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TAXONOMIC DESCRIPTION
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Pseudomonas floridensis sp. nov., a bacterial pathogen isolated from tomato

Sujan Timilsina,[1,2] Gerald V. Minsavage,[1] James Preston,[3] Eric A. Newberry,[1] Matthews L. Paret,[4] Erica M. Goss,[1,5] Jeffrey B. Jones[1] and Gary E. Vallad[2,*]

Abstract

An unusual fluorescent pseudomonad was isolated from tomato exhibiting leaf spot symptoms similar to bacterial speck. Strains were fluorescent, oxidase- and arginine-dihydrolase-negative, elicited a hypersensitive reaction on tobacco and produced a soft rot on potato slices. However, the strains produced an unusual yellow, mucoid growth on media containing 5 % sucrose that is not typical of levan. Based on multilocus sequence analysis using 16S rRNA, *gap1*, *gltA*, *gyrB* and *rpoD*, these strains formed a distinct phylogenetic group in the genus *Pseudomonas* and were most closely related to *Pseudomonas viridiflava* within the *Pseudomonas syringae* complex. Whole-genome comparisons, using average nucleotide identity based on blast, of representative strain GEV388^T and publicly available genomes representing the genus *Pseudomonas* revealed phylogroup 7 *P. viridiflava* strain UASW0038 and *P. viridiflava* type strain ICMP 2848^T as the closest relatives with 86.59 and 86.56 % nucleotide identity, respectively. *In silico* DNA–DNA hybridization using the genome-to-genome distance calculation method estimated 31.1 % DNA relatedness between GEV388^T and *P. viridiflava* ATCC 13223^T, strongly suggesting the strains are representatives of different species. These results together with Biolog GEN III tests, fatty acid methyl ester profiles and phylogenetic analysis using 16S rRNA and multiple housekeeping gene sequences demonstrated that this group represents a novel species member of the genus *Pseudomonas*. The name *Pseudomonas floridensis* sp. nov. is proposed with GEV388^T (=LMG 30013^T=ATCC TSD-90^T) as the type strain.

The genus *Pseudomonas* consists of a large group of Gram-reaction-negative, rod-shaped bacteria that are motile and highly diverse in nature. The members of the genus *Pseudomonas* are found in different environmental niches including soil, water, plants and animals [1, 2]. More than 140 species of the genus *Pseudomonas* have validly published names, and the genus includes some major plant pathogens [3–5]. Traditionally, speciation of pseudomonads was based on only a few phenotypic characteristics. For taxonomic purposes, DNA–DNA hybridization (DDH) is regarded as the gold standard for differentiation of species [6]. Although, DDH provides higher resolution for taxonomy, the approach is laborious and it is not feasible to establish a central database [7]. The availability of DNA sequencing technologies and databases have laid the foundation for sequence-based taxonomy [3]. Sequence analysis of 16S rRNA genes and conserved housekeeping genes is frequently used for characterization and classification of members within the genus *Pseudomonas* [8, 9]. Several schemes have been proposed for multilocus sequence analysis and characterization of strains belonging to the genus *Pseudomonas* [10–12]. Recently, whole-genome sequences (WGS) have been used for comparative and taxonomic purposes. Multiple pairwise comparative measures like average nucleotide identity and genome-to-genome distance calculations have been utilized for species differentiation using WGS [13–16]. Unlike conventional DDH approaches, WGS can be used along with phenotypic characteristics to compare a wide range of bacteria from multiple sources to provide a clear taxonomic designation [17].

Author affiliations: [1]Deparment of Plant Pathology, University of Florida, Gainesville, FL 32611, USA; [2]Gulf Coast Research and Education Center, University of Florida, Gainesville, FL 32611, USA; [3]Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611, USA; [4]North Florida Research and Education Center, University of Florida, Quincy, FL 32251, USA; [5]Emerging Pathogens Institute, University of Florida, Gainesville, FL 32610, USA.
***Correspondence:** Gary E. Vallad, gvallad@ufl.edu
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Abbreviations: ANI, average nucleotide identity; ANIb, ANI based on BLAST; DDH, DNA-DNA hybridization; ICBR, Interdisciplinary Center for Biotechnology Research; IMG, Integrated Microbial Genomes; M-JGI, Microbiome Samples-Joint Genome Institute; NCBI, National Center for Biotechnology Information; UF, University of Florida; WGS, whole-genome sequences.
The GenBank/EMBL/DDBJ accession number for the genome sequence of GEV388^T is MUIO00000000 (SRR5224169). The accession numbers for the housekeeping gene sequences of *P. floridensis* used in this study are KY612169–KY612204, and those for the 16S rRNA gene sequences are KY614190–KY614192.
Six supplementary figures and two supplementary tables are available with the online version of this article.

