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

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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 ω7c in summed 8 (40.82%), 16:1 ω6c/ 16:1 ω6c 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 dihidrolase 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 comsumption 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 28ºC for seven days, and tubes with positive growth were streaked in cetrimide agar and incubated at 28ºC 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%

118 respectively, and for *gyrB* gene 86%, 87.8% and 89%, respectively. These values are
119 similar or lower than those found among several species of genus *Pseudomonas.* For
120 example, in the case of *rpoD* gene, *P. jessenii* showed about 92% identity with respect
121 to *P. vancouverensis, P.moorei* and *P. mohnii; P. reinekii* showed 94% with respect to
122 *P.moorei* and *P. mohnii*, *P.moorei* and *P. mohnii* showed 96% identity and *P.*
123 *koreensis* and *P. moraviensis* 93.7% identity. In the *rpoB* gene *P. vancouverensis* and
124 *P. mohnii* have 95.6% identity. *P.moorei* and *P. mohnii*, *P. jessenii* and *P. reinekii*, *P.*
125 *koreensis* and *P. moraviensis* and *P. vancouverensis*, *P. jessenii* and *P. reinekii*
126 showed about 97% identity. All these species showed values ranging from 89% to
127 97% in the *gyrB* gene among them. Therefore the results of the *rpoD, rpoB* and *gyrB*
128 gene analysis also suggested that strain LMT03^T belongs to an undescribed species of
129 *Pseudomonas*.
130 DNA-DNA hybridization was carried out by the method of Ezaki *et al*. (1989),
131 following the recommendations of Willems *et al*. (2001). LMT03^T was hybridized
132 with *P. argentinensis* CH01^T and *P. argentinensis* PA01, and after four replicates less
133 than 52% hybridization was obtained in both cases. LMT03^T showed a mean value of
134 51% (47/56 reciprocal values) with respect to CH01^T and 46% (42/50 reciprocal
135 values) with respect to PA01. Therefore the strain LMT03^T represents a different
136 species within genus *Pseudomonas* when the recommendation of a threshold value of
137 70% DNA-DNA similarity for definition of a bacterial species is considered (Wayne
138 *et al.*, 1987).
139 For base composition analysis, DNA was prepared according to Chun & Goodfellow
140 (1995). The mol % G+C content of DNA was determined using the thermal
141 denaturation method (Mandel & Marmur, 1968). The G+C content of strain LMT03^T
142 was 57.8 mol %. These values are similar to those obtained for *P. argentinenis* and
143 related species (Peix *et al.*, 2005).
144 The cellular fatty acids were analysed by using the Microbial Identification System
145 (MIDI; Microbial ID) Sherlock 6.1 and the library RTSBA6 according to the technical
146 instructions provided by this system (Sasser, 1990). *P. punonensis* LMT03^T was
147 grown on TSA plates (Becton Dikinson, BBL) for 24h at 28°C as was previously
148 described for *P. argentinensis* CH01^T, *P. straminea* IAM 1598^T and *P. flavescens*
149 DSM12071^T. The major fatty acids of strain LMT03^T were 18:1 ω7c in summed 8
150 (40.82%), 16:1 ω6c/ 16:1 ω6c in summed feature 3 (23.72%) and $C_{16:0}$ (15.20%). As

