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Diversity of bacteria associated with corn roots inoculated with Canadian woodland soils, and description of *Pseudomonas aylmerense* sp. nov.

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Abstract

Bacteria associated with corn roots inoculated with soils collected from the Canadian woodlands were isolated and characterized. Genus-level identification based on 16S rRNA sequence analysis classified the 161 isolates in 19 genera. The majority (64%) of the isolates were affiliated with the genus *Pseudomonas*. Further analysis of the *Pseudomonas* isolates based on BLASTn and *rpo*D-*rpo*B-*gyr*B concatenated gene phylogeny revealed three unique clusters that could not be assigned to known species. This study reports the taxonomic description of one of the distinct lineages represented by two strains (S1E40^T and S1E44) with *P. lurida* LMG 21995^T, *P. costantinii* LMG 22119^T, *P. palleroniana* LMG 23076^T, *P. simiae* CCUG 50988^T and *P. extremorientalis* LMG 19695^T as the closest taxa. Both strains showed low ANIm (<90%) and genome-based DNA-DNA hybridization (<50%) values, which unequivocally delineated the new strains from the closest relatives. These findings were supported by multilocus

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sequence analysis (MLSA) and DNA fingerprinting. In addition, growth characteristics and biochemical tests revealed patterns that differed from the related species. Strains S1E40^T and S1E44 are Gram-negative, aerobic, rod-shaped and motile by at least one flagellum; and grew optimally at 30 °C. The predominant polar lipid is phosphatidylethanolamine while the major respiratory quinone is ubiquinone-9. Based on phenotypic and genotypic data presented here, strains S1E40^T and S1E44 represent a novel species for which the name *Pseudomonas aylmerense* sp. nov. is proposed. The type strain is S1E40^T (= LMG 30784^T = DOAB 703^T = HAMI 3696^T) with a G + C content of 61.6%.

Keywords: Systematics, Microbiology

1. Introduction

Pseudomonas species are ubiquitous and complex group of Gram-negative proteobacteria with over 253 documented species ([1]; http://www.bacterio.net/pseudomonas.html). These species are classified into two intrageneric clusters (IG): *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* [2]. Each IG is further divided into several groups/subgroups [2]. Although some members of the *P. fluorescens* intrageneric cluster are phytopathogens e.g. *P. syringae* and *P. savastoni* [3], in general, these fluorescent pseudomonads are recognized to play significant roles in envrionmental and agricultural sustainability. This is partly because of the versatility of this bacterial group to colonize diverse environmental spheres, e.g. plant rhizosphere, water and soils [4]. In addition, an ability to adapt in new environments and to exhibit a wide spectrum of phenotypic traits (including active secondary metabolites) make this group a desirable research focus. For example, some *Pseudomonas* spp. are described as xenobiotic-degraders [5, 6, 7], plants growth promoters [8, 9] and biocontrol agents [10, 11, 12, 13]. A variety of mechanisms are employed by fluorescent pseudomonads to achieve these significant ecological functions while assuring their environmental fitness [14, 15, 16]. Some members of the *Pseudomonas fluorescens* group (*P. putida* KT24440 and *P. chlororaphis* D-TR133) can produce chemically diverse secondary metabolites such as siderophores [8, 17] and antibiotics [13, 18] which are important for competitive and cooperative interactions in the rhizosphere. Given the multiplicity of ecological functions and the impact *Pseudomonas* spp. have in sustainable agriculture, it is of particular interest to characterize existing or new isolates. As such, members of this genus are routinely being isolated and characterized from various environments leading to the discovery of new species.

The cultivable *Pseudomonas* population in the Canadian woodland is under-investigated and poorly documented. As such, we initiated a prospective study to collect soil samples from a woodland in Aylmer, Québec, Canada. The rhizosphere

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soils of Canadian native legumes were targeted because previous studies show that they harbor unique and novel *Bradyrhizobium* genotypes [19, 20] using soybean as trap hosts. This article reports the characterization of bacteria isolated from rhizosphere soils of a Canadian woodland, and the description of a novel species, *Pseudomonas alymerense* sp. nov., within the genus *Pseudomonas*.

2. Materials and methods

2.1. Soil collection and isolation of bacterial strains

Soil samples were collected from three sites in Gatineau and Luskville, Québec, Canada. Root-zone soils were collected to a depth of 10 cm from the rhizospheres of three native legumes, *Amphicarpea bracteata* (soil sample 1; S1; Latitude 45°22′48.21″N, Longitude 75°48′25.52″W), *Desmodium canadense* (soil sample 2; S2; Lat. 45°22′48.13″N, long. 75°48′37.36″W) and *Desmodium glutinosum* (soil sample 3; S3; Lat. 45°31′58.48″N, Long. 75°59′47.29″W) plants. Where required, soil samples were pooled and stored at 4 °C until use. Two corn cultivars, early Golden Bantam (E) and Bt4223 (Bt) were used as trap plants. The experimental set-up was done as reported previously [19] with modifications. Corn seeds were surface disinfected in 95% ethanol (Sigma-Aldrich, Canada) for 30 seconds followed by commercial bleach (Clorox; 5.25% NaOCl) for the same time. Disinfected corn seeds were rinsed three times in autoclaved sterile double distilled de-mineralized water. Three seeds were sown in pots containing sterile vermiculite and incubated under a controlled environment (25 °C, 16h/8h photoperiod) for 25 days. Corn seedlings were inoculated with a 10% suspension of the three soil samples (S1, S2, and S3). Pots were manually irrigated with autoclaved Hoagland solution [21]. A total of forty-two pots were used.

Bacterial isolation was done from one gram of root tissues homogenized in 0.85% NaCl [22], serially diluted to 1/1000 and 1 ml plated on King's B medium [23]; (KB; 10 g/L proteose peptone #2, 1.5 g/L anhydrous K_2HPO_4, 1.5 g/L $MgSO_4$). The plates were incubated at 28 °C for 48 h [22]. Single colonies were generated and used for downstream analyses.

2.2. PCR amplifications, DNA sequencing and phylogenetic analyses of isolates

Single colonies of all the 161 isolates obtained were inoculated in liquid Luria-Bertani medium (LB; Difco™, USA) with shaking at 250 rpm for 16 h and used for DNA extraction. Genomic DNA was extracted using the Wizard SV Genomic DNA Purification System Kit (Promega, Canada) and quantified as reported previously [24]. For genus-level identification, 16S rRNA PCR amplification and sequencing were performed as indicated previously [22] using primer pair 16S27

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(5-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5-TACGGYTACCTTGT TACGACTT-3′) [25].