Pseudomonas syringae represents a complex group of strains causing various diseases in a wide range of plant hosts [18]. In the case of plant-pathogenic pseudomonads, infra-subspecific taxonomy has been described by using the pathovar designation. The plant pathogenic *P. syringae* group includes species with validly published names including *Pseudomonas viridiflava*, *P. cichorii*, *P. avellanae*, *P. cannabina*, *P. congelans*, *P. caricapapayae*, *P. amygdali*, *P. tremae*, *P. ficuserectae* and *P. savastanoi* [4]. Multilocus sequence analysis/typing schemes along with DDH have been used to describe 13 phylogroups and nine genomospecies of the *P. syringae* complex [6, 11, 19–21]. Whole-genome sequencing and phylogenetic approaches are currently developing to characterize taxonomically the strains of the genus *Pseudomonas* isolated from different hosts [6, 21, 22].

In Florida, on tomato (*Solanum lycopersicum* L.), pathogenic strains of *P. syringae*, *P. viridiflava* and *Pseudomonas corrugata* were described previously and, more recently, *P. cichorii* strains were reported [23–25]. An outbreak of leaf spot was observed in a tomato field in central Florida in 2010 and 2011. Foliar symptoms included dark brown to black, raised lesions with a yellow halo (Fig. S1, available in the online version of this article). As the disease symptoms were similar to bacterial speck caused by *P. syringae*, bacterial isolations from the diseased tissue were made on nutrient agar (NA; Difco) and King's medium B (KMB) [26]. A total of eight fluorescent strains belonging to the genus *Pseudomonas* were isolated from eight different symptomatic plants in the field. Pathogenicity of bacterial strains was confirmed by spray inoculation of tomato foliage with bacterial suspensions adjusted to approximately 10^8 c.f.u. ml^{-1}. The plants were bagged with clear polyethylene bags and incubated in a growth chamber at 28 °C for 48 h. Disease symptoms similar to natural infection were observed 48 h post inoculation. The standard LOPAT tests, which consist of determining levan production on 5 % sucrose medium, oxidase activity, potato soft rot, arginine dihydrolase assay and tobacco hypersensitivity, were conducted for preliminary characterization of all strains representing the genus *Pseudomonas* [27].

Based on the LOPAT tests of eight strains belonging to the genus *Pseudomonas* isolated during this study, the strains caused potato soft rot and a hypersensitive reaction on tobacco. The strains were also oxidase- and arginine-dihydrolase-negative similar to *P. viridiflava.* However, during the levan assay, the strains produced a yellow-coloured biofilm, uncharacteristic of *P. viridiflava* and distinct from the typical white levan reaction produced by levan-positive strains of the genus *Pseudomonas*. Similar atypical strains, which produced a yellowish biofilm, were reported from diseased tomato plants in Spain [28] and were identified as *P. viridiflava* based on 16S rRNA gene sequence identity.

The biofilm produced by the novel strains of the genus *Pseudomonas* was mucoid and yellowish on NA amended with 5 % sucrose. A lectin, concanavalin A (ConA), was reported to bind to levan-like compounds [29]. Thus, in order to study the composition of the mucoid growth, the biofilm of the novel strain GEV388^T was stained using a FITC-labelled fluorescent ConA [30]. A known levan-producing strain of *P. syringae* (GEV1421) was used as a positive control, and type strains of *P. cichorii* (ATCC 10857^T) and *P. viridiflava* (ATCC 13223^T) were used as negative controls. Interestingly, fluorescent binding between the FITC-labelled ConA and the biofilm of the atypical strain of the genus *Pseudomonas* and levan-positive *P. syringae* strains was observed. However, it was not observed with levan-negative *P. viridiflava* and *P. cichorii* (Fig. S2). Thus, the results suggest that the atypical strains of the genus *Pseudomonas* produce a polysaccharide biofilm but do not utilize sucrose to produce levan.