151 expected, all the relatives clustering in the same phylogenetic group that strain
152 LMT03^T shared similar fatty acid profiles (Table 1), although slight differences were
153 found in the amounts of $C_{10:0}$ 3OH, $C_{12:0}$ 3OH and $C_{16:0}$. Therefore LMT03^T has the
154 three fatty acids typically present in genus *Pseudomonas* according to Palleroni
155 (2005) which are $C_{10:0}$ 3OH, $C_{12:0}$ and $C_{12:0}$ 3OH.
156 The strain LMT03^T was cultivated for 24h in TSA plates (Becton Dikinson, BBL) at
157 28°C to obtain the cell mass required for quinone analysis that was carried out by the
158 Identification Service and Dr. Brian Tindall at DSMZ (Braunschweig, Germany) from
159 freeze dried cells using the methods described by Tindall (1990a; 1990b). The novel
160 isolate LMT03^T contained Q9 as major quinone (96%) and low levels of Q8 (4%).
161 The presence of Q9 as major quinone is in agreement with the results obtained in the
162 species of genus *Pseudomonas* (Palleroni, 2005).
163 For pigment analysis, cells were grown in King B agar and nutrient agar, and testing
164 for pigment production and spectral characteristics was performed by extraction with
165 methanol according to Hildebrand *et al*. (1994), using a visible-UV Kontron Uvikon
166 860 spectrophotometer. The spectral analysis of the methanol-extracted yellow
167 pigment of strain LMT03^T revealed a major peak at 446 nm, the same absorbance
168 position of the yellow pigment of *P. flavescens* (Hildebrand et al. 1994) and slightly
169 different to that of the closest relative *P. argentinensis*, whose major peak was at 442
170 nm (Peix et al., 2005), revealing high similarity of yellow-insoluble pigments in this
171 phylogenetic subcluster of genus *Pseudomonas*. As for the fluorescent pigment
172 analysis, the spectral study of supernatants from King's B broth cultures revealed a
173 peak at 334 nm, which is also in the range found for absorption peaks of other
174 fluorescent Pseudomonas species such as *P. argentinensis* (Peix *et al.*, 2005) or *P.*
175 *flavescens* (Hildebrand *et al.*, 1994).
176 The physiological and biochemical tests were performed as previously described
177 (Peix *et al.*, 2005). Additionally API 20NE, API ID32GN and API 50CH
178 (BioMérieux, France) were used following the manufacturer's instructions. The
179 results of API 20NE and API ID32GN and API 50CH were recorded after 48h
180 incubation at 28ºC. Phenotypic characteristics of the new species are reported below
181 in the species description and the differences with respect to the closest *Pseudomonas*
182 species are recorded in Table 2. The phenotypic characteristics of strain LMT03^T
183 support its classification within genus *Pseudomonas* since it is a motile Gram negative
184 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, translucid and usually 0.5 to 2.5 mm in diameter within 2 days growth at 25°C. It grows at 5ºC but not at 41ºC 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.

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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.

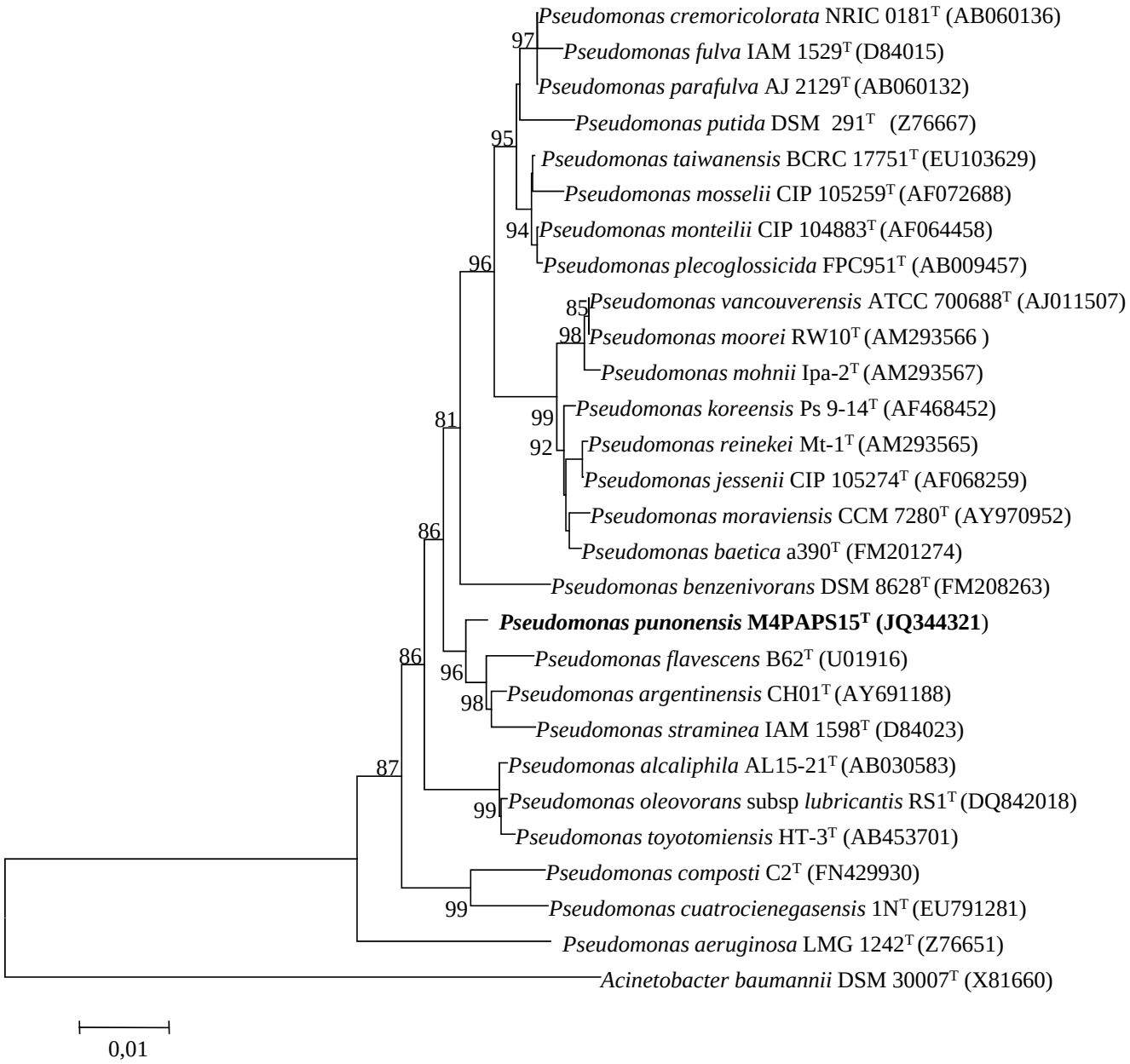
Figure 2. Neighbour-joining tree based on concatenated partial *rpoD, rpoB* and *gyrB* gene sequences of *Pseudomonas punonensis* LMT03^T and closely related *Pseudomonas* species. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 2 nt substitutions per 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 ω7c/16:1 ω6c. Summed feature 8: C18:1 ω7c.

