***Pseudomonas punonensis* sp. nov., a novel species isolated from grasses in Puno region (Peru)**

Elena Ramos^{1‡}, Martha-Helena Ramírez-Bahena^{2,3‡}, Angel Valverde^{2,3†}, Encarna Velázquez^{3,4}, Doris Zúñiga¹, Carmen Velezmoro¹, Alvaro Peix^{2,3*}

1. Laboratorio de Ecología Microbiana y Biotecnología Marino Tabusso, Dpto. de Biología. Universidad Nacional Agraria La Molina. Peru.

2. Instituto de Recursos Naturales y Agrobiología. IRNASA-CSIC, Salamanca. Spain.

3. Unidad Asociada Grupo de Interacción Planta-Microorganismo Universidad de Salamanca-IRNASA (CSIC)

4. Departamento de Microbiología y Genética. Universidad de Salamanca. Spain.

[†]Present address: Institute for Microbial Biotechnology and Metagenomics, University of the Western Cape, Bellville 7535, Cape Town, South Africa.

[‡]These authors contributed equally to this work

***Corresponding author:** Alvaro Peix. Instituto de Recursos Naturales y Agrobiología, IRNASA-CSIC, c/Cordel de Merinas 40-52, 37008 Salamanca, Spain. E-mail: alvaro.peix@csic.es

Running title: *Pseudomonas punonensis* sp. nov.

Keywords: *Pseudomonas*/ taxonomy/ grasses /Peru/Altiplano

Contents List Category: New taxa Gram negative bacteria (Proteobacteria)

Accession numbers for strain LMT03^T(=M4PAPS15^T) gene sequences: JQ344321 for 16S rRNA, JX435103 for *rpoD*, JX435104 for *rpoB* and JX435105 for *gyrB*

Summary

During a study of “tunta” (frozen-dry potato) production process in Peru a strain named LMT03^T was isolated from the grasses straw in which the potato are dried. This strain was classified into genus *Pseudomonas* on the basis of the 16S rRNA gene sequence analysis, and the closest related species is *Pseudomonas argentinensis* CH01^T with 99.3% identity in this gene and 96%, 92% and 86% identities in *rpoB*, *rpoD* and *gyrB* genes, respectively. The strain shows a polar single flagellum, like other related yellow pigment producing pseudomonads. The major quinone was Q9. The major fatty acids were 18:1 ω 7c in summed 8 (40.82%), 16:1 ω 6c/ 16:1 ω 6c in summed feature 3 (23.72%) and C_{16:0} (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 48h incubation on nutrient agar medium. Nitrate reduction was negative. It does not hydrolyse aesculin. The G+C DNA content was 57.8 mol %. DNA-DNA hybridization results showed lower than 52% relatedness with respect to the type strain of *Pseudomonas argentinensis* CH01^T. These results together with other phenotypic characteristics support the definition of a new species within genus *Pseudomonas*, for which the name *P. punonensis* sp. nov. is proposed. The type strain is LMT03^T = M4PAPS15^T (LMG 26839^T, CECT 8089^T).

Bitter potatoes (*Solanum juzepczukii* and *Solanum curtilobum*) play a definitive role in the balance of the fragile ecosystem of the Altiplano, because they can resist frost, (up 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, as bitter potatoes can not be consumed fresh due to its high content of glycoalkaloids, process named “tunta”. The "tunta", elaborated in the southern highlands of Peru and northern Bolivia, is traditionally obtained from frozen potato tubers in the cold bitter frost, its immersion in river pools for periods between 15 to 20 days, drying, shelling and a final freeze. The strain LMT03^T was isolated during a process for evaluating the microbiological quality control of this manufacturing chain. The organism 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, 10g of these grasses were submerged in 90 mL peptone water 0.1% and shaken thoroughly. 1 mL aliquots were inoculated into asparagine broth tubes and incubated at 28°C for seven days, and tubes with positive growth were streaked in cetrimide agar and incubated at 28°C for 48h. The strain LMT03^T was classified into 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.