Partial 16S rRNA sequences (>1342 nt) were edited and assigned to taxonomic classes using Classifier, Ribosomal Database Project 10 (RDP Classifier; [26]), a naïve Bayesian tool based upon taxonomic classifications in Bergey's Taxonomic Outline of the Prokaryotes [27]. Phylogenetic analysis was performed as previously described [22] using 107 representative type strains of closely-related bacteria affiliated to strains obtained in this study based on RDP classifications. Maximum likelihood algorithm was implemented in MEGA 7 [28] using general time reversible (GTR) with 1000 bootstrap replicates.

Species-level identification of the *Pseudomonas* strains was done by sequencing and analyzing three highly discriminatory housekeeping genes: the RNA polymerase sigma factor (*rpo*D), RNA polymerase beta subunit (*rpo*B) and DNA gyrase subunit B (*gyr*B) as previously reported [2]. The genes were sequenced and BLASTn [29] performed to identify closely-related *Pseudomonas* type strains as previously reported [30]. Maximum likelihood phylogenetic inference was performed in CLC Genomic Workbench Version 11.0 (Qiagen, Canada) using general time reversible (GTR) with rate variation (+G) and topology variation (+T) (GTR + G + T) with 1000 bootstrap. The best-fit substitution model is based on corrected Akaike minimum theoretical information criterion. Strains that clustered into distinct and unique clades from their closest related species were identified as potential novel genotypes. Phylogenetic diversity, based on *rpo*D, the most discriminatory housekeeping gene [2], was computed using split-tree 4 [31].

Detailed MLSA phylogeny of strains (S1E40^T and S1E44) of the proposed *P. aylmerense* sp. nov. was performed as previously described [2, 30, 32]. Partial sequences of 16S rRNA, *rpo*D, *rpo*B and *gyr*B were concatenated using Geneious 10.1.2 (http://www.geneious.com/), were aligned with the MUSCLE algorithm [33] and phylogenetic trees were inferred as indicated above. Evolutionary reconstructions were done using neighbor-joining, minimum-evolution and maximum likelihood algorithms with 1000 bootstrap replicates.

2.3. Genome-based DNA-DNA hybridization (DDH) of the proposed *P. aylmerense* sp. nov.

The whole-genome sequences of strains S1E44 and *P. palleroniana* LMG 23076^T were determined by paired-end sequencing using Illumina MiSeq technology (Genome-Quebec, Montreal, Canada), and de novo assembly was performed using ABySS version 1.5.2 [34] at different k-mer values (75−113) as previously reported [35]. Genome data of strain S1E40^T (MAUE00000000), *P. lurida* LMG 21995^T (PDJB00000000), *P. costantinii* LMG 22119^T (MDDR00000000), *P. simiae*

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CCUG 50988^T (MDFH00000000), and *P. extremorientalis* LMG 19695^T (MDGK00000000) were retrieved from GenBank. Genome-sequence-based digital DDH (dDDH; [36]) and MUMmer-based average nucleotide identity (ANIm; [37]) were employed between two strains of *P. aylmerense* and five phylogenetically closely related *Pseudomonas* species to confirm the taxonomic position. The dDDH values were calculated using the genome-to-genome distance calculator (GGDC) version 2.1 (http://ggdc.dsmz.de; [36]). ANIm similarity values were computed as described by Kurtz et al. [37] and implemented in JSpecies [38].

2.4. Chemo-taxonomy of strains S1E40 and S1E44

The total fatty acid compositions of the two strains (S1E40^T and S1E44) and those of five closely related taxa were determined by Keystone labs Inc. (Alberta, Canada) using Agilent Technologies 6890N gas chromatography (Santa Clara, CA, USA). The bacteria were cultured on Tryptic Soy Broth medium (TSB, Bacto ™) agar and incubated overnight at 28 °C and fatty acid profiles were generated using the Microbial Identification System, Sherlock TSBA60 Library Version 6.0 (MIDI, USA). Polar lipids identification and respiratory quinones of the type strain of *P. aylmerense* (strain S1E40^T) were performed by the Identification Service, Leipniz-Institut-Deutsche Sammlung von Mikroorganismen und Zekllkulturen GmbH, Braunschweig, Germany (DSMZ).

2.5. Morphology and phenotypic characterization

Cell morphology of strains S1E40^T and S1E44 was investigated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Cultures were grown and processed as previously reported [30, 39]. Briefly, SEM samples were adsorbed to Poly-L-Lysine coated silicon wafers (Ted Pella Inc., Redding, CA, USA), fixed in 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1M sodium Cacodylate buffer pH 7.2, dehydrated through a graded ethanol series, and critical point dried (Biodynamics Research Corp., Rockville, MD, USA). Wafers were gold sputter coated to a thickness of 8 nm in an Emitech K550V (EM Technologies Ltd., Ashford, Kent, England) and imaged on a Quanta 600 SEM operating at 20 kV (FEI Company TM, Brno, Czech Republic). TEM was performed as previously described using phosphotungstic acid 1% (pH 7.0) as a negative stain [40], observations were made with an Hitachi H-7000 (Hitachi, Tokyo, Japan) and images were captured with an Orius TM SC 200 CCD camera (Gatan Inc., Pleasanton, USA). Gram status was determined using the 3% KOH test [41]; and the production of fluorescent pigments was evaluated on King's B medium (KB; [23]) and visualized using an ultraviolet (wavelength, 365 nm) transilluminator Spectroline®(Fisher Scientific, Canada).

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Optimal growth for strains S1E40^T and S1E44 was evaluated at different temperatures, 5 °C−40 °C, with an interval of 5 °C for 24 hrs. Saline tolerance was determined by supplementing the LB medium with 0, 1, 2, 4, 6 and 8% (w/v) NaCl as previously described [30, 32]. The Schaeffer and Fulton [42] method was used to determine whether the strains were spore-forming. Strain motility was demonstrated using triphenyl tetrazolium [43] in semisolid medium (per liter: 3.0 g beef extract, 10.0 g pancreatic digest of casein, 5.0 g NaCl, 4.0 g agar).

Comparative phenotypic analyses were performed, in triplicate, on the two strains of the proposed *P. aylmerense* sp. nov. and the type strains of the five closest related species (*P. lurida* LMG 21995^T, *P. costantinii* LMG 22119^T, *P. palleroniana* LMG 23076^T, *P. simiae* CCUG 50988^T and *P. extremorientalis* LMG 19695^T) based on 16S rRNA and MLSA phylogenies and BLAST results. The type strains were obtained from Belgian (LMG) and Swedish (CCUG) bacterial collections. Carbon utilization and enzymatic tests of the two strains in parallel with the five closest *Pseudomonas* type strains were performed, in triplicate, using Biolog plates PM1 & PM2A (Hayward, CA) and API NE (BioMérieux, USA) respectively according to the manufacturers' instructions.

