The genus *Pseudomonas* Migula 1894 is a genus of gammaproteobacteria that belongs to the family *Pseudomonadaceae* (Moore *et al.*, 2006). It is one of the most complex bacterial genera and, to date, it has the most species with validly published names of all genera of Gram-negative bacteria (Mulet *et al.*, 2012). More than 120 species with validly published names with standardized descriptions are included in the genus *Pseudomonas* (Pascual *et al.*, 2012). The species are divided into two main lineages on the basis of four concatenated housekeeping genes, the *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* lineages, and an external group named *Pseudomonas putida* (Mulet *et al.*, 2012) that might represent an independent genus on the basis of phylogenetic analysis (Pascual *et al.*, 2012). The first lineage comprises the *P. fluorescens*, *P. syringae*, *P. lutea*, *P. putida*, *P. anguilliseptica* and *P. stutzeri* groups. The genus *Pseudomonas* continues to expand and, since 2013, nine novel species isolated from a wide variety of environments have been described: *Pseudomonas chengduensis* (Tao *et al.*, 2014), *P. formosensis* (Lin *et al.*, 2013b), *P. guangdongensis* (Yang *et al.*, 2013), *P. guariconensis* (Toledo *et al.*, 2013), *P. guguanensis* (Liu *et al.*, 2013), *P. helmanticensis* (Ramirez-Bahena *et al.*, 2014), *P. kunmingensis* (Xie *et al.*, 2014), *P. punonensis* (Ramos *et al.*, 2013) and *P. sagittaria* (Lin *et al.*, 2013a).

In the course of screening bacterial soil isolates for the production of novel bioactive compounds with pharmaceutical applications, a newly isolated strain, F-278,770\(^1\), was identified as producing cytotoxic compounds against several human tumour cell lines. This strain was isolated from a soil sample collected from the Tejeda, Almijara and Alhama Natural Park, Granada, Spain (36° 55’ 53.99” N 3° 51’ 45.34” W, 885 m above sea level) in November 2011. The soil pH was 7.87 in distilled water and soil humidity was 11.3 %. The soil sample was dispersed in sterile diluent (VL70 medium without added growth substrates or vitamins) by stirring with a magnetic bar, serially diluted, plated on gellan gum-solidified VL70 medium containing 0.05 % (w/v) d-xylose as carbon source (Sait *et al.*, 2002; Joseph *et al.*, 2003; Davis *et al.*, 2005) and then incubated at 18 °C and 60 % relative humidity in the dark for 2 weeks.

### Pseudomonas granadensis sp. nov., a new bacterial species isolated from the Tejeda, Almijara and Alhama Natural Park, Granada, Spain

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During the course of screening bacterial isolates as sources of as-yet unknown bioactive compounds with pharmaceutical applications, a chemo-organotrophic, Gram-negative bacterium was isolated from a soil sample taken from the Tejeda, Almijara and Alhama Natural Park, Granada, Spain. Strain F-278,770\(^1\) was oxidase- and catalase-positive, aerobic, with a respiratory type of metabolism with oxygen as the terminal electron acceptor, non-spore-forming and motile by one polar flagellum, although some cells had two polar flagella. Phylogenetic analysis of the 16S rRNA, gyrB, rpoB and rpoD genes revealed that strain F-278,770\(^1\) belongs to the *Pseudomonas koreensis* subgroup (*Pseudomonas fluorescens* lineage), with *Pseudomonas moraviensis*, *P. koreensis*, *P. baetica* and *P. helmanticensis* as its closest relatives. Chemotaxonomic traits such as polar lipid and fatty acid compositions and G+C content of genomic DNA corroborated the placement of strain F-278,770\(^1\) in the genus *Pseudomonas*. DNA–DNA hybridization assays and phenotypic traits confirmed that this strain represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas granadensis* sp. nov. is proposed. The type strain is F-278,770\(^1\) (=DSM 28040\(^T\) =LMG 27940\(^T\)).

