzenivorans DSM 8628T (FM208263)
Pseudomonas punonensis LMT03T (JQ344321)
Pseudomonas flavescens B62T (U019116)
Pseudomonas argentinensis CH01T (AY691188)
Pseudomonas straminea IAM 1598T (D84023)
Pseudomonas alcaliphila AL15-21T (AB030583)
Pseudomonas oleovorans subsp. lubricantis RS1T (DQ842018)
Pseudomonas toytomienosis HT-3T (AB453701)
Pseudomonas composti C2T (FN429930)
Pseudomonas cuatrocienegasensis 1NT (EU791281)
Pseudomonas aeruginosa LMG 1242T (Z76651)
Acinetobacter baumannii DSM 30007T (X81660)
Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of P. punonensis LMT03T and closely related Pseudomonas species. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 1 nt substitution per 100 nt.
fluorescent *Pseudomonas* species such as *P. argentinensis* (Peix et al., 2005) or *P. flavescens* (Hildebrand et al., 1994).

The physiological and biochemical tests were performed as previously described (Peix et al., 2005). Additionally API 20NE, API 32GN and API 50CH (bioMérieux) were used following the manufacturer’s instructions and results were recorded after 48 h incubation at 28 °C. Phenotypic characteristics of strain LMT03\(^T\) are reported below in the species description and the differences with respect to the closest *Pseudomonas* species are recorded in Table 2. The phenotypic characteristics of strain LMT03\(^T\) support its classification within the genus *Pseudomonas* since it is a motile Gram-negative rod, strictly aerobic, catalase-positive (weak) and oxidase-positive and produces a fluorescent pigment typical of this genus (Hildebrand et al., 1994). Nevertheless, as was stated by Palleroni (2005) these characteristics do not allow an absolute differentiation of the genus *Pseudomonas* from other rRNA groups of aerobic ‘pseudomonads’. The analysis of the 16S rRNA genes and that of chemotaxonomic characteristics such as fatty acids and ubiquinone composition are necessary for this purpose (Palleroni, 2005).

Therefore, from the analysis of all phylogenetic, chemotaxonomic and phenotypic data, it can be concluded that LMT03\(^T\) represents a novel species of the genus *Pseudomonas*, for which we propose the name *Pseudomonas punonensis* sp. nov.

**Description of *Pseudomonas punonensis* sp. nov.**

*Pseudomonas punonensis* (pu.no.nen’sis. N.L. fem. adj. *punonensis* of or belonging to Puno, a region of Peru where the type strain was isolated).

Gram-negative, strictly aerobic, non-spore-forming rod-shaped cells 1.2–1.3 μm long and 0.4–0.5 μm wide, motile by a single polar flagellum. Colonies on nutrient agar are circular convex, yellow, translucent and usually 0.5–2.5 mm in diameter within 2 days growth at 25 °C. Grows at 5 °C but not at 41 °C; the pH range for growth is 5–9. A diffusible fluorescent pigment is produced on King B medium. Strictly aerobic with oxidative metabolism is 5–9. A diffusible fluorescent pigment is produced on King B medium. Strictly aerobic with oxidative metabolism

*Fig. 2.* Neighbour-joining tree based on concatenated partial *rpoD*, *rpoB* and *gyrB* gene sequences of *P. punonensis* LMT03\(^T\) and closely related *Pseudomonas* species. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 2 nt substitutions per 100 nt.
Table 1. Cellular fatty acid composition (%) of P. punonensis LMT03<sup>T</sup>, its closest related species and the type species of the genus *Pseudomonas*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
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<tr>
<td>10:0 3-OH</td>
<td>4.83</td>
<td>2.40</td>
<td>3.91</td>
<td>3.74</td>
<td>3.6</td>
</tr>
<tr>
<td>11:0 3-OH</td>
<td>0.93</td>
<td>0.10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>12:0 2-OH</td>
<td>ND</td>
<td>0.99</td>
<td>0.21</td>
<td>ND</td>
<td>3.7</td>
</tr>
<tr>
<td>12:0 3-OH</td>
<td>4.54</td>
<td>2.58</td>
<td>3.57</td>
<td>3.55</td>
<td>4.5</td>
</tr>
<tr>
<td>10:0</td>
<td>0.14</td>
<td>0.09</td>
<td>0.20</td>
<td>ND</td>
<td>tr</td>
</tr>
<tr>
<td>11:0</td>
<td>ND</td>
<td>0.09</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>12:0</td>
<td>8.31</td>
<td>7.88</td>
<td>9.58</td>
<td>9.23</td>
<td>4.8</td>
</tr>
<tr>
<td>13:0</td>
<td>ND</td>
<td>0.08</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>14:0</td>
<td>0.56</td>
<td>0.69</td>
<td>0.78</td>
<td>0.71</td>
<td>1.3</td>
</tr>
<tr>
<td>15:1&lt;i&gt;10&lt;/i&gt;6c</td>
<td>0.15</td>
<td>0.14</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>15:0</td>
<td>ND</td>
<td>0.97</td>
<td>ND</td>
<td>ND</td>
<td>tr</td>
</tr>
<tr>
<td>16:0</td>
<td>15.20</td>
<td>19.69</td>
<td>17.63</td>
<td>19.75</td>
<td>20.5</td>
</tr>
<tr>
<td>17:1</td>
<td>ND</td>
<td>0.73</td>
<td>0.54</td>
<td>0.31</td>
<td>ND</td>
</tr>
<tr>
<td>17:0</td>
<td>0.27</td>
<td>0.52</td>
<td>0.36</td>
<td>ND</td>
<td>tr</td>
</tr>
<tr>
<td>18:0</td>
<td>0.58</td>
<td>0.51</td>
<td>0.52</td>
<td>0.78</td>
<td>tr</td>
</tr>
<tr>
<td>Summed features*</td>
<td>3</td>
<td>23.72</td>
<td>21.34</td>
<td>22.40</td>
<td>22.39</td>
</tr>
</tbody>
</table>

*Summed feature 3: C<sub>16:1</sub>ω7c/C<sub>16:1</sub>ω6c. Summed feature 8: C<sub>18:1</sub>ω7c.

lactate, valerate, 3-hydroxybenzoate, 4-hydroxybutyrate, l-alanine, l-serine and l-proline are positive. Assimilation of N-acetylglucosamine, maltose, l-rhamnose, inositol, salicin, melibiose, l-fucose, sorbitol, glycogen, erythritol, l-xylene, d-malate, adonitol, methyl β-D-xyloside, methyl β-D-glucoside, methyl β-D-mannoside, dulcitol, amygdalin, arbutin, cellobiose, lactose, trehalose, melezitose, raffinose, starch, inulin, xyliot, gentiobiose, caprate, adipate, phenylacetate, l-histidine, 2- and 5-ketogluconate, suberate and 3-hydroxybenzoate are negative. Assimilation of D-ribose and propionate is weak.

The type strain is LMT03<sup>T</sup> (=LMG 26839<sup>T</sup> = CECT 8089<sup>T</sup>), isolated from straw in Peru. DNA G+C content of the type strain is 57.8 mol%.

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