2.6. BOX- and ERIC DNA fingerprinting

BOX-PCR fingerprinting was performed using BOX_A1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) [44]; and ERIC-PCR was done with primers ERIC-1R/ERIC2 (5′-CACTTAGGGGTCCTCGAATGTA-3′/5′-AAGTAAGTGACTGGGGTGAGCG-3′) as described by Versalovic *et al.* [45]. Amplified products were electrophoretically separated on 1.5% agarose gels in 0.53 Tris-borate-EDTA buffer. Fingerprint banding similarities were analyzed using GelCompar II 5 (Applied Maths, Kortrijk, Belgium). Jaccard's similarity coefficients and unweighted pair-group method using arithmetic averages (UPGMA) were used for cluster analysis.

2.7. Hypersensitivity test and growth in artificial minimal medium

Strain S1E40^T was tested for the ability to induce hypersensitivity response (HR) on *Nicotiana benthamiana* seedlings. The assay was conducted as previously reported [46] using 10^8 colony forming units of each of the bacterial strains. Plants were observed daily for symptoms associated with a HR response. The bacterial strains were also inoculated in M9 minimal (M9M) medium, a nutrient-poor medium reported [46] to mimic nutrient availability in plant cells. The growth of strains S1E40^T and S1E44 was monitored in liquid M9M medium with 10 mM fructose (28 °C, 235 rpm) as previously described [46]. The optical density of aliquots

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were measured at 595 nm, in triplicate, at 24-hour intervals using the BMG FLUOstar OPTIMA microplate reader (BMG, Canada).

2.8. Inhibition of fungal mycelial growth and metabolite profiling

In vitro antagonistic activity of the two strains against *F. graminearum* DAOMC 178148 was evaluated using the dual culture bioassay. Briefly, a plug of *F. graminearum* (5 mm in diameter) was transferred from a 48-hr old fungal culture to the center of a 90 mm Petri dish containing glucose-casamino acid-yeast (GCY; glucose 15 g/l, casamino acids 1.5 g/l, yeast extract 1.0 g/L, KH_2PO_4 1.5 g/l, $MgSO_4.7H_2O$ 1.0 g/L, agar 15 g/L) medium. A bacterial strain (S1E40^T or S1E44) was streaked on both sides of the fungal plug at equidistance and incubated at 30 °C and monitored daily for 7 days. Inhibition rate (%) was calculated using the formula: $([G_r\text{-}G_{r+b}]/G_r)$ x100, where G_r is radial growth of *F. graminearum* alone, and G_{r+b} is the radial growth of *F. graminearum* in the presence of bacterial strain S1E40^T or S1E44. The assay was repeated twice. A PCR was performed as previously reported [47] to assess if the strains possess genes of known antifungal compounds (phenazine-1-carboxylic acid (PCA) and 2,4-diacetophloroglucinol (phl)).

Since the type strain (S1E40^T) of proposed *P. aylmerense* sp. nov. inhibited the mycelial growth of *F. graminearum in vitro* and did not possess PCA or phl genes, we initiated an exploratory study on the metabolites secreted in three media (GCY, KB and LB). All culture extracts were fractionated using C_{18} Thermo Hypersil SPE cartridges in various solvents of decreasing polarity (deionized H_2O, H_2O:MeOH (1:1), and MeOH); H_2O:MeOH and MeOH fractions were reconstituted to a concentration of 500 μg/mL for analysis using ultra-high performance liquid chromatography coupled high-resolution mass spectrometry (UPLC-HRMS) (Thermo Ultimate 3000 UPLC coupled to a Thermo LTQ Orbitrap XL high-resolution mass spectrometer). Chromatography was performed on a Phenomenex C_{18} Kinetex column (50 mm × 2.1 mm ID, 1.7 μm) with a 10 min H_2O: ACN (0.1 % formic acid) gradient at flow rate of 0.35 mL/min: 5% ACN to 95% ACN by 4.5 min, held until 8.0 min, returning to 5% ACN by 9 min and held until 10 min to equilibrate. The HRMS was operated in ESI^+ mode (100−2000 m/z range) using the following parameters: sheath gas (40), auxiliary gas (5), sweep gas (2), spray voltage (4.2 kV), capillary temperature (320 °C), capillary voltage (35 V), and tube lens (100 V). MS/MS experiments on selected extracts were carried out using alternating full scan (30000 resolution, 100−1600 *m/z* range) and MS/MS scan events (7500 resolution, 35 eV CID, 1.0 *m/z* isolation width, 0.25 activation Q, and 30 ms activation time) for specified $[M + H]^+$ ions. Data preprocessing was carried out using mzMine2 withpreviously described parameters [48] using a noise limit threshold of 5.0 E^5 for mass detection. Redundant variables associated with MeOH blanks were removed from the resulting data matrix prior to processing

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using the *muma* package in the R environment [49]. A short list of pseudomolecular ions contributing most to the metabolite phenotype in PCA (highest loading values along PC1 and PC2) was compiled and used to query UPLC-HRMS data files to establish relationships (metal/solvent adduct formation, neutral water loss, dimerization, etc.) for all pseudomolecular ion signals associated with the same retention time.

2.9. Nucleotide sequence accession numbers

One hundred and sixty-one 16S rRNA gene sequences generated in this study were deposited in the GenBank with accession numbers MH463671−MH463773 (*Pseudomonas* strains) and MH465137−MH465194 (non-*Pseudomonas*). GenBank accession numbers for housekeeping genes are MH494082−MH494170 (*rpo*D), MH487734−MH487822 (*rpo*B) and MH544493−MH544581 (*gyr*B). The whole-genome shotgun (wgs) data of the proposed *P. aylmerense* strain S1E44 and the type strain of *P. palleroniana* LMG 23076^{T} are deposited at DDBJ/EMBL/GenBank under the accession number PYWW00000000 and PYWX00000000 respectively. The wgs versions described in this paper are the first versions, PYWW01000000 and PYWX01000000.

3. Results and discussion

We isolated and characterized bacteria associated with corn roots inoculated with soils collected from rhizosphere of native legumes from Canadian woodlands. Genus-level identification based on 16S rRNA gene sequence analysis classified 161 isolates into 19 genera (Fig. 1). The majority (64%) of the isolates were affiliated with the genus *Pseudomonas* (Fig. 1). This is not surprising since KB medium [23] selectively favors the growth of most fluorescent pseudomonads [50]. Other genera with >3% of the recovered strains were *Stenotrophomonas* (7.45%), *Lelliottia* (6.83%), *Rahnella* (3.73%) and *Enterobacter* (3.73%).