Fatty acids	*P. punonensis*	*P. argentinensis*	*P. straminea*	*P. flavescens*	*P. aeruginosa*
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 ω6c	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

	P. punonensis	*P. argentinensis*	*P. straminea*	*P. flavescens*	*P. aeruginosa*
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	+	+	-	+	+


Pseudomonas cremoricolorata NRIC 0181^{T} (AB060136)
97
Pseudomonas fulva IAM 1529^{T} (D84015)
Pseudomonas parafulva AJ 2129^{T} (AB060132)
Pseudomonas putida DSM 291^{T} (Z76667)
95
Pseudomonas taiwanensis BCRC 17751^{T} (EU103629)
Pseudomonas mosselii CIP 105259^{T} (AF072688)
94
Pseudomonas monteilii CIP 104883^{T} (AF064458)
96
Pseudomonas plecoglossicida FPC951^{T} (AB009457)
85
Pseudomonas vancouverensis ATCC 700688^{T} (AJ011507)
98
Pseudomonas moorei RW10^{T} (AM293566)
Pseudomonas mohnii Ipa-2^{T} (AM293567)
81
99
Pseudomonas koreensis Ps 9-14^{T} (AF468452)
92
Pseudomonas reinekei Mt-1^{T} (AM293565)
Pseudomonas jessenii CIP 105274^{T} (AF068259)
Pseudomonas moraviensis CCM 7280^{T} (AY970952)
86
Pseudomonas baetica a390^{T} (FM201274)
Pseudomonas benzenivorans DSM 8628^{T} (FM208263)
***Pseudomonas punonensis* M4PAPS15^{T} (JQ344321**)
86
Pseudomonas flavescens B62^{T} (U01916)
96
98
Pseudomonas argentinensis CH01^{T} (AY691188)
Pseudomonas straminea IAM 1598^{T} (D84023)
