Pseudomonas canadensis sp. nov., a biological control agent isolated from a field plot under long-term mineral fertilization

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Abstract

The bacterial strain 2-92\textsuperscript{T}, isolated from a field plot under long-term (>40 years) mineral fertilization, exhibited in vitro antagonistic properties against fungal pathogens. A polyphasic approach was undertaken to verify its taxonomic status. Strain 2-92\textsuperscript{T} was Gram-reaction-negative, aerobic, non-spore-forming, motile by one or more flagella, and oxidase-, catalase- and urease-positive. The optimal growth temperature of strain 2-92\textsuperscript{T} was 30°C. 16S rRNA gene sequence analysis demonstrated that the strain is related to species of the genus Pseudomonas. Phylogenetic analysis of six housekeeping genes (dnaA, gyrB, recA, recF, rpoB and rpoD) revealed that strain 2-92\textsuperscript{T} clustered as a distinct and well separated lineage with Pseudomonas simiae as the most closely related species. Polar lipid and fatty acid compositions corroborated the taxonomic position of strain 2-92\textsuperscript{T} in the genus Pseudomonas. Phenotypic characteristics from carbon utilization tests could be used to differentiate strain 2-92\textsuperscript{T} from closely related species of the genus Pseudomonas. DNA–DNA hybridization values (wet laboratory and genome-based) and average nucleotide identity data confirmed that this strain represents a novel species. On the basis of phenotypic and genotypic characteristics, it is concluded that this strain represents a separate novel species for which the name Pseudomonas canadensis sp. nov. is proposed, with type strain 2-92\textsuperscript{T} (=LMG 28499\textsuperscript{T}=DOAB 798\textsuperscript{T}). The DNA G+C content is 60.30 mol%.

Species of the genus Pseudomonas are aerobic, Gram-reaction-negative gammaproteobacteria, ubiquitous in agricultural soils, and are well adapted to grow in the rhizosphere. This genus includes species that are of significant environmental importance such as plant growth promoters, xenobiotic degraders and biocontrol agents [1–3]. The fluorescent pseudomonads are uniquely capable of synthesizing many metabolites that play a role in maintaining soil health leading to bioprotection of crops against pathogens [4, 5].

In 2009, we started a prospective study focused on cultivable members of the genus Pseudomonas in a soil ecosystem under long-term (>40 years) applications of inorganic fertilizer (NPK). Soil samples were collected from a corn-alfalfa rotation plot located at Woodslee, Ontario, Canada (42.22 N 82.73 W). Triplicates of bulk soil (10 g each) were suspended in 90 ml 0.85 % NaCl and vortexed vigorously. The soil suspensions were serially diluted in 0.85 % NaCl, plated on King’s B (KB) agar (Sigma-Aldrich) and incubated at 28°C for 48 h to isolate fluorescent pseudomonads. Colonies were screened for fluorescence under UV light. Single colonies were obtained after repeated streaking and plated on Pseudomonas-F agar medium (BD Difco). A total of 148 bacterial isolates were obtained of which 99 % fluoresced under UV light. All isolates were evaluated for in vitro antagonistic activities against the following fungal pathogens: Fusarium graminearum, Rhizoctonia solani and Gaeumannomyces graminis. Twelve of the isolates were able to inhibit the mycelial growth of at least two of the fungal pathogens. All 12 biological control isolates were identified as members of the genus Pseudomonas on the basis of phenotypic features and 16S rRNA sequence analyses. Of the three strains (2–92, 2–36 and 2–114) that potently inhibited the growth of R. solani and G. graminis, two were assigned to known species of the genus Pseudomonas: Pseudomonas simiae 2–36 and Pseudomonas extremorientalis 2–114. Strain 2–92\textsuperscript{T} did not match any known species of the genus Pseudomonas.

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Keywords: Pseudomonas canadensis sp. nov.; biological control; genome sequencing; ANIm; digital DNA-DNA hybridization (ddDH); polar lipids.

Abbreviations: DDH, DNA-DNA hybridization; ML, maximum-likelihood.