High quality paired-end partial *rpo*D, *rpo*B and *gyr*B gene sequences obtained from 89 *Pseudomonas* strains (86.4%) were used as markers for species-level identification [2]. The majority (68.5%) of the *Pseudomonas* strains were assigned to the *P. fluorescens* group, 19.1% to the *P. putida* group, 10.1% to the *P. aeruginosa* group and 3.4% to the *P. oleovorans/P. anguilliseptica* group (Fig. 2). The phylogenetic diversity of the population was 1.919 with an average distance of 0.1844 and 415 informative sites. The *P. fluorescens* group accounted for 43.3% (0.834) of the phylogenetic diversity with an average distance of 0.0977 and estimated proportion of invariant sites of 0.4736. Strains affiliated with *P. marginalis* and *P. lurida* dominated the *P. fluorescens* group while *P. montellii* was dominant within the *P. putida* group. *P. nitroreducens/P. nitritireducens* were the dominant strains for the

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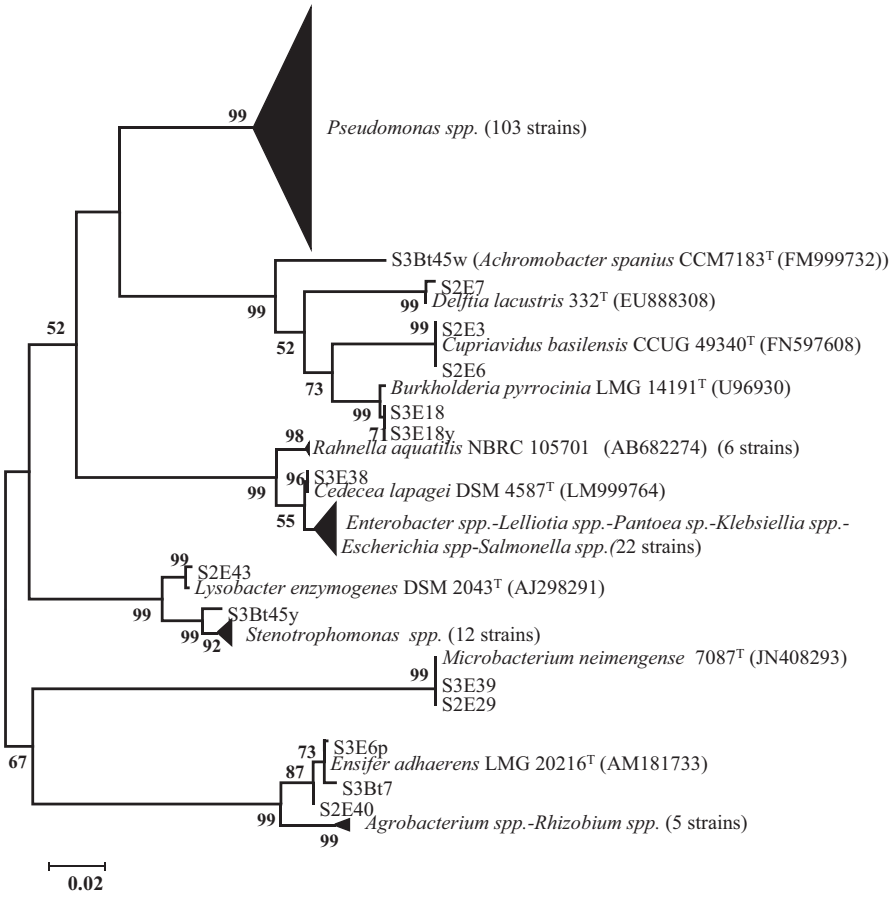

Fig. 1. Maximum likelihood tree based on partial 16S rRNA sequences (1342−1344 nt) showing the phylogenetic affiliations of the isolates into nineteen different bacterial genera. Tree was implemented in MEGA 7 [28] using general time reversible substitution model with 1000 bootstrap replicates. Bootstrap values ≥50% are shown at nodes.

P. aeruginosa group. Based on *rpo*D-*rpo*B-*gyr*B concantenated gene sequences as well as BLAST analyses, all strains clustered closely with validly published type strains within the genus *Pseudomonas* except for eleven strains constituting three potential novel genotypes (square brackets, Fig. 2). These novel genotypes clustered uniquely as indicated by their branching from the main phylogenetic trunk. One of these novel genotypes consists of strains S1E40^T and S1E44, representatives of the proposed *P. aylmerense* sp. nov. These strains (S1E40^T and S1E44) were characterized further using a polyphasic approach.

BLAST and phylogenetic analyses of the 16S rRNA of strains S1E40^T and S1E44 of the proposed *P. aylmerense* were employed to determine the closest related *Pseudomonas* species. Strains S1E40^T and S1E44 had identical 16S rRNA sequences. The neighbor-joining (NJ) tree (Fig. 3) of 16S rRNA gene sequences revealed that the closest related species of *Pseudomonas* to the novel strains were members of the *P. fluorescens* subgroup: *P. lurida* DSM 15835^T (99.58%), *P. costantinii* CFBP

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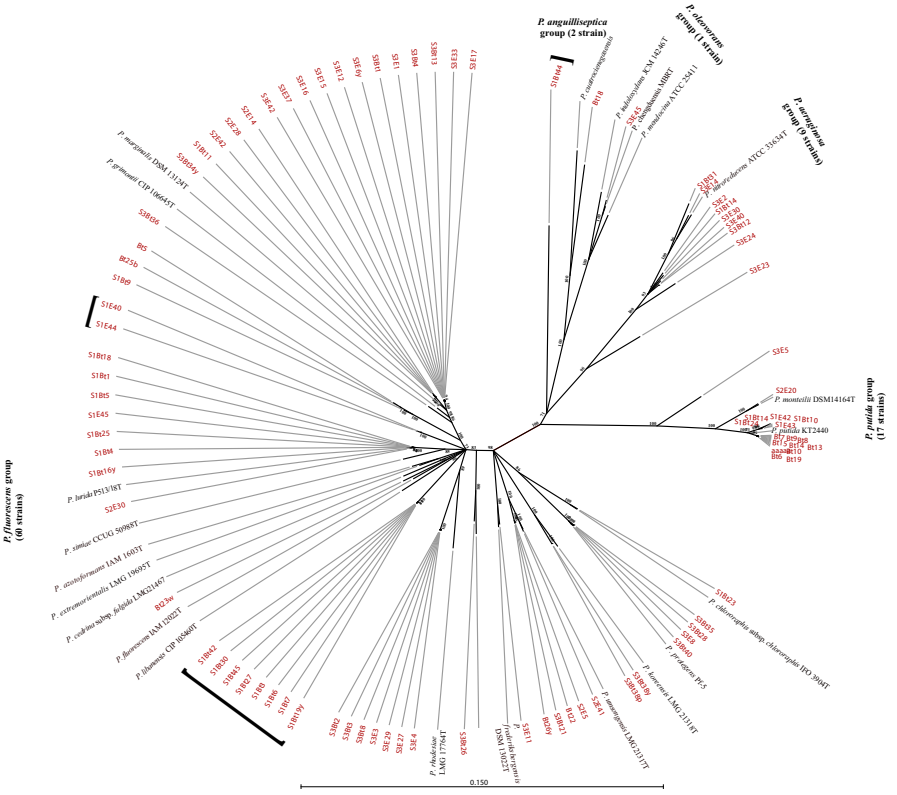