A Gram reaction of the strains was determined using a standard potassium hydroxide (KOH) test [31]. An overnight culture of GEV388^T was grown at 28 °C in nutrient broth (Difco Laboratories), and the bacterial cell morphological characteristics were observed in a Tencai G2 Spirit TWIN 120 kV electron microscope at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida (UF). The bacterial cells were determined to be Gram-reaction-negative and rod shaped, measuring 1.75–3.6 µm long and ~0.75 µm wide with lophotrichous flagella (Fig. S3).

Genomic DNA was extracted from eight strains, including GEV388^T, using the CTAB (cetyl trimethylammonium bromide) method [32] to amplify conserved genes for sequence analysis. The 16S rRNA gene was amplified using primers 63f and 1387r [33] specific for members of the genus *Pseudomonas*. Three representative strains were amplified for sequence comparison of the 16S rRNA gene. The resulting products were sequenced using Sanger sequencing at the ICBR, UF. Consensus sequences derived from forward and reverse sequence reads were submitted to the National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequences of the atypical strains belonging to the genus *Pseudomonas* were compared phylogenetically with sequences of type strains representing different species of the genus *Pseudomonas*. The sequences were aligned using the MUSCLE program within MEGA v. 7 [34, 35]. Maximum-likelihood and maximum-parsimony phylogenetic trees using 16S rRNA gene sequences were reconstructed using MEGA v. 7 [35]. The robustness of the topology was estimated using 1000 bootstrap replicates. Comparative analysis determined that the three representative atypical strains of the genus *Pseudomonas* had identical 16S rRNA gene sequences. Phylogenetically, the strains were closely related to *P. viridiflava* (Fig. S4). The sequence identities between the atypical strains and the type strain *P. viridiflava* ATCC 13223^T (accession number: NR_114482) and the levan-positive *P. viridiflava* strains LPPA 144 and LPPA 366 (accession numbers: AY180968.1 and AM182934.1) reported from Spain were 98.81, 98.89 and 99.05 %, respectively. Similarly, the atypical strains shared 98.42 % sequence identity with *P. cichorii* ATCC 10857^T based on 16S rRNA gene sequences.

The novel strains of the genus *Pseudomonas* were further characterized phylogenetically using multiple housekeeping gene sequences. The housekeeping genes *gap1*, *gltA*, *gyrB* and *rpoD* were sequenced from all eight strains along with the type strain *P. viridiflava* ATCC 13223^T as described strains [12]. The amplification products were sequenced at ICBR, UF, and the sequences were submitted to NCBI. Reference sequences were extracted from NCBI and the Plant-Associated and Environmental Microbe database (PAMDB; http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl) [36] for phylogenetic comparison. The model for phylogenetic analysis was determined using the Akaike Information Criterion (AIC) statistic within jModelTest v. 2 [37]. Based on AIC, a general time-reversible model with gamma-distributed invariant sites was used. A maximum-likelihood phylogenetic tree was reconstructed using concatenated gene sequences in MEGA v. 7. The eight atypical strains of the genus *Pseudomonas* were identical based on the four housekeeping gene sequences (Fig. S5). However, the strains were phylogenetically distinct from previously described species of the genus *Pseudomonas* with *P. viridiflava* as the closest relative. The sequence identity of the atypical strains with *P. viridiflava* ATCC 13223^T based on *gap1*, *gltA*, *gyrB* and *rpoD* was 90.21, 94.12, 89.85 and 91.75 % respectively.