The cells were stained according to the Gram procedure described by Doetsch (1981). Motility was checked by phase-contrast microscopy after growing them in nutrient agar medium at 22°C for 48 h. The flagellation type was determined by electron microscopy after 48h incubation in TSA at 22°C as was previously described (Rivas *et al.*, 2007). Strain LMT03^T is Gram negative, rod-shaped (0.4-0.5 x 1.2-1.3 µm) and motile by a single polar flagellum (Figure S1 is available at IJSEM on-line). Cells grew as round translucent yellow coloured colonies on nutrient agar.

For 16S rDNA 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'-

GCGTCGATCATCTTGCCG-3') designed in this study for amplification of *gyrB* gene.

The sequences obtained were compared with those from the GenBank using the BLASTN (Altschul *et al.*, 1990) and EzTaxon (Chun *et al.*, 2007) programs. For phylogenetic analysis sequences were aligned using the Clustal_X software (Thompson *et al.*, 1997). The distances were calculated according to Kimura's two-parameter model (Kimura, 1980). Phylogenetic trees of 16S rRNA were inferred using the neighbour-joining analysis (NJ, Saitou & Nei, 1987), and maximum likelihood (ML; Rogers & Swofford, 1998). MEGA5 software (Tamura *et al.*, 2011) was used for all analyses.

The comparison of the 16S rRNA gene sequence of strain LMT03^T against the type strains of bacterial species recorded in the EzTaxon database showed that the new strain belong to genus *Pseudomonas* being *P. argentinensis* CH01^T its closest relative with 99.3% identity (11 different nucleotides). Other related species are *P. straminea* IAM 1598^T and *P. flavescens* B62^T with 98.8% (17 different nucleotides) and 98.5% (22 different nucleotides) identities, respectively. The remaining species of genus *Pseudomonas* presented identities lower than 98.5%. All the species showing more than 97% identity in the 16S rRNA gene as well as the type species of the genus, *P. aeruginosa* LMG 1242^T, were included in the phylogenetic analysis. The NJ phylogenetic tree (figure 1) showed that strain LMT03^T occupied a branch related with a cluster formed by *P. argentinensis* CH01^T, *P. straminea* IAM 1598^T and *P. flavescens* B62^T. Similar results were obtained after ML phylogenetic analysis (data not shown).

Additionally 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 LMT03 as a separated species within the *P. straminea* group. The concatenated *rpoD*, *rpoB* and *gyrB* genes phylogenetic tree (figure 2) showed that LMT03^T cluster together with the type strains of *P. argentinensis*, *P. straminea* and *P. flavescens*, being *P. argentinensis* the closest related species (figure 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 *rpoB* gene, the identities were 95.8%, 90.5% and 90.7%

respectively, and for *gyrB* gene 86%, 87.8% and 89%, respectively. These values are similar or lower than those found among several species of genus *Pseudomonas*. For example, in the case of *rpoD* gene, *P. jessenii* showed about 92% identity with respect to *P. vancouverensis*, *P. moorei* and *P. mohnii*; *P. reinekii* showed 94% 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. *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 LMT03^T 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). LMT03^T was hybridized with *P. argentinensis* CH01^T and *P. argentinensis* PA01, and after four replicates less than 52% hybridization was obtained in both cases. LMT03^T showed a mean value of 51% (47/56 reciprocal values) with respect to CH01^T and 46% (42/50 reciprocal values) with respect to PA01. Therefore the strain LMT03^T represents a different species within 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). The mol % G+C content of DNA was determined using the thermal denaturation method (Mandel & Marmur, 1968). The G+C content of strain LMT03^T was 57.8 mol %. These values are similar to those obtained for *P. argentinensis* and related species (Peix *et al.*, 2005).

The 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). *P. punonensis* LMT03^T was grown on TSA plates (Becton Dickinson, BBL) for 24h at 28°C as was previously described for *P. argentinensis* CH01^T, *P. straminea* IAM 1598^T and *P. flavescens* DSM12071^T. The major fatty acids of strain LMT03^T were 18:1 ω 7c in summed 8 (40.82%), 16:1 ω 6c/ 16:1 ω 6c in summed feature 3 (23.72%) and C_{16:0} (15.20%). As

expected, all the relatives clustering in the same phylogenetic group that strain LMT03^T shared similar fatty acid profiles (Table 1), although slight differences were found in the amounts of C_{10:0} 3OH, C_{12:0} 3OH and C_{16:0}. Therefore LMT03^T has the three fatty acids typically present in genus *Pseudomonas* according to Palleroni (2005) which are C_{10:0} 3OH, C_{12:0} and C_{12:0} 3OH.