Fig. 2. Maximum likelihood tree based on partial *rpo*D-*rpo*B-*gyr*B concatenated sequences (1713-1719nt) showing the phylogenetic affiliations of 89 *Pseudomonas* strains to four different *Pseudomonas* groups. Tree was implemented in CLC Genome Workbench (Qiagen) using the best substitution model (GTR + G + T; loglikelihood = -10,488.71) with 1000 bootstrap replicates. Bootstrap values ≥70% are shown at nodes. Numbers in brackets indicate the number of isolates; and single square brackets indicate potential novel species.

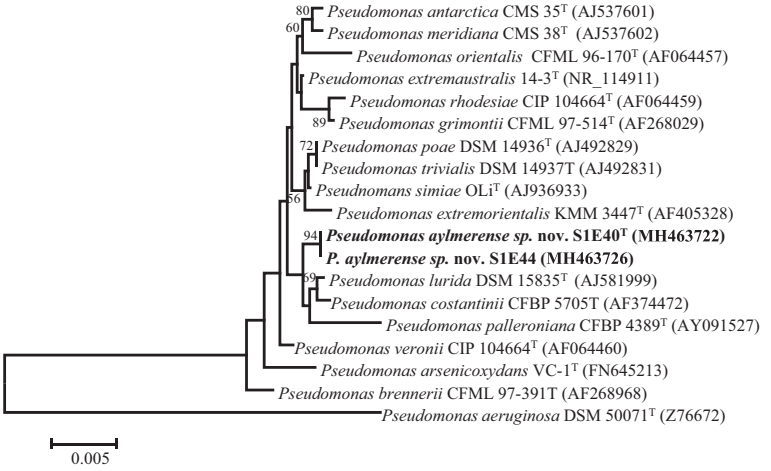

Fig. 3. Neighbor-joining phylogenetic tree based on partial 16S rRNA gene sequences (=1342−1344 nt) of closely related *Pseudomonas* species (EzBiocloud.net) of *P. aylmerense* sp. nov. with 1000 bootstrap values. Bootstrap values greater than 50% are shown at nodes.

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5705^T (99.44%), *P. simiae* CCUG 50988^T (99.40%), *P. palleroniana* CFBP 4389^T (99.14%), and *P. extremorientalis* KMM 3447^T (99.02%). These type strains and the strains of the proposed *P. aylmerense* sp. nov. clustered consistently together. The tree topology was similar using the minimum evolution algorithm or the maximum likelihood (ML) algorithm (data not shown).

Previous studies [2, 30, 32] indicated that 16S rRNA gene sequences show low taxonomic resolution at the species level. Combining 16S rRNA gene sequence data and other housekeeping genes such as *rpo*D, *gyr*B and *rpo*B are routinely used to refine interspecific phylogenetic positions of species of the genus *Pseudomonas* [2, 30, 32]. A MLSA (=3753−3777 nt) was performed on concatenated sequences of 16S rRNA and three housekeeping genes (*rpo*D, *rpo*B and *gyr*B; [2]). A phylogenetic tree of the concatenated sequences using the maximum likelihood (Fig. 4) showed strains of the proposed *P. aylmerense* sp. *nov*. forming a distinct lineage within known *Pseudomonas species* supported by a 100% bootstrap value.

Genome-based DNA relatedness corroborated results from MLSA. The generated whole genome sequences for strains *P. aylmerense* S1E44 (6.99 Mb, G + C = 61.63%) and *P. palleroniana* LMG 23076^T (6.12 Mb, G + C = 60.5) consist of 58 and 91 contigs with protein-coding sequences of 6290 and 5499 respectively. dDDH and ANIm values between the type of the proposed type strain of *P. aylmerense* (S1E40^T) and strain S1E44 were 99.8% and 99.9% respectively, confirming high similarity. Table 1 shows the dDDH and ANI values calculated between strain S1E40^T and the closest related *Pseudomonas* type strains. The ANIm values ranged from 89.3% to 88.3% with *P. lurida* LMG 21995^T showing the highest percent similarity. ANIm values of all the closely related species were below the

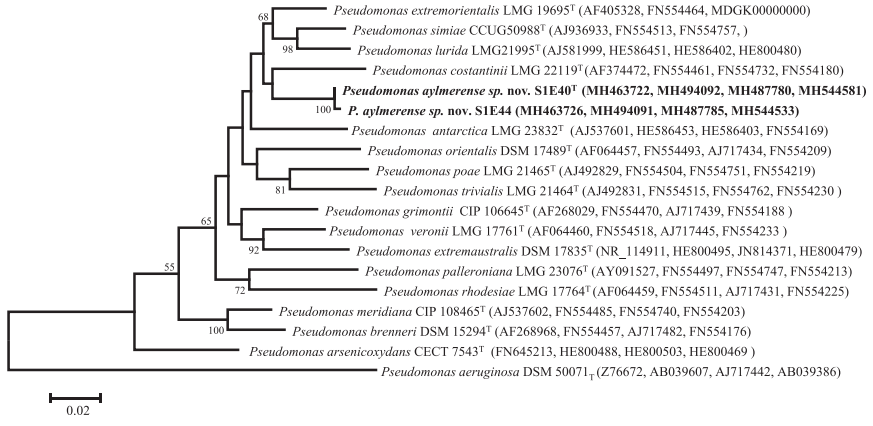

Fig. 4. Maximum-likelihood phylogenetic tree based on concatenated partial 16S rRNA (= 1342−1344 nt) and housekeeping *rpo*D (701−717 nt), *rpo*B (=915−917 nt), *gyr*B (=795−799 nt) gene sequences (3753−3777 nt total). Tree had the highest log likelihood (-13,068.8802) and was inferred using the General Time Reversible substitution model with 1000 bootstrap values. Bootstrap values greater that 50 are shown at nodes. *P. aeruginosa* was used as outgroup.

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Table 1. Genome-based DNA-DNA hybridization (dDDH) and MuMmer-based average nucleotide identity (ANIm) values between strains of *P. aylmerense* and five closely related type strains of *Pseudomonas* species; and G + C content of each strain.