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**Abbreviations:** FAME, fatty acid methyl ester; HGT, horizontal gene transfer; ML, maximum-likelihood; MLSA, multilocus sequence analysis; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA, rpoB, rpoD and gyrB gene sequences of strain F-278,770\(^1\) are HG764746, HG764747, HG764748 and HG764749, respectively.

Six supplementary figures and two supplementary tables are available with the online Supplementary Material.
Strain F-278,770T was isolated on tryptic soy agar medium (TSA; BD Biosciences) to verify its purity. Cell suspensions in TSA supplemented with 20% glycerol were maintained at −80 °C.

Reference strains used for taxonomic comparisons were *Pseudomonas baetica* CECT 7770T, *P. jessenii* DSM 17150T, *P. koreensis* LGM 21318T, *P. moroeti* DSM 12647T, *P. moraviensis* LGM 24280T, *P. reinekei* DSM 18361T and *P. vancouverensis* DSM 17555T. Strain F-278,770T and the reference strains were routinely grown aerobically on TSA or in TSB for 24 or 48 h at 28 °C in the dark for inoculum or sample preparation.

Strain F-278,770T was Gram-negative according to a 3% KOH assay (Powers, 1995) and oxidase- and catalase-positive on tests with 1% (w/v) *N*,*N*,*N*,*N*-tetramethyl-p-phenylenediamine dihydrochloride and 3% (v/v) hydrogen peroxide solutions, respectively. It showed an aerobic metabolism and was able to grow on TSA at 4−37 °C but not at 45 °C and on nutrient agar (NA) prepared in the laboratory ingredient by ingredient and supplemented with 0.5−5% (w/v) NaCl but not with 6% (w/v) NaCl. Cells were rod-shaped (0.3−0.7 µm wide and 1.1−2.2 µm long), non-spore-forming (Schaeffer & Fulton, 1933) and motile by one polar flagellum, although some cells had two polar flagella (Fig. S1, available in the online Supplementary Material) (Heimbrook et al., 1989). After 48 h of incubation at 28 °C on TSA plates, colonies of strain F-278,770T were circular (approx. 3−5 mm in diameter), convex with regular edges, white−yellow and mucoid (Fig. S2). Strain F-278,770T produced neither fluorescein nor pyocyanin on King B and King A medium, respectively (King et al., 1954).

Commercial miniaturized API 20E, API 20NE and API ZYM galleries (bioMérieux) and carbon utilization tests based on Biolog GN2 MicroPlates were used according to the manufacturer’s instructions. API 20E and API 20NE strips and Biolog GN2 plates were examined after 48 h and API ZYM strips after 4 h of incubation. Miniaturized test results were recorded in triplicate. Using the API 20NE and API 20E systems, strain F-278,770T was positive for hydrolysis of gelatin, citrate utilization and assimilation of glucose, arabinose, mannose, mannotol, *N*-acyethylglucosamine, potassium gluconate, capric acid, malic acid and trisodium citrate. Acid was produced from glucose, melibiose and arabinose. After three trials, the activity of arginine dihydrolase was variable between API galleries, always showing a negative result in API 20E. In the API ZYM system, our isolate was positive for esterase C4, esterase C8, leucine arylamidase, acid phosphatase and naphthol AS-Bl-phosphohydrolase, whereas the test for valine arylamidase displayed a weak result. In the Biolog GN2 system after 48 h of incubation, strain F-278,770T oxidized 50 compounds, while 13 were oxidized weakly. They are listed in the species description. Differential physiological and biochemical characteristics are summarized in Table 1. Phenotypic properties of the reference strains were in general accordance with results given in the literature.

For fatty acid methyl ester (FAME) analysis, fatty acids were extracted, saponified and methylated according to standard protocols (MIDI Microbial Identification System; Sasser, 1990), and individual FAMEs were identified using the Microbial Identification System using the TSBA50 library (MIDI).