The strain LMT03^T was cultivated for 24h 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 and Dr. Brian Tindall at DSMZ (Braunschweig, Germany) from freeze dried cells using the methods described by Tindall (1990a; 1990b). The novel isolate LMT03^T contained Q9 as major quinone (96%) and low levels of Q8 (4%). The presence of Q9 as major quinone is in agreement with the results obtained in the species of 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 LMT03^T 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 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 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 ID32GN and API 50CH (BioMérieux, France) were used following the manufacturer's instructions. The results of API 20NE and API ID32GN and API 50CH were recorded after 48h incubation at 28°C. Phenotypic characteristics of the new species 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 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 genus *Pseudomonas* to other ribosomal RNA 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 new species within 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 of 1.2-1.3 µm in length and 0.4-0.5 µm in diameter, motile by a single polar flagellum. Colonies morphology on nutrient agar are circular convex, yellow, translucent and usually 0.5 to 2.5 mm in diameter within 2 days growth at 25°C. It grows at 5°C but not at 41°C and pH range for growth is 5 to 9. A diffusible fluorescent pigment is produced on King B medium. Strictly aerobic with oxidative metabolism and no fermentation of sugars in peptone media. The major quinone was Q9. The major fatty acids were 18:1 ω7c in summed 8, 16:1 ω6c/ 16:1 ω6c in summed feature 3 and C_{16:0}. Oxidase positive and catalase weakly positive. The arginine dihydrolase system is not present. Urease, indol and β-galactosidase are not produced. Nitrate reduction and esculine hydrolysis were negative. Assimilation of glucose, L-arabinose, mannitol, glycerol, galactose, fructose, mannose, D-sucrose, turanose, gluconate, caprate, malate, citrate, itaconate, malonate, acetate, lactate, valerate, 3-hydroxybenzoate, 4-hydroxybutyrate, L-alanine, L-serine and L-proline was positive. Assimilation of N-acetyl-glucosamine, D-maltose, L-rhamnose, inositol, salicine, melibiose, L-fucose, sorbitol, glycogen, erythritol, L-xylose, D-xylose, adonitol, methyl-β-D-xyloside, methyl-β-D-glucoside, methyl-β-D-mannoside, dulcitol, amygdaline, arbutine, cellobiose, lactose, trehalose, melezitose, raffinose, starch, inulin, xylitol, gentiobiose, caprate, adipate, phenylacetate, L-histidine, 2 and 5-keto-gluconate, suberate, 3-hydroxybenzoate was negative. Assimilation of D-ribose and propionate is weak. G+C base composition

was 57.8 mol%. The type strain is LMT03^T (LMG 26839^T, CECT 8089^T) that was isolated from straw in Peru.

Acknowledgements

This research was granted by Projects PROCOM 255-2008-CONCYTEC-OAJ and PROCOM 273-2009-CONCYTEC-OAJ (Lima, Peru) as well as Junta de Castilla y León (Regional Spanish Government). MHRB is recipient of a JAE-Doc researcher contract from CSIC. We thank Association of tunta producers “Los Aymaras” by the collection of samples and Dr. J. Euzeby by his valuable help in providing the correct ethymology for the name of the new taxon. We also thank P. Pérez and M. J. Cuesta for their help in pigment spectral analysis.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D.J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403-410.

Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA sequences. *Int J Syst Bacteriol* **45**, 240-245.

Chun, J., Lee, J.H., Jung, Y., Kim, M., Kim, S., Kim, B.K. & Lim, Y.W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259-2261.

Clark, L.L., Dajcs, J.J., McLean, C.H., Bartell, J.G. & Stroman, D.W. (2006). *Pseudomonas otitidis* sp. nov., isolated from patients with otic infections. *Int J Syst Evol Microbiol* **56**, 709–714.