The GenBank accession numbers of draft genomes used in this study and from which the sequences of six genes (dnaA, gyrB, recA, recF, rpoB and rpoD) were extracted are AYTDO0000000, MDDQQ000000, MDOR0000000, MDGK0000000, MDFK0000000, MDFI0000000 and MDFJ0000000 for strain 2-92\textsuperscript{T}, Pseudomonas azotoformans LMG 21611\textsuperscript{T}, P. costantinii LMG 22119\textsuperscript{T}, P. extremientalis LMG 19695\textsuperscript{T}, P. poae LMG 21465\textsuperscript{T}, P. salomonii LMG 22120\textsuperscript{T}, P. simiae CCUG 50988\textsuperscript{T} and P. trivialis LMG 21464\textsuperscript{T}, respectively. The 16S rRNA gene sequence of strain 2-92\textsuperscript{T} is also deposited in GenBank with accession number HQ403142.

Two supplementary figures and two supplementary tables are available with the online Supplementary Material.
In the present study, the taxonomic status of strain 2–92T was investigated using electron microscopy, phenotypic tests, chemotaxonomic traits, analyses of the 16S rRNA gene and six house-keeping genes (dnaA, gyrB, recA, recF, rpoB and rpoD), DNA–DNA hybridization and DNA G+C content determination as well as analysis of draft genome sequences. Based on this polyphasic characterization, a novel species, *Pseudomonas canadenensis* sp. nov. is proposed.

Cells of strain 2–92T were purified on *Pseudomonas*-F agar medium (BD Difco), and cell suspensions in Luria–Bertani (LB; BD Difco) broth supplemented with 30 % (v/v) glycerol were maintained at –80 °C. Cells were routinely grown overnight in liquid LB with shaking or on LB agar medium and incubated at 28 °C. Strain 2–92T was Gram-negative based on the 3 % KOH assay [6] and oxidase-positive based on API 20 NE strips (BioMérieux). It was catalase-positive based on 3 % (v/v) hydrogen peroxide solution. Cell growth was tested at different temperatures (5–40 °C, in steps of 1 °C below 5 °C then at intervals of 5 °C), and salt tolerance (NaCl) was determined in the range 0–6 % (w/v) as described by González et al. [7]. Strain 2–92T grew at 4 °C, showed optimal growth at 30 °C and did not grow at 40 °C. This strain was tolerant to different NaCl concentrations up to 4 % and was non-spore-forming based on the Schaeffer and Fulton method [8]. Cell morphology was investigated using scanning (SEM) and transmission (TEM) electron microscopy. Bacteria were cultured in LB broth overnight at 28 °C, processed as described by Greco-Stewart et al. [9] and imaged using a Philips XL-30 ESEM scanning electron microscope (data not shown). TEM was performed as previously reported by Hayat and Miller using a Hitachi H7000 microscope (Fig. 1). Cells were rod-shaped with sizes in the range of 0.5–0.6 µm wide and 2.1–2.6 µm long, consistent with species of the genus *Pseudomonas* [3], and had one or more polar flagella. Motility was demonstrated using triphenyltetrazolium [11] in semisolid medium (per litre: 3.0 g beef extract, 10.0 g pancreatic digest of casein, 5.0 g NaCl, 4.0 g agar). Fluorescent pigment was produced on KB medium [12]. After 48 h of incubation at 28 °C on KB, the colonies of 2–92T were white–yellowish, circular (mean 4 mm in diameter) and convex with regular margins.

The cellular fatty acid composition of 2–92T was determined by Keystone labs (Alberta Canada) using an Agilent Technologies 6890 N gas chromatograph. Strain 2–92T and closely related type strains were grown, in parallel, on trypticase soy broth (TSB) agar (TSBA; 30 g l–1 TSB, 15 g l–1 agar; BD Biosciences) at 28.0 °C for 24 h, and fatty acid extraction and analyses were performed according to the recommendations of the Microbial Identification (MIDI) system. The profiles were generated and identified using the Microbial Identification System, Sherlock TSBA60 Library version 6.0 (MIDI). Profiles of strain 2–92T were compared with those of closely related species of the *Pseudomonas fluorescens* subgroup generated under the same standardized conditions (Table S1, available in the online Supplementary Material). The major cellular fatty acid peaks (Table S1) of strain 2–92T were C16:0 (31.3 %), C16:1ω6c/C16:1ω7c (19.9 %; summed feature 3), C18:1ω7c/C18:1ω6c (14.4 %; summed feature 8), C17:0 cyclo (9.2 %), C12:0 2-OH (7.7 %), C12:0 3-OH (6.1 %) and C10:0 3-OH (3.2 %). The presence of C10:0 3-OH and C12:0 3-OH fatty acids in the profile of strain 2–92T is consistent with the classification as a *sensu stricto* pseudomonad [13]. In addition, the polar lipids of strain 2–92T were determined by the Identification Service of the DSMZ (Braunschweig, Germany). The assay identified major amounts of phosphatidylethanolamine, diphasatidylglycerol and phosphatidylglycerol (Fig. S1), which is consistent with species of the genus *Pseudomonas* [14]. Minor amounts of phosphatidylcholine, unidentified phospholipid and unidentified lipid were also detected (Fig. S1).