The major cellular fatty acids of strain F-278,770T were characterized as summed feature 3 (C16:1ω7c and/or iso-C15:0 2-OH; 35.6%), C16:0 (23.7%) and C18:1ω7c (12.4%), along with smaller amounts of C10:0 3-OH, C12:0 2-OH, C12:0 3-OH and C17:0 cyclo (Table S1). Our isolate shared a similar FAME profile with the reference type strains of species of *Pseudomonas* analysed in this survey and the profile was consistent with previously published results (Tzrsová et al., 2006; Cámara et al., 2007; López et al., 2012), supporting the classification of our isolate in the genus *Pseudomonas* (Oyaizu & Komagata, 1983; Vancanneyt et al., 1996).

Analysis of polar lipids was carried out by the Identification Service of the DSMZ (Braunschweig, Germany). The identified polar lipid components of strain F-278,770T included major amounts of phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol, which is in agreement with data published previously for species of the genus *Pseudomonas* (Moore et al., 2006; Pascual et al., 2012). In addition, three unidentified phospholipids, an unidentified aminolipid and an unidentified lipid were detected in small amounts (Fig. S3).

Genomic DNA was extracted with the DNeasy Blood &Tissue kit (Qiagen) and the concentration and purity of each sample were determined by measuring the *A*260 and the *A*260/*A*320 ratio, respectively. For amplification of nearly full-length 16S rRNA genes, the primers FD1 (5′-AGAGTTTGATCTGGCTCAG-3′) and RP2 (5′-ACGCTACCTTGTTACGACTT-3′) were used (Weisburg et al., 1991). PCR mixtures included 5.0 µl PCR buffer (10×), 4.0 µl MgCl2 (25 mM), 1.0 µl dNTPs (10 mM each), 1.0 µl each forward and reverse primer (10 µM), 0.3 µl Taq polymerase (5 U µl−1; Qiogene) and 5.0 µl DNA in a total volume of 50 µl. The thermal cycling program was described previously (Pascual et al., 2010). PCR products were purified and sequenced using the above primers and the internal primers 926F (5′-AACCTYAAAKGAATTGACGG-3′) and 1100R (5′-GGTTGCGTCTGGT-3′) at Secugen S.L. (Madrid, Spain). Multilocus sequence analyses (MLSA) based on three (*rpoB*, *rpoD* and *gyrB*) and four (*rpoB*, *rpoD*, *gyrB* and 16S rRNA) housekeeping genes, accepted as a useful tool to determine more precisely phylogenetic relationships among species of the genus *Pseudomonas* (Mulet et al., 2012), were employed here. Partial coding genes of strain F-278,770T were amplified and sequenced according to Ait Tayeb et al. (2005) and Pascual et al. (2012). The remaining sequences analysed in this paper were obtained from public databases and their accession numbers are mapped on the phylogenetic trees. Phylogenetic analyses were performed according to Pascual et al. (2010, 2012). The
Table 1. Comparative phenotypic characteristics among strain F-278,770T and related type strains

Strains: 1, F-278,770T; 2, *P. baetica* CECT 7720T; 3, *P. helmanticensis* OHA11T; 4, *P. jessenii* DSM 17150T; 5, *P. koreensis* LMG 21318T; 6, *P. moorei* DSM 12647T; 7, *P. moraviensis* LMG 24280T; 8, *P. reinekei* DSM 18361T; 9, *P. vancouverensis* DSM 17555T. Data were obtained in this study except for the Biolog GN2 profiles of *P. baetica* CECT 7720T (López et al., 2012), *P. koreensis* LMG 21318T (Kwon et al., 2003) and *P. moraviensis* LMG 24280T (Tvrzová et al., 2006) and all data for *P. helmanticensis* OHA11T (Ramírez-Bahena et al., 2014). +, Positive; −, negative; ND, no data available; V, variable (different results in three different trials); W, weakly positive.