Doetsch, R.N. (1981). Determinative Methods of Light Microscopy. In Manual of Methods for General Bacteriology. pp. 21-33. Edited by P. Gerhardt, R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg & G.B. Phillips. Washington: American Society for Microbiology.

Ezaki, T., Hashimoto, Y. & Yabuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224-229.

Hildebrand, D. C., Palleroni, N.J., Hendson, M., Toth, J. & Johnson, J.L. (1994). *Pseudomonas flavescens* sp. nov., isolated from walnut blight cankers. *Int. J. Syst. Bacteriol.*, 44: 410-415.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111-120.

Mandel, M. & Mamur, J. (1968). Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195-206.

Mulet, M., Bennasar, A., Lalucat, J. & García-Valdés, E. (2009). An *rpoD*-based PCR procedure for the identification of *Pseudomonas* species and for their detection in environmental samples. *Mol Cell Probes* **23**: 140–147.

Mulet, M., Lalucat, J. & García-Valdés, E. (2010). DNA sequence-based analysis of the *Pseudomonas* species. *Environ Microbiol* **12**: 1513–1530.

Palleroni, N.J. (1984). Genus I. *Pseudomonas* Migula 1894. In *Bergey's Manual of Systematic Bacteriology*. Vol I, pp. 141-171. Edited by N.R. Krieg, & J.G. Holt. Baltimore, USA: Williams and Wilkins Cop.

Palleroni, N.J. (2005). Genus I. *Pseudomonas* Migula 1894, 237^{AL} (Nom. Cons., Opin. 5 of the Jud. Comm. 1952, 121). In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, part B, pp. 323-379. Edited by D. R. Boone, D. J. Brenner, R. W. Castenholz, G. M. Garrity, N. R. Krieg & J. T. Staley. New York: Springer.

- Peix, A., Berge, O., Rivas, R., Abril, A. & Velázquez, E. (2005).** *Pseudomonas argentinensis* sp. nov., a novel yellow pigment-producing bacterial species, isolated from rhizospheric soil in Córdoba, Argentina. *Int J Syst Evol Microbiol* **55**, 1107-1112.
- Rivas, R., García-Fraile, P., Mateos, P.F., Martínez-Molina, E. & Velázquez, E. (2007).** Characterization of xylanolytic bacteria present in the bract phyllosphere of the date palm *Phoenix dactylifera*. *Lett Appl Microbiol* **44**, 181-187.
- Rogers, J.S. & Swofford, D.L. (1998).** A fast method for approximating maximum likelihoods of phylogenetic trees from nucleotide sequences. *Syst Biol* **47**, 77-89.
- Saitou, N. & Nei, M. (1987).** A neighbour-joining method: a new method for reconstructing phylogenetics trees. *Mol Biol Evol* **4**, 406-425.
- Sasser, M. (1990).** Identification of bacteria by gas chromatography of cellular fatty acids, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar S. (2011).** MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol Evol* **28**, 2731-2739.
- Tayeb, L., Ageron, E., Grimont, F., & Grimont, P.A.D. (2005).** Molecular phylogeny of the genus *Pseudomonas* based on *rpoB* sequences and application for the identification of isolates. *Res Microbiol* **156**: 763–773.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Res* **25**, 4876-4882.
- Tindall, B.J. (1990a).** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst. Appl. Microbiol.* **13**, 128-130

320

321 **Tindall, B.J. (1990b).** Lipid composition of *Halobacterium lacusprofundi*. *FEMS*
322 *Microbiol. Letts.* **66**, 199-202

323

324 **Uchino, M., Kosako, Y., Uchimura, T. & Komagata, K. (2000).** Emendation of
325 *Pseudomonas straminea* Iizuka and Komagata 1963. *Int J Syst Bacteriol* **50**, 1513-
326 1519.

327

328 **Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O.,**
329 **Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt,**
330 **E., Starr, M.P. & Trüper, H.G. (1987).** Report of the ad hoc committee on
331 reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* **37**, 463-
332 464.

333

334 **Willems, A., Doignon-Bourcier, F., Goris, J., Coopman, R., De Lajudie, P. &**
335 **Gillis, M. (2001).** DNA-DNA hybridization study of *Bradyrhizobium* strains. *Int J Syst*
336 *Evol Microbiol* **51**, 1315-1322.