API 20 NE strips (BioMérieux) and carbon utilization tests based on Biolog PM1 and PM2A MicroPlates were performed according to the instructions of the manufacturers (Table 1). In API 20 NE assays performed in parallel with the 7 closely related type strains, strain 2–92T assimilated potassium gluconate, capric acid, adipic acid and trisodium citrate, and was positive for arginine dihydrolase, urease, gelatinase and oxidase but negative for indole production, β-glucosidases and β-galactosidase. Using Biolog assays, in a parallel study with closely related strains, strain 2–92T could be differentiated from *P. simiae* CCUG 50988T (phylogenetically closest species) by its ability to assimilate sucrose, trehalose, melibiose, L-rhamnose, L-phenylalanine, D-xylitol, N-acetyl-D-glucosamine, itaconic acid and putrescine as well as its inability to reduce nitrates (Table 1). Also, strain 2–92T could be differentiated from the type strains *Pseudomonas azotoformans* LMG 21611T, *P. costantinii* LMG 22119T, *P. extremorientalis* LMG 19695T, *P. poae* LMG 21465T, *P. salomonii* LMG 22120T, *P. simiae* CCUG 50988T and *P. trivialis* LMG 21464T [15–19] by using several phenotypic characteristics (Table 1).

Genomic DNAs were extracted using a Wizard SV Genomic DNA Purification kit (Promega), and the purity of each
sample was determined by agarose gel electrophoresis. The DNA concentrations were determined fluorimetrically using a FLUOstar OPTIMA micro-plate reader (BMG-LABTECH) with picogreen chemistry (Invitrogen). PCR amplifications of Pseudomonas were retrieved from GenBank. Sequences of strain 2-92 were compared with other species by pairwise distance calculations; multiple sequence alignments and phylogenetic analysis of the 16S rRNA gene sequences were performed using MUSCLE [23] and MEGA5.0 [24], respectively. Phylogenetic analysis was performed on almost-complete 16S rRNA gene sequences using maximum-likelihood (ML) with a general time reversible best-fit substitution model as implemented in jModelTest version 2 [25]. The topological robustness of the trees was evaluated by bootstrap analysis based on 1000 replicates. The 16S rRNA gene tree confirmed that strain 2-92ª was unique and a member of the genus Pseudomonas (Fig. 2). Pairwise sequence similarity values were between 98.8 and 99.5 % with several type strains of the P. fluorescens group [26], for example P. simiae CCUG 50988ª, P. salomonii LMG 22120, P. extremorientalis KMM 3447ª and P. azotoformans LMG 2161ª [2]. Phylogenies reconstructed using neighbour-joining and minimum-evolution algorithms showed similar topologies (Fig. S2).

Consistent with previous reports [7, 26] 16S rRNA gene sequences showed low resolution at the intrageneric level. Housekeeping genes such as rpoD, gyrB and rpoB have been used routinely to refine interspecific phylogenetic positions of species of the genus Pseudomonas [7, 26, 27]. Partial gene fragments of dnaA, gyrB, recA, recF, rpoB and rpoD were retrieved from de novo-assembled draft genomes obtained in this study from seven closely related species of the genus Pseudomonas. The whole-genome sequences were determined by paired-end sequencing using an Illumina MiSeq instrument with TrueSeq V3 chemistry (Génome-Québec, Montreal, Canada). De novo assembly was performed using ABySS version 1.5.2 [28] at different k-mer values (75–113) as previously reported [29]. The GenBank accession numbers of the draft genomes used in this study are AYTD00000000 [30], MDDQ00000000, MDDR00000000, MDGK00000000, MDFK00000000, MDFI00000000, MDFH00000000 and MDFJ00000000 for strain 2-92ª, P. azotoformans LMG 2161ª, P. costantinii LMG 2211ª, P. extremorientalis LMG