87
Pseudomonas alcaliphila AL15-21^{T} (AB030583)
Pseudomonas oleovorans subsp *lubricantis* RS1^{T} (DQ842018)
99
Pseudomonas toyotomiensis HT-3^{T} (AB453701)
Pseudomonas composti C2^{T} (FN429930)
99
Pseudomonas cuatrocienegasensis 1N^{T} (EU791281)
Pseudomonas aeruginosa LMG 1242^{T} (Z76651)
Acinetobacter baumannii DSM 30007^{T} (X81660)
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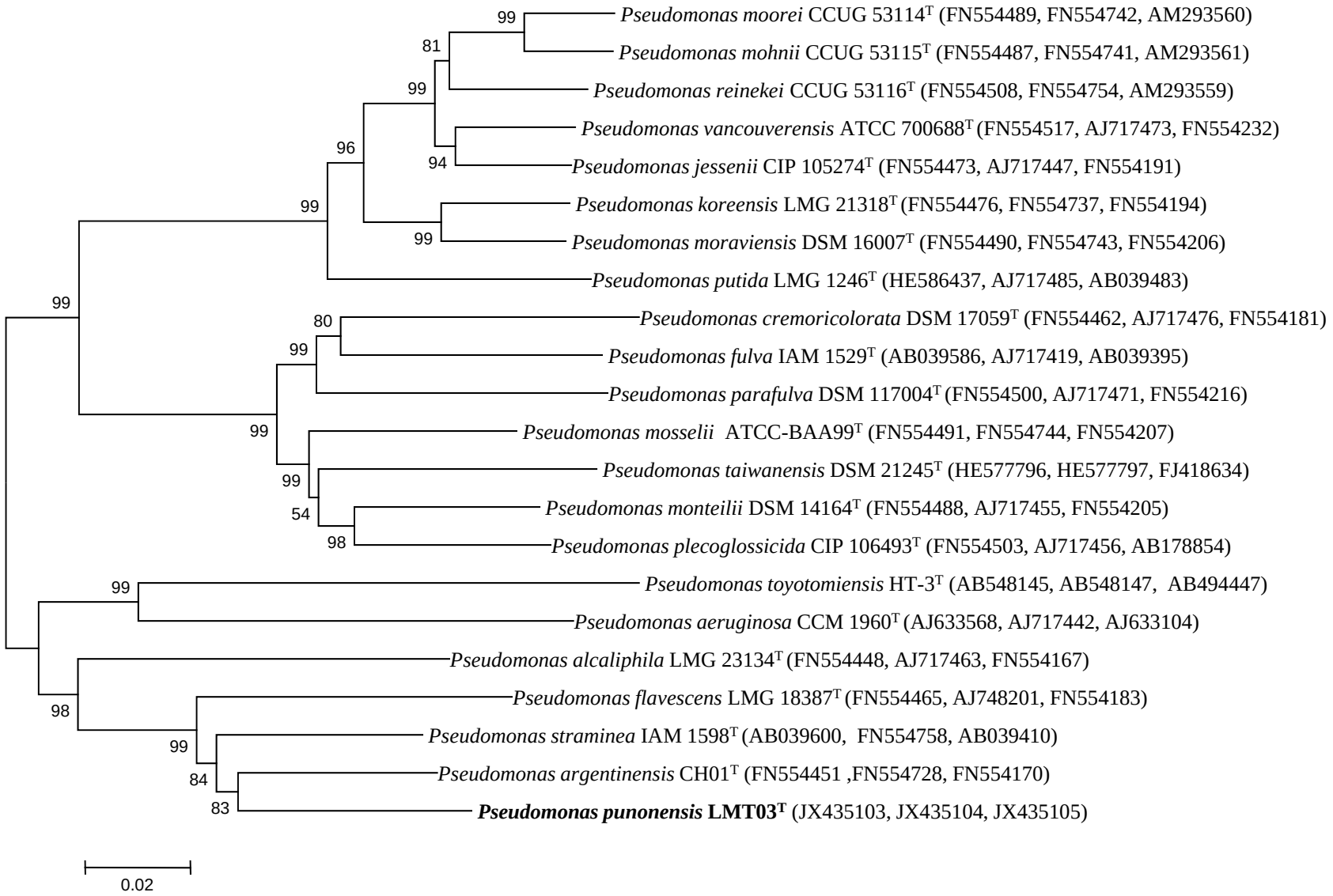
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Pseudomonas moorei CCUG 53114T (FN554489, FN554742, AM293560)
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Pseudomonas mohnii CCUG 53115T (FN554487, FN554741, AM293561)
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Pseudomonas reinekei CCUG 53116T (FN554508, FN554754, AM293559)
Pseudomonas vancouverensis ATCC 700688T (FN554517, AJ717473, FN554232)
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Pseudomonas jessenii CIP 105274T (FN554473, AJ717447, FN554191)
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Pseudomonas koreensis LMG 21318T (FN554476, FN554737, FN554194)
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Pseudomonas moraviensis DSM 16007T (FN554490, FN554743, FN554206)
Pseudomonas putida LMG 1246T (HE586437, AJ717485, AB039483)
99
80
Pseudomonas cremoricolorata DSM 17059T (FN554462, AJ717476, FN554181)