Berge *et al.* [11] used housekeeping gene sequences to characterize the plant pathogenic *P. syringae* complex into 13 different phylogroups [11]. The study amplified different regions of the *gltA* gene (*cts*) and *rpoD* gene in combination with the *gap1* and *gyrB* genes. Thus, to further elucidate the phylogenetic relationship of the atypical strains of the genus *Pseudomonas* with other known strains belonging to the genus *Pseudomonas*, a representative, GEV388^T, was compared with strains representing 13 phylogroups of the *P. syringae* complex using *cts*, *rpoD*, *gap1* and *gyrB*. Additionally, previous studies suggested that two gene loci, *gyrB* and *rpoD*, provided sufficient phylogenetic information for characterization of members of the genus *Pseudomonas* [9]. Thus, to incorporate all species of the genus *Pseudomonas* previously analysed with varying multilocus sequence typing schemes, the *gyrB* and *rpoD* genes were concatenated and analysed phylogenetically. Maximum-likelihood and maximum-parsimony phylogenetic trees were reconstructed using the two loci as described previously for multilocus sequence analysis.

Phylogenetic comparison with representative phylogroups of plant-pathogenic members of the genus *Pseudomonas*, based on *cts*, *rpoD*, *gap1* and *gyrB*, showed that the representative GEV388^T strain does not belong to any predefined phylogroup of the genus *Pseudomonas* (Fig. S6) and most likely represents a novel species of the genus *Pseudomonas*. However, the strain was closely related phylogenetically to phylogroups 7 and 8 that encompasses *P. viridiflava*. Following further assessment of the phylogenetic relationships based on *gyrB* and *rpoD*, the atypical strains were determined to be phylogenetically close to *Pseudomonas asturiensis* and *P. viridiflava* (Fig. 1). However, the sequence identity of the atypical strains was 89.92 and 89.34 % for *gyrB* and *rpoD*, respectively, with *P. asturiensis* LMG 26898^T, and 89.92 and 91.75 % for *gyrB* and *rpoD*, respectively, with *P. viridiflava* ATCC 13223^T.

Based on preliminary phenotypic and extensive phylogenetic analysis using single and multiple genes, the atypical strains most likely grouped as representatives of a novel species group within the genus *Pseudomonas*. All eight strains of this novel *Pseudomonas* group were characterized phenotypically using the Biolog GENIII MicroPlate system along with type strains of other closely related species of the genus *Pseudomonas*: *P. viridiflava* ATCC 13223^T, *P. syringae* LMG 1247^T, *P. cichorii* ATCC 10857^T, *P. asturiensis* LMG 26898^T and *P. amygdali* ATCC 33614^T (Table 1). Bacterial cells from overnight bacterial cultures grown on NA were suspended in Biolog inoculation fluid at the recommended concentration, and 100 µl of suspension was added to each well of the GENIII MicroPlate and incubated at 28 °C for 48 h. Metabolic reactions were read using a Biolog plate reader. Although results suggested that the atypical strains have a carbon-source utilization pattern similar to those of *P. viridiflava* and *P. syringae*, the strains could not be associated with either at the species level. The Biolog GENIII assay differentiated strain GEV388^T from other closely related type strains of species of the genus *Pseudomonas* based on differences in the utilization of various carbon sources like mannitol, sorbitol and galactonic acid. Bacterial growth of the eight strains was observed in tubes of broth incubated at temperatures ranging from 4 to 37 °C in LB medium, a pH ranging between pH 5 and 8 and with a salinity based on NaCl ranging from 0 to 4 % (w/v).

Contrary to the bacterial strain reaction during the standard LOPAT levan assay, no reaction was observed with sucrose during the Biolog assay for the eight novel strains. Thus, the novel strains representing the genus *Pseudomonas* were plated on Ayers minimum medium supplemented with 1 % sucrose [38]. A levan-positive *P. syringae* and a levan-negative *P. viridiflava* strain were used as controls. Biofilm production, by any strain, was not observed in the minimum medium suggesting that the novel strains do not utilize sucrose. On the basis of these results, the strains could be confirmed as levan negative. However, the strains continued to produce a yellowish, viscous biofilm when plated on NA supplemented with 5 % sucrose.