337

338 **Xiao, Y.P., Hui, W., Wang, Q., Roh, S.W., Shi, X.Q., Shi, J.H. & Quan, Z.X.**
339 **(2009).** *Pseudomonas caeni* sp. nov., a denitrifying bacterium isolated from the sludge
340 of an anaerobic ammonium-oxidizing bioreactor. *Int J Syst Evol Microbiol* **59**, 2594-
341 2598.

Figure legends:

Figure 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *Pseudomonas 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.

Figure 2. Neighbour-joining tree based on concatenated partial *rpoD*, *rpoB* and *gyrB* gene sequences of *Pseudomonas 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 (in percentage) of *P. punonensis* LMT03^T, its closest related species and the type species of the genus *Pseudomonas*. Data for *P. argentinensis* CH01^T (LMG22563^T), *P. straminea* IAM 1598^T and *P. flavescens* B62^T (LMG18387^T) were obtained in the same conditions by Peix *et al.* (2005). Data for *P. aeruginosa* KCTC1750^T are from Xiao *et al.* (2009) in the same conditions. nd: no detected, tr: traces. Summed feature 3: C16:1 ω 7c/16:1 ω 6c. Summed feature 8: C18:1 ω 7c.

Fatty acids	<i>P. punonensis</i>	<i>P. argentinensis</i>	<i>P. straminea</i>	<i>P. flavescens</i>	<i>P. aeruginosa</i>
10:0 3OH	4.83	2.40	3.91	3.74	3.6
11:0 3OH	0.93	0.10	nd	nd	nd
12:0 2OH	nd	0.09	0.21	nd	3.7
12:0 3OH	4.54	2.58	3.57	3.55	4.5
10:0	0.14	0.09	0.20	nd	tr
11:0	nd	0.09	nd	nd	nd
12:0	8.31	7.88	9.58	9.23	4.8
13:0	nd	0.08	nd	nd	nd
14:0	0.56	0.69	0.78	0.71	1.3
15:1 ω 6c	0.15	0.14	nd	nd	nd
15:0	nd	0.97	nd	nd	tr
16:0	15.20	19.69	17.63	19.75	20.5
17:1	nd	0.73	0.54	0.31	nd
17:0	0.27	0.52	0.36	nd	tr
18:0	0.58	0.51	0.52	0.78	tr
Summed feature 3	23.72	21.34	22.40	22.39	20.0
Summed feature 8	40.82	41.52	39.73	38.51	38.9

Table 2. Differential phenotypic characteristics among *P. punonensis* LMT03^T, its phylogenetically closest related species and the type species of this genus *P. aeruginosa*. The type strains of *P. argentinensis*, *P. straminea* and *P. flavescens* were included in this study as reference and the data obtained coincide with those previously published in Peix *et al.*, (2005), Uchino *et al.*, (2000) and Hildebrand *et al.*, (1994). Data for *P. aeruginosa* are from Palleroni (1984 and 2005).[‡]Data from Xiao *et al.* (2009). [¥]Data from Clark *et al.* (2006) for the type strain ATCC 10145^T. +: positive, -: negative, v: variable, w: weak. *The production of catalase is very weak

	<i>P. punonensis</i>	<i>P. argentinensis</i>	<i>P. straminea</i>	<i>P. flavescens</i>	<i>P. aeruginosa</i>
Catalase	w*	+	+	+	+
Non fluorescent	+	+	+	+	-
yellow pigment					
Growth at:					
4°C	+	-	+	+	-
37°C	+	+	w	-	+
Nitrate reduction	-	+	-	-	+
Acid from:					
Glucose	-	-	-	+	+ [‡]
Assimilation of:					
D-malate	+	+	-	+	v
Trehalose	-	+	-	+	-
Sucrose	+	-	-	+	-
Turanose	+	-	-	-	- [¥]
Valerate	+	+	-	-	+
L-histidine	-	+	v	+	+
L-alanine	+	+	-	+	+

0.01

Figure S1. Electron micrograph of strain LMT03^T showing the polar flagellum. Bar (1 cm), 0.1 μm