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Pseudomonas fulva IAM 1529T (AB039586, AJ717419, AB039395)
Pseudomonas parafulva DSM 117004T (FN554500, AJ717471, FN554216)
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Pseudomonas mosselii ATCC-BAA99T (FN554491, FN554744, FN554207)
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Pseudomonas taiwanensis DSM 21245T (HE577796, HE577797, FJ418634)
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Pseudomonas monteilii DSM 14164T (FN554488, AJ717455, FN554205)
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Pseudomonas plecoglossicida CIP 106493T (FN554503, AJ717456, AB178854)
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Pseudomonas toyotomiensis HT-3T (AB548145, AB548147, AB494447)
Pseudomonas aeruginosa CCM 1960T (AJ633568, AJ717442, AJ633104)
Pseudomonas alcaliphila LMG 23134T (FN554448, AJ717463, FN554167)
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Pseudomonas flavescens LMG 18387T (FN554465, AJ748201, FN554183)
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Pseudomonas straminea IAM 1598T (AB039600, FN554758, AB039410)
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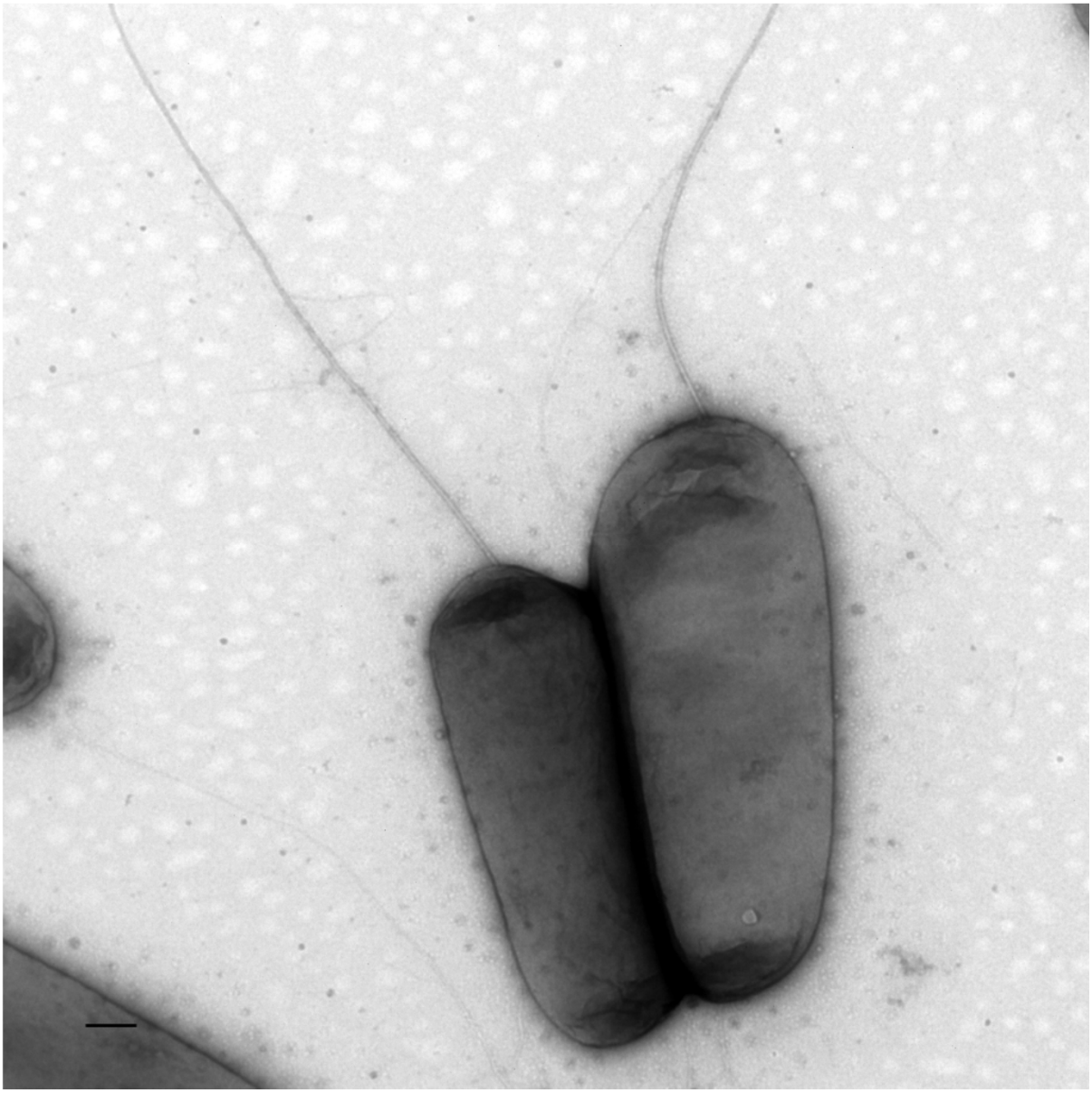

Figure S1. Electron micrograph of strain LMT03^{T} showing the polar flagellum. Bar (1 cm), 0.1 μm