The strains of the novel *Pseudomonas* group were sent to the Microbial Identification System (MIDI, Newark, DE, USA) laboratories to determine the cellular fatty acid composition. The major fatty acids of all strains were summed as feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) (Table S1). Other fatty acids detected included $C_{10:0}$ 3-OH, $C_{12:0}$, $C_{12:0}$ 2-OH, $C_{12:0}$ 3-OH, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}\omega7c$ 11-methyl. The fatty acid profiles of the atypical strains were closely related to those of *P. cichorii*, *P. viridiflava* and *P. syringae* pv. *tagetis*. The results confirmed that the atypical strains belong to the *P. syringae* complex of plant-pathogenic bacteria. However,

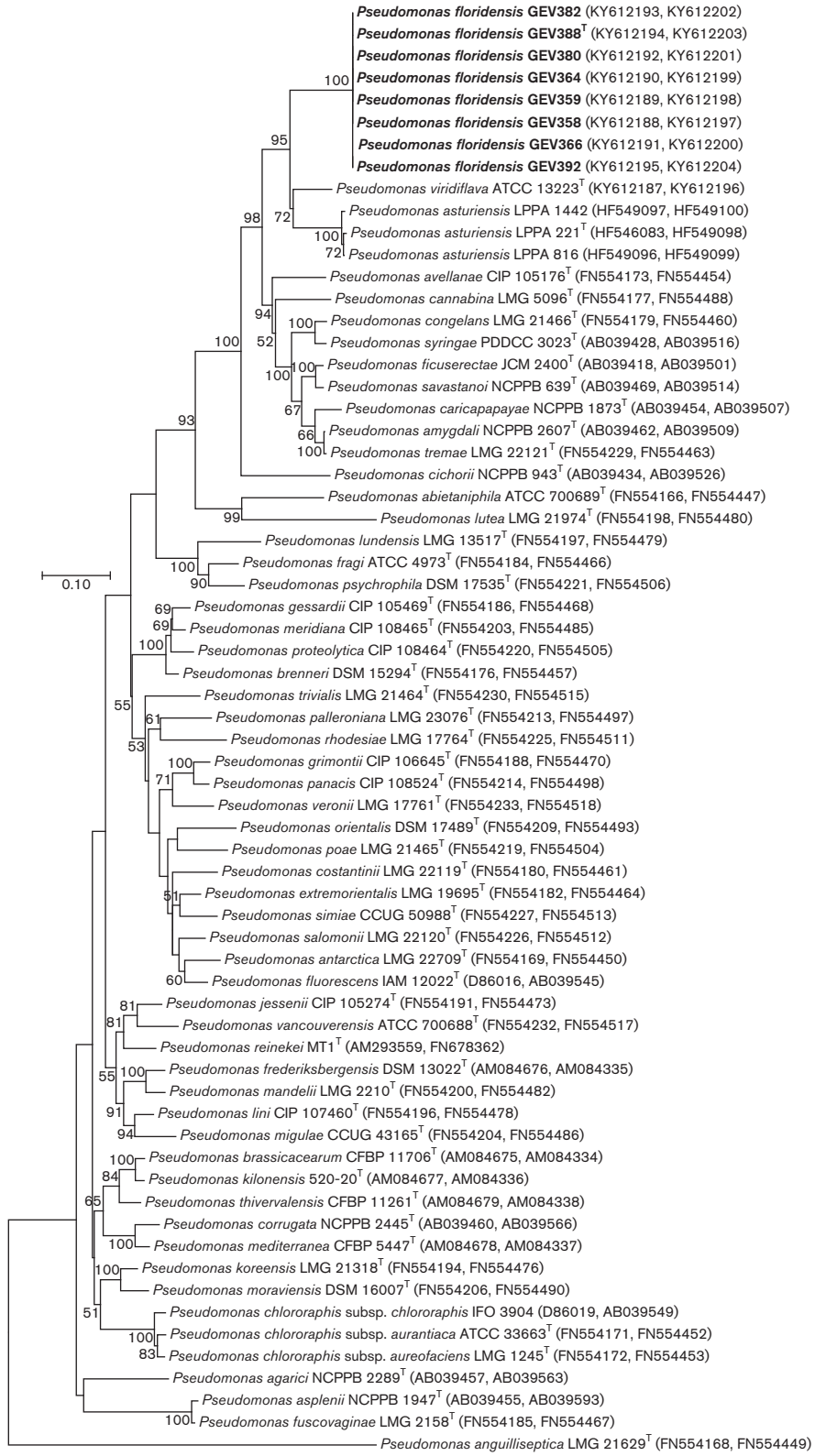

Fig. 1. Maximum-likelihood tree based on concatenated sequences of *gyrB* and *rpoD* genes using the general time-reversible model with gamma-distributed invariant sites, showing the position of *Pseudomonas floridensis* sp. nov. strains. Bar, substitutions per site. Bootstrap values (>50 %, based on 1000 replicates) are indicated as percentages at branching points. Accession numbers for each sequence are listed along with the strain.