***Pseudomonas* type strains**	**dDDH (%)**	**ANIm (%)**	**G + C content (%)**
P. aylmerense sp. nov. S1E40^T	100.0	100.0	61.63
P. aylmerense sp. nov. S1E44	99.8	99.9	61.63
P. lurida LMG 21995^T	35.8	89.3	60.61
P. simiae CCUG 50988^T	35.1	89.0	60.30
P. costantinii LMG 22119^T	34.3	88.6	59.39
P. extremorientalis LMG 19695^T	35.8	88.4	60.93
P. palleroniana LMG 23076^T	32.8	88.3	60.61

Reciprocal values are identical. Cut-off values: 70% for dDDH and 95% for ANIm.

threshold level (<95 %) for species definition as reported by Richter and Rosselló-Móra [38]. Similarly, the dDDH values were highest between S1E40^T and *P. lurida* LMG 21995^T, and all values were clearly below the species cut-off of 70% [36]. The ANIm and dDDH values indicate that strains S1E40^T and S1E44 represent a distinct and authentic novel species within the genus *Pseudomonas*. In addition, the two strains of *P. aylmerense* sp. nov (61.63%) could be differentiated from their closest taxa (59.39−60.93%) by G + C content (Table 1).

Classical DNA fingerprinting methods (BOX- and ERIC-PCRs) were employed to further confirm the uniqueness of strains of the proposed *P. aylmerense*. DNA fingerprints of strains S1E40^T and S1E44 using BOX- (data not shown) and ERIC-PCRs (Fig. 5) in parallel with the closest *Pseudomonas* type strains were similar. Both techniques showed that strains S1E40^T and S1E44 are a unique lineage resulting in a distinct clustering with high cophenetic correlation coefficients (Fig. 5). This corroborates the strong evidence from phylogenetic analyses and genome-based data that the strains of *P. aylmerense* sp. nov. are a distinct lineage. In addition, DNA fingerprint patterns show differences between strains S1E40^T and S1E44, as such this approach could be used to differentiate the two strains.

The cellular fatty acid, polar lipids and quinones compositions of strains S1E40^T and S1E44 were consistent with the classification as a *sensu stricto* pseudomonad [51]. The major cellular fatty acid of strains S1E40^T and S1E44 were summed feature 3 ($C_{16:1}\omega6c/C_{16:1}\omega7c$, 30.28%), $C_{16:0}$ (30.28%) and summed feature 8 ($C_{18:1}\omega6c/C_{18:1}\omega7c$; 16.41%). Polar lipids analysis identified the new strains to have major amounts of phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol with traces of phospholipid. This is consistent with authentic species within the genus *Pseudomonas* [52]. Analysis of respiratory quinones of the novel strains showed the presence of Q9 (87%) and Q8 (10%). Q9 as the major

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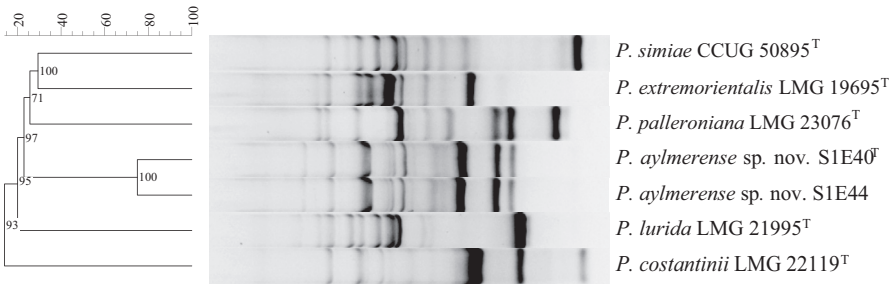

Fig. 5. Dendogram of DNA fingerprints of strains (S1E40^T and S1E44) of the proposed *Pseudomonas aylmerense* based on ERIC primers. Unweighted pair-group method using arithmetic averages (UPGMA) was used for clustering with Jaccard's similarity coefficients. Numbers on the nodes are cophenetic correlation coefficients.

ubiquinone of strains S1E40^T and S1E44 is consistent with *Pseudomonas* species [53, 54].

Results of the carbon utilization assays (Biolog and API 20NE) for the two strains of the proposed *P. aylmerense* were similar. A summary of the differential characteristics of strains S1E40^T and S1E44 from the closest type strains of *Pseudomonas* species, run in parallel, are shown in Table 2. Strains S1E40^T and S1E44 could be differentiated from *P. lurida* LMG21995^T, *P. costantinii* LMG 22119^T, *P. simiae* CCUG 50988^T, and *P. extremorientalis* LMG 19695^T by their inability to assimilate L-aribitol. The inability of strains S1E40^T and S1E44 to utilize L-homoserine and N-acetyl-L-glutamic acid could differentiate them from *P. palleroniana* LMG 23076^T. Strains of the proposed *P. aylmerense* sp. nov. could be differentiated from *P. lurida* LMG 21995^T by their inability to utilize L-rhamnose; and from all the closest species except *P. extremorientalis* LMG 19695^T by the ability to utilize laminarin. The two strains of proposed *P. aylmerense* sp. nov. gave a positive reaction for catalase.

Strains (S1E40^T and S1E44) of proposed *P. aylmerense* are Gram-reaction-negative based on the 3 % KOH assay [41]. The two strains also had an optimum growth at 30 °C with minimal or no growth at 5 °C or 40 °C. These two representative strains tolerated concentrations of NaCl (w/v) up to 4%, and above this threshold the growth significantly decreased (data not shown). The strains were non-spore-forming.

Cell morphology studies showed that strains S1E40^T and S1E44 are rod-shaped (mean dimensions, 1.6 μm long × 0.5 μm wide) with at least one flagellum (Fig. 6). Motility was demonstrated in semisolid medium using triphenyl tetrazolium [43]. The colonies of the strains were white-yellowish, convex and circular with a mean diameter of 4.6 mm and regular margins after 48 hrs. On KB medium [23] colonies of strains S1E40^T and S1E44 produced a fluorescent yellowish-green pigment, when observed under UV light (wavelength 365 nm), which is generally attributed to the presence of pyoverdines [8]. The draft genome sequences of both strains of proposed *P. aylmerense* showed an elaborate pyoverdine gene cluster. Fluorescent pseudomonads produce

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Table 2. Differential phenotypic characteristics of the two strains of proposed *P. alymerense* sp. nov. and type strains of closely related *Pseudomonas* species. Strains S1E40^T and S1E44 could be differentiated from all the closest species by assimilation of laminarin with the exception of *P. extremeorientalis* that exhibited a weak reaction. Also, strains of *P. aylmerense* could be differentiated from all the other species by their inability to utilize L-arabitol and 2-Deoxy-D-ribose with the exception of *P. palleroniana*.