| Nitrate reduction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-------------------|---|---|---|---|---|---|---|---|
| Aesculin hydrolysis|   |   |  |  |   |   |   |   |
| Gelatinase        |   | + | + |   |   | + | + |   |

**Table 1.** Phenotypic characteristics that differentiate P. canadensis sp. nov. 2–92ª from closely related species of the genus Pseudomonas

Strains: 1, P. canadensis sp. nov. 2–92ª (=LMG 28499ª); 2, P. simiae CCUG 50988ª; 3, P. extremorientalis KMM 3447ª; 4, P. costantinii CFBP 5705ª; 5, P. salomonii LMG 22120ª; 6, P. poae DSM 14936ª; 7, P. trivialis DSM 14937ª; 8, P. azotoformans LMG 2161ª. P. canadensis sp. nov. 2–92ª can be distinguished from the reference taxa by its ability to utilize melibiose and L-rhamnose, except for P. trivialis that showed a variable reaction for L-rhamnose. P. canadensis sp. nov. can be differentiated from P. simiae by its ability to assimilate sucrose, melibiose, L-rhamnose, L-phenylalanine, N-acetyl-d-glucosamine, itaconic acid and putrescine. +, Positive reaction; -, negative reaction; w, weakly positive reaction; v, variable reaction.
A ML tree of dnaA-gyrB-recA-recF-rpoB-rpoD concatenated genes (~6 kb) was inferred using the general time reversible substitution model and implemented in MEGAS (Fig. 3). In the dnaA-gyrB-recA-recF-rpoB-rpoD concatenated tree, strain
MUMmer-based average nucleotide identity (ANIm) values were computed using the program JSpecies [37]. Reciprocal values are given in parentheses. Genome-to-genome digital DDH (dDDH) values were computed using the program based on 16S rRNA gene phylogeny and multi-locus sequence analysis Pseudomonas [37]. The dDDH values between strain 2-92 previously described by Ramisse [37] were employed to confirm the taxonomic position of strain 2-92 which have been proposed to replace wet-lab DDH [34, 36], described by Kurtz [34]. ANIm similarity values were computed as Wet-lab and genome-based DDH values between Table 2. Hybridizations were performed in triplicates of concatenated genes computed with Geneious (www. genomias.com; [31]; Table S2). Phylogenies reconstructed using the neighbour-joining and minimum-evolution algorithms showed similar topologies (data not shown).

DNA–DNA hybridization (DDH) studies were employed as previously described by Ramisse et al. [32] between strain 2-92 and seven closely related species of the genus Pseudomonas (Table 2). Hybridizations were performed in triplicates with reciprocal reactions. DDH values of 49.0, 35.6 and 46.7 % between strain 2-92 and P. simiae CCUG 50988T, P. extremorientalis LMG 19695T and P. salomonii LMG 22120T, respectively (Table 2), were clearly below the threshold level (<70 %) for species definition [33]. Also, genome-sequence-based digital DDH (dDDH; [34]) and MUMmer-based average nucleotide identity (ANIm; [35]), which have been proposed to replace wet-lab DDH [34, 36], were employed to confirm the taxonomic position of strain 2-92. The dDDH values were calculated using the genome-to-genome distance calculator (GGDC) version 2.1 (http://ggdc.dsmz.de; [34]). ANIm similarity values were computed as described by Kurtz et al. [35] and implemented in JSpecies [37]. The dDDH values between strain 2-92 and the seven closely related species of the genus Pseudomonas were all below the threshold of 70 % for species delineation (Table 2) as recommended by Meier-Kolthoff et al. [34]. For example, strain 2-92T had dDDH values of 45.60 and 33.45 % with P. simiae CCUG 50988T and P. salomonii LMG 22120T, respectively. Similarly, the ANIm values (88.1–90.7 %) between strain 2-92 and P. simiae CCUG 50988T (92.0 %) or the other closely related species were below the threshold level (<95 %) for species definition as reported by Richter and Rosselló-Móra [37] (Table 2). The DNA G+C content of strain 2-92T was 60.3 mol% using the HPLC method [38], which is well within the range reported for species of the genus Pseudomonas [3]. The DNA G+C content was confirmed by whole-genome sequencing and analysis of strain 2-92T (AYTD00000000; 6.4 Mb genome size) [30].