Table 1. Selected phenotypes of the atypical strains of the genus *Pseudomonas* isolated from tomato in Florida and representative strains of closely related species of the genus *Pseudomonas*

Strains: 1, *Pseudomonas floridensis* sp. nov. GEV388^T; 2, *P. viridiflava* ICMP 2848^T; 3, *P. syringae* LMG 1247^T; 4, *P. cichorii* ATCC 10857^T; 5, *P. amygdali* LMG 2123^T; 6, *P. asturiensis* LMG 26898^T. –, No significant reaction; +, positive reaction; w, intermediate reaction. All strains were negative for production of arginine dihydrolase, positive for utilization of glycerol and D-mannitol, and induced a hypersensitive reaction on tobacco.

Characteristic	1*	2	3	4	5	6
Levan	–	–	+	–	+	–
Oxidase	–	–	–	+	–	–
Potato rot	+	+	–	–	–	–
Growth at pH 5	+	+	+	+	+	w
Carbon Sources (Biolog GENIII)						
D-Sorbitol	+	+	+	–	w	–
L-Galactonic acid	+	–	w	–	–	+
D-Fucose	w	+	+	–	–	+
D-Arabitol	+	+	w	+	+	+
Troleandomycin	+	–	w	–	w	w
Sucrose	–	–	+	–	w	–

*The other 7 strains isolated from tomato in this study gave identical reactions for these tests as GEV388^T.

the profiles did not support further taxonomic classification of GEV388^T to any of the predefined species of the genus *Pseudomonas*. Variations in fatty acids like $C_{12:0}$, $C_{16:0}$, $C_{18:0}$ and summed feature 8 were observed compared with the *P. viridiflava* strains. Similarly, $C_{17:0}$ fatty acid found in the closely related *P. savastanoi*, *P. putida* and *P. asturiensis* [4], was not found in any of the atypical strains.

The genome of strain GEV388^T was sequenced, and the sequence deposited in the NCBI Genbank database. A genomic library was prepared using a Nextera DNA library preparation kit and was sequenced using an Illumina MiSeq platform at ICBR, UF. The sequences yielded 449 014 reads with an average of 241 bp paired-end reads (108.14 Mb). The raw sequences were assembled using *de novo* genome assembly in CLC Genomics workbench v. 5.0 (CLC Bio-Qiagen) yielding 287 contigs $\geq$500 bp (N50=60 718 bp) with 17.72× coverage. The assembly yielded a genome size of 6 103 769 nucleotides. The DNA G+C content was 59.17 %, within the range observed for species of the genus *Pseudomonas* [39]. The draft genome was annotated using the Integrated Microbial Genomes and Microbiome Samples-Joint Genome Institute (IMG/M-JGI) platform [40].

The draft genome of GEV388^T was used to calculate the average nucleotide identity (ANI) and genome distance by computing *in silico* DDH with multiple type strains of species of the genus *Pseudomonas*. For comparative purposes, pairwise ANI between GEV388^T and all publicly available genomes belonging to the genus *Pseudomonas* (>1800 genomes) (data not shown) in the IMG database was calculated using the IMG/M-JGI web tool (https://img.jgi.doe.gov/mer/). Additionally, pairwise ANI based on BLAST (ANIb) was calculated between GEV388^T and phylogenetically closely related species of the genus *Pseudomonas* using jSpecies v1.2.1 [41]. A web-based tool, genome-to-genome distance calculator v2.1, was used to compute the *in silico* DDH between the novel strain GEV388^T and other reference strains [42].

Collectively, the highest ANIb for GEV388^T was observed with the *P. viridiflava* species group (Table S2). Based on current classification, *P. viridiflava* encompasses phylogroups 7