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*Reported as negative by Cámara et al. (2007).
†Reported as negative by Kwon et al. (2003).
‡Reported as positive by Tvrzová et al. (2006).
P. granadensis F-278,770T (HG764746)

P. vancouverensis Dha-51T (AJ011507)

P. moraviensis DSM 16007T (AY970952)

P. koreensis Ps 9-14T (AF468842)

P. jessenii CIP 105274T (AF068259)

P. moorei RW10T (AM293566)

P. umsorgensis Ps 3-10T (AF468840)

P. baetica JLF-a390T (FM201274)

P. mohnii IpA-2T (AM293566)

P. umsongensis Ps 3-10T (AF468450)

P. baetica JLF-a390T (FM201274)

P. palleroniana CFBP 4389T (AY091527)

P. lurida DSM 15835T (AJ581999)

P. cedrina subsp. cedrina CFBP 5705T (AF374472)

P. cedrina subsp. fulgida DSM 14938T (AJ942830)

P. chlororaphis DSM 50083T (Z76673)

P. fragi ATCC 4973T (AF094733)

P. deceptivus DSM 9987T (AF064180)

P. azotoformans IAM 1603T (D84009)

P. gessardii CIP 105469T (AF074384)

P. mucidolens IAM 12406T (D84017)

P. libanensis DSM 14936T (AJ942830)

P. synxantha IAM 12356T (D84027)

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sequence lengths of each alignment ranged from 1362 to 1420 nt (16S rRNA gene), 2424 to 2803 nt (three concatenated genes) and 3817 to 4222 nt (four concatenated genes). The sequence lengths of strain F-278,770T sequenced in this study were 1419, 1119, 768 and 916 nt for the 16S rRNA, rpoB, rpoD and gyrB genes, respectively. According to the EzTaxon-e database (Kim et al., 2012), the closest relatives of strain F-278,770T on the basis of the 16S rRNA gene sequence were P. vancouverensis DhaA-51T, P. reinekei MT1T, P. moorei RW10T, P. jessenii CIP 105274T and P. koreensis Ps 9-14T, sharing 16S rRNA gene sequence similarity of 99.50, 99.50, 99.49, 99.29 and 99.28 %, respectively. This result is consistent with the pairwise nucleotide similarity values of 16S rRNA gene sequences calculated in this study as p-distances (Table S2). In the 16S rRNA gene sequence tree based on the neighbour-joining (NJ) algorithm (Fig. 1), strain F-278,770T grouped with the type strains of P. vancouverensis, P. reinekei, P. moraviensis, P. koreensis, P. jessenii, P. moorei, P. umsongensis, P. baetica, P. helmanticensis, P. mohnii, P. asplenii, P. fuscovaginae and P. agarici, these 14 taxa forming a well-defined clade. When the maximum-parsimony (MP) and maximum-likelihood (ML) algorithms were used, the same 14 taxa were clustered as a monophyletic group, although the relative position of the taxa was not exactly identical (Figs S5 and S6). This lack of stability in the relative taxonomic position of species of the genus Pseudomonas when using the 16S rRNA gene sequence is in agreement with the low bootstrap values found in the internal nodes. Currently, phylogenetic analyses based on MLSA are considered more likely to diagnose the correct evolutionary pattern among species of the genus because MLSA uses a larger number of nucleotides than a single gene analysis and mitigates possible confounding effects of horizontal gene transfer (HGT) on phylogenetic reconstructions (Pascual et al., 2010). We checked these genes to detect possible recombination events with the RDP4 Beta 4.16 program (Martin et al., 2010; Pascual et al., 2010) and did not detect any signal of HGT in the sequences that could distort the phylogenetic signal. Given that there is no agreement within the scientific community whether to include or exclude the 16S rRNA gene sequence in MLSA applied to the genus Pseudomonas (Mulet et al., 2012; Ramírez-Bahena et al., 2014), we analysed MLSA based on three (rpoB, rpoD and gyrB) and four (rpoB, rpoD, gyrB and 16S rRNA) concatenated gene sequences. By concatenating three housekeeping genes, strain F-278,770T was placed into the P. koreensis subgroup in all three trees reconstructed with the NJ, MP and ML algorithms, with its closest relatives being P. moraviensis, P. koreensis, P. baetica and P. helmanticensis (Figs 2, S5 and S6). The phylogenetic relationships among the species displayed high bootstrap values, suggesting that they form robust clades. In addition to the P. koreensis subgroup, other subgroups such as the P. jessenii and P. mandelii subgroups could also be clearly resolved (Figs 2, S5 and S6), corroborating the previous phylogenetic delineations of the genus Pseudomonas (Mulet et al., 2012). When the 16S rRNA gene was included in the MLSA (four concatenated genes), similar results were obtained (Figs S4–S6), given that the low capacity of the 16S rRNA gene to differentiate recently diversified species is buffered by the faster evolutionary rates of the other three housekeeping genes (Pascual et al., 2010).