Characteristics	1	2	3	4	5	6	7
Nitrate Reduction	−	−	−	−	+	−	−
Gelatine hydrolases	+	+	+	−	+	−	+
Assimilation of:							
L-Arabinose	+	+	+	+	+	+	+
D-Serine	−	−	−	+	−	−	−
D-Sorbitol	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	w	w
L-Rhamnose	−	−	+	+	−	−	w
Sucrose	−	−	−	+	−	+	+
M-Tartaric Acid	+	+	+	+	+	−	+
Adonitol	−	−	+	+	+	+	w
Tyramine	−	−	−	+	+	−	+
Dextrin	−	−	−	+	−	−	w
Laminarin	+	+	−	−	−	w	−
L-Arabitol	−	−	+	+	+	+	−
2-Deoxy-D-Ribose	−	−	+	+	+	+	−
I-Erythritol	+	+	−	+	+	−	+
Xylitol	−	−	+	+	w	+	−
N-Acetyl-L-glutamic Acid	−	−	−	−	+	+	+
L-Homoserine	−	−	−	−	−	−	+
L-Phenylalanine	−	−	+	+	−	−	+
D,L-Octopamine	−	−	−	−	+	−	+

1-2 *P. aylmerense* sp. nov.: 1, S1E40^T; 2, S1E44; 3, *P. lurida* LMG 21995^T; 4, *P. costantinii* LMG 22119^T; 5, *P. simiae* CCUG 50889^T; 6, *P. extremorientalis* LMG 19695^T; 7, *P. palleroniana* LMG 23076^T. +, positive reaction/strong growth observed; −, negative reaction/no growth; w: weak reaction/slow growth in triplicate assays.

pyoverdines as a mechanism for ecological fitness and they also can play important role in biological control of fungal plant pathogens [15, 55, 56].

S1E40^T and S1E44 did not provoke HR responses on *N. benthamiana* (data not shown), suggesting a non-compatible interaction. This was confirmed in artificial M9M medium, reported to mimic nutrients availability in plant cells [46] with the strains of the proposed *P. aylmerense* not showing observable growth after 96 h.

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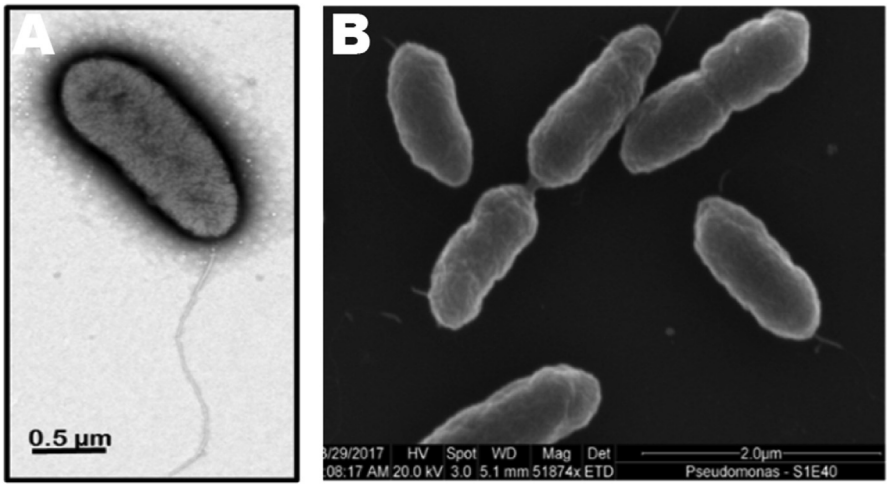

Fig. 6. Transmission electron (A) and scanning electron (B) micrographs of the type strain of *Pseudomonas aylmerense* S1E40^{T} cells grown overnight. The images depict rod-shaped cells with, at least, one flagellum.

The strains S1E40^{T} and S1E44 inhibited mycelial growth of *F. graminearum* DOAMC 178148 in *in vitro* dual cultures on GCY medium with inhibition rates ranging from 29.7.4%−33.9%. *In vitro* inhibition is generally attributed to the secretion of antifungal compounds such as phenazine-1-carboxylic acid (PCA) [15, 57, 58, 59] and 2,4-diacetophloroglucinol (phl) [58, 60, 61]. PCR amplifications of PCA and phl genes did not indicate the presence of these genes, suggesting that strains S1E40^{T} and S1E44 might produce other as yet unknown, antifungal compounds.

An exploratory study was initiated to better understand the secondary metabolites produced in different media by the type strain (S1E40^{T}) of proposed *P. aylmerense* sp. nov. Most notable differences in UPLC-HRMS profiles between control and extracts of strain S1E40^{T} were observed from MeOH fractions. PCA analysis performed using a data matrix of 133 variables (pseudomolecular ions) representing all MeOH fractions, yielded robust models explaining 90−98% of the total variance of the individual media trials in the first two PCs. A distinct metabolite phenotype was observed for the type strain of proposed *P. aylmerense* sp. nov. following 48 h growth on KB and GCY media and by 96 h growth on LB medium. Four structurally related secondary metabolites (confirmed by MS/MS experiments of parent $[M + H]^{+}$ ions) were observed to increase from 24 to 96 h of growth on all three media (Table 3). Database query using Antibase [62] of $[M + H]^{+}$, $[M + Na]^{+}$ and MS/MS fragments did not return any known matches, suggesting that these structurally related secondary metabolites may represent new chemistry, not previously observed. Further characterization of these unknown metabolites is warranted.

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Table 3. Retention time (RT) and observed pseudomolecular ions and MS/MS fragment ions (from [M + H]+ parent ion) for four secondary metabolites observed to accumulate from 24 − 96 hrs in three culture media (King B, GCY and Luria-Bertina) inoculated with the type strain of *Pseudomonas aylmerense* S1E40^T. Each metabolite exhibited similar fragment losses from parent [M + H]+ ion (within a 5 ppm difference); confirming shared structural similarity between the four secondary metabolites.

RT	[M + H]+	[M + Na]+	[M + H−H2O]+	[2M + H]+	[M + H]+ fragments (MS/MS)			
5.18	452.276	474.258	434.266	903.544	434.266	391.223	354.3	311.257
5.39	466.293	488.275	448.282	931.578	448.282	405.24	368.316	325.274
5.45	454.293	476.275	436.282	907.578	436.282	393.24	356.315	313.274
5.82	494.324	516.306	476.314	987.641	476.314	433.272	396.347	353.305
		Average Δm/z ([M + H]+ − [fragment]+):			18.0106	61.0528	97.9775	141.019

Based on polyphasic characterization, strains S1E40^T and S1E44 represent a novel species within the genus *Pseudomonas*, for which the name *Pseudomonas aylmerense* sp. nov. is proposed with S1E40^T (= LMG 30784^T = DOAB 703^T = HAMI 3696^T) as type strain. Table 4 presents the formal proposal of the new species *P. aylmerense* with the Taxonumber TA00596.