Based on the data from genotypic and phenotypic analyses presented in this study, we propose that the novel strain represents a novel species, named Pseudomonas canadensis sp. nov.

**DESCRIPTION OF PSEUDOMONAS CANADENSIS SP. NOV.**

Pseudomonas canadensis (ca.nad.en’sis N.L. fem. adj. canadensis from or originating from Canada, the country where strain 2-92T was isolated).

**Table 2.** Wet-lab and genome-based DDH values between P. canadensis sp. nov. and the type strains of closely related species of the genus Pseudomonas based on 16S rRNA gene phylogeny and multi-locus sequence analysis

Reciprocal values are given in parentheses. Genome-to-genome digital DDH (dDDH) values were computed using the program GGDC 2.1 [34]. MUMmer-based average nucleotide identity (ANIm) values were computed using the program JSpecies [37].

| Type strain                  | Wet-lab DDH (%) | dDDH (%) | ANIm (%) |
|-----------------------------|-----------------|----------|----------|
|                            | 2-92T | 2-92T | 2-92T |
| P. canadensis 2-92T         | 100   | 100   | 100     |
| P. simiae CCUG 50988T       | 49.0±2.1 (48.4)| 45.60 (45.50) | 92.02 (92.09) |
| P. trivialis LMG 21465T     | 45.1±2.8 (48.5)| 45.40 (45.07) | 88.2 (87.98) |
| P. poae LMG 21465T          | 38.5±3.8 (42.0)| 32.15 (34.01) | 88.1 (87.9) |
| P. salomonii LMG 22120T     | 46.7±3.2 (47.2)| 33.45 (34.01) | 88.7 (88.54) |
| P. costantinii LMG 22119T   | 48.3±3.1 (50.1)| 33.70 (33.10) | 88.8 (88.4) |
| P. azotofermans LMG 21611T  | 45.0±4.0 (51.0)| 40.20 (40.09) | 90.68 (90.74) |
| P. extremorientalis LMG 19695T | 35.6±4.2 (40.0)| 40.40 (40.4) | 90.74 (90.68) |
Cells are aerobic, Gram-reaction-negative, non-spore-forming rods (approx. 0.5 µm wide and 2.3 µm long), motile with one or multiple polar flagella. After 48 h on KB, colonies are white-yellowish and circular (average 4 mm in diameter), convex with regular margins and produce fluorescent pigments. Tolerant to different NaCl concentrations up to 4%, and grows at 4°C with optimal growth at 30°C and no growth at 40°C. Based on Biolog PM1 and PM2A Microplate assays, utilizes N-acetyl-d-glucosamine, succinic acid, L-aspartic acid, L-proline, D-alanine, D-mannose, D-gluconic acid, L-lactic acid, D-mannitol, L-glutamic acid, DL-malic acid, Tween 20, D-fructose, acetic acid, α-D-glucose, L-asparagine, α-ketoglutaric acid, L-glutamine, adenosine, citric acid, fumaric acid, bromosuccinic acid, propionic acid, butyric acid, malonic acid, quinic acid, succinamic acid, D,L-saccharic acid, D,L-succinate, methylpyruvate, L-mevalonate, D,L-isoascorbic acid, D,L-glucosaminic acid and D,L-asparagine. The DNA G+C content of strain 2-92\textsuperscript{T} is 60.3 mol%.

The type strain is 2-92\textsuperscript{T} (=DOAB 798\textsuperscript{T}=LMG 28499\textsuperscript{T}), isolated from a soil sample from Woodslee, Ontario, Canada. The DNA G+C content of strain 2-92\textsuperscript{T} is 60.3 mol%.

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### Conflicts of interest
The authors declare that there are no conflicts of interest.

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