The highest interspecific sequence similarities found between strain F-278,770T and its phylogenetic neighbours were 99.5 % with P. moorei–P. reinekei–P. vancouverensis (16S rRNA gene), 95.1 % with P. moraviensis (MLSA of three concatenated genes) and 96.6 % with P. moraviensis (MLSA of four concatenated genes) (Table S2). These values were lower than the maximum interspecific similarity found among the remaining species of the genus Pseudomonas analysed in this study: 100 % (16S rRNA gene), 96.8 % (MLSA of three concatenated genes) and 97.8 % (MLSA of four concatenated genes). Moreover, the highest interspecific sequence similarity found in the MLSA based on these four genes between strain F-278,770T and its phylogenetic neighbours (96.6 %) was lower than the threshold value (97 %) established for strains belonging to the same species in the genus Pseudomonas (Mulet et al., 2010) (Table S2). Consequently, a priori, our strain could not be assigned phylogenetically to any described species. However, because the maximum interspecific 16S rRNA gene sequence similarity found between strain F-278,770T and type strains of other species of the genus Pseudomonas exceeded the recently updated threshold value (98.65 %) that differentiates prokaryotic species (Kim et al., 2014), we employed DNA–DNA hybridizations to corroborate whether our isolate represented a novel taxon in the genus Pseudomonas.

DNA–DNA hybridizations were measured by the plate method (Urdiaín et al., 2008), combining the hydroxyapatite method with non-radioactive detection of released DNA. The hybridization temperature was 70 °C. DNA–DNA hybridization values were determined in triplicate between the novel strain and all the reference strains used in this study. Reciprocal hybridizations assay were also performed. DNA–DNA hybridization values between strain F-278-770T and its closest relatives (Table 2) were clearly below the accepted threshold value of 70 % for species delineation (Wayne et al., 1987), confirming that strain F-278,770T represents a unique species.

Analysis of the molar G+C content of genomic DNA was carried out by the Identification Service of the DSMZ using an HPLC method according to Tamaoka & Komagata (1984) and Mesbah et al. (1989). The DNA G+C content
of strain F-278,770ᵀ was 60.3 mol%. This value is within the range reported for members of the genus *Pseudomonas* (Moore *et al.*, 2006).

Comparison of the morphological, physiological, chemotaxonomic and phylogenetic characteristics of strain F-278,770ᵀ with those of its closest phylogenetic neighbours supports the conclusion that this strain represents a novel species belonging to the *P. koreensis* subgroup of the genus *Pseudomonas*, for which we propose the name *Pseudomonas granadensis* sp. nov.

**Description of Pseudomonas granadensis** sp. nov.

*Pseudomonas granadensis* (gra.na.den'sis. N.L. fem. adj. *granadensis* denizen of the province of Granada in south-east Spain, where the type strain was isolated).