Table 4. Digital Protologue table of *Pseudomonas alymerense* sp. nov. strain S1E40^T = HAMBI 3696^T.

Taxonumber	TA00596
Date of the entry	08/07/2018
Draft number/date	6
Version	Draft
Species name(Give the binomial species name)	*Pseudomonas aylmerens*
Genus name	*Pseudomonas*
Specific epithet	*aylmerense*
Species status	sp. nov.
Species etymology	ayl.mer.en'se. N.L. neut. adj. aylmerense of or belonging to Aylmer, Québec, Canada
Authors	Tchagang CF, Xu R, Overy D, Blackwell B, Chabot D, Hubbard K, Doumbou CL, Bromfield ESP and Tambong JT
Title	Diversity of bacteria associated with corn roots inoculated with Canadian woodland soils, and description of *Pseudomonas aylmerense* sp. nov.
Journal	Heliyon
E-mail of the corresponding author	james.tambong@agr.gc.ca
Submitter	JAMES TAMBONG

(*continued on next page*)

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Table 4. (*Continued*)

E-mail of the submitter	james.tambong@agr.gc.ca
Has the taxon been subjected to emendation?	No
Designation of the type strain	S1E40
Strain collection numbers	HAMBI 3696 = DOAB 703
16S rRNA gene accession number	MH463722
Alternative housekeeping genes: Gene [Accession number]	rpoD [MH494092], rpoB [MH487780], gyrB [MH544581]
Genome accession number [RefSeq]	MAUE00000000
Genome status	draft
Genome size	6.98 Mb
GC mol %	61.6
Data on the origin of the sample from which the strain had been isolated	
Country of origin	CAN
Region of origin	Quebec
Date of isolation	06/06/2007
Source of isolation	woodland soil suspension using corn as trap plants
Sampling date	30/05/2007
Geographic location	Aylmer
Latitude	45°2248.21″N
Longitude	75°48′25.52″W
Depth	10 cm
Number of strains in study	2
Growth medium, incubation conditions [Temperature, pH, and further information] used for standard cultivation.	King's B or Luria-Bertani medium, Optimal growth temperature is at 30 °C for 48 h.
Is a defined medium available?	Yes
Alternative medium 1	Nutrient agar
Conditions of preservation	25 % glycerol at -80 °C or freeze-dried.
Gram stain	NEGATIVE
Cell shape	rod
Cell size (length or diameter)	0.5−0.6 μm wide × 1.6−1.8.3 μm long
Motility	motile
If motile	flagellar
Sporulation (resting cells)	none
Colony morphology	Colonies are white-yellowish and circular (average 5 mm in diameter) on KB after 48 h, and produce fluorescent pigments.
Temperature range	10 −35 °C
Lowest temperature for growth	10

(*continued on next page*)

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Table 4. (*Continued*)

Highest temperature for growth	35 °C
Temperature optimum	30°C
Lowest NaCl concentration for growth	0
Highest NaCI concentration for growth	6
Salinity optimum	2
Relationship to O_2	aerobe
Carbon source used [class of compounds]	Biolog PM1 & PM2A plates and API 20NE strips
Positive tests with BIOLOG	L-Arabinose, D-saccharic acid, N-acetyl-D-glucosamine, succinic acid, D-Galactose, L-aspartic acid, L-proline, D-alanine, D-mannose, D-trehalose, D-sorbitol, glycerol, D, L-α-glycerol-phosphate, D-xylose, L-lactic acid, D-mannitol, L-glutamic acid, D-galactonic-γ-lactone, DL- malic acid, D-ribose, tween 20, D-fructose, acetic acid, α-D-glucose, L-asparagine, D-glucosaminic acid, tween 40, α-keto-glutaric acid, uridine, L-glutamine, m-tartaric acid , α-hydroxy glutaric acid-γ-lactone, adenosine, citric acid, m-inositol, D-threonine, fumaric acid, bromo-succinic acid, propionic acid, mucic acid, inosine, L-serine, L-alanine, L-alanyl-glycine, methyl pyruvate, L-malic acid, pyruvic acid, 2-aminoethane, dextrin, gelatine, glycogen, laminarin, D-arabitol, i-erythritol, γ −aminobutyric acid, caproic acid, citramalic acid, D-glucosamine, 4-hydroxybenzoic acid, β-hydroxy butyric acid, itaconic acid, malonic acid, quinic acid, succinamic acid, L-alaninamide, L-arginine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-ornithine, L-pyroglutamic acid, L-valine, D,L-carnitine, putrescine
Negative tests with BIOLOG	dulcitol, D-serine, L-fucose, L-rhamnose, maltose, D-melibiose, thymidine, D-aspartic, 1,2-propanediol, α-D-lactose, lactulose, sucrose, β-methyl-D glucoside, adonitol, maltotriose, 2-deoxy adenosine, glycolic acid, glyoxylic acid, D-cellobiose, tyramine, D-psicose, L-lyxose, glucuronamide, D-arabinose, gentiobiose, L-glucose, lactitol, palatinose, D-raffinose, salicin, L-sorbose, stachyose and xylitol
Positive tests with API	Glucose, mannose, mannitol, N-acetyl-glucosamine, potassium gluconate, malate and trisodium citrate. Arginine dihydrolase, urease, and gelatinase.
Negative tests with API	β-glucosidase, β-galactosidase, glucose fermentation, nitrate reduction, maltose assimilation and phenylacetic acid.
Oxidase	positive

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Table 4. (*Continued*)

Catalase	positive
Quinone type	Q9 (87%) and Q8 (10%).
Major fatty acids	summed feature 3 (C16:1ω6C/C16:1ω7C, 30.28%/), C16:0 (30.28%) and summed feature 8 (C18:1ω6C/C18:1ω7C; 16.41%)
Habitat	Soil
Biotic relationship	free-living
Miscellaneous, extraordinary features relevant for the description	Polar lipids include major amounts of phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol with traces of phospholipid.

Declarations

Author contribution statement

Caetanie F. Tchagang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Renlin Xu: Performed the experiments.

David Overy, Barbara Blackwell, Denise Chabot, Keith Hubbard, Cyr-Lézin Doumbou, Eden S.P. Bromfield: Contributed reagents, materials, analysis tools or data.

James T. Tambong: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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