Cells are Gram-negative rods, 0.3–0.7 μm wide and 1.1–2.2 μm long, non-spore-forming, motile by one polar flagellum, although some cells have two polar flagella. Chemo-organotrophic, aerobic, having a respiratory type of metabolism with oxygen as the terminal electron acceptor, oxidase- and catalase-positive. After 48 h of incubation at 28 °C on TSA, colonies are white–yellow, circular (approx. 3–5 mm in diameter), convex with regular edges and mucoid. Produces neither fluorescein on King B medium nor pyocyanin on King A medium. Growth is observed at 4–37 °C (optimum 28 °C) and on media supplemented with 0.5–5 % (w/v) NaCl (optimum 0.5 %). In API 20NE and API 20E tests, positive for hydrolysis of gelatin and citrate utilization, and variable between API galleries for activity of arginine dihydrolase. Assimilates glucose, arabinose, mannose, mannitol, N-acetylglucosamine, potassium gluconate, capric acid, malic acid and trisodium citrate. Acid is produced from glucose, melibiose and arabinose. In the API ZYM system, positive for esterase C₄, esterase C₈, leucine arylamidase, acid phosphatase and naphthol AS-Bl-phosphohydrolase, showing weak activity for valine arylamidase. The following substrates are oxidized (Biolog GN2): Tween 40 and 80, N-acetyl-D-glucosamine, L-arabinose, D-arabitol, D-fructose, D-galactose, x-D-glucose, D-mannitol, D-mannose, trehalose, methyl pyruvate, monomethyl succinate, cis-aconitic acid, citric acid, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, β-hydroxybutyric acid, D-glucosaminic acid, β-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D- and L-alanine, L-allyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, hydroxy-L-proline, L-leucine, L-proline, L-pyroglutamic acid, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, uridine,
Table 2. DNA–DNA hybridization between strain F-278,770T and the type strains of closely related species

Close relatives were identified by phylogenetic analyses of the 16S rRNA gene and MLSA. Reciprocal hybridization values are given in parentheses.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DNA–DNA hybridization with strain F-278,770T (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. granadensis</em> F-278,770T</td>
<td>100</td>
</tr>
<tr>
<td><em>P. baetica</em> CECT 7720T</td>
<td>60.8 ± 4.1 (61.5)</td>
</tr>
<tr>
<td><em>P. koreensis</em> LMG 21318T</td>
<td>62.9 ± 3.5 (62.1)</td>
</tr>
<tr>
<td><em>P. moorei</em> DSM 12647T</td>
<td>46.9 ± 2.7 (46.2)</td>
</tr>
<tr>
<td><em>P. moraviensis</em> LMG 24280T</td>
<td>63.2 ± 3.3 (58.2)</td>
</tr>
<tr>
<td><em>P. reinekei</em> DSM 18361T</td>
<td>47.2 ± 4.2 (51.7)</td>
</tr>
<tr>
<td><em>P. vancouverensis</em> DSM 17555T</td>
<td>57.4 ± 1.7 (61.1)</td>
</tr>
</tbody>
</table>

2-aminoethanol and glycerol. Oxidation of dextrin, gentiobiose, d-psicose, acetic acid, formic acid, α-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, L-ornithine, L-phenylalanine, D-serine, putrescine and DL-α-glycerol phosphate is weak. The other organic substrates included in the Biolog GN2 microplate are not oxidized. The most abundant FAMEs are summed feature 3 (C15:0 7c and/or iso-C15:0 2-OH), C16:0 and C18:1 ω7c. Polar lipids consist of major amounts of phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol plus small amounts of three unidentified phospholipids, an unidentified aminolipid and an unidentified lipid.

The type strain is F-278,770T (=DSM 28040T=LMG 27940T), isolated from a soil sample collected from the Tejeda, Almijara and Alhama Natural Park, Granada, Spain. The DNA G+C content of the type strain is 60.3 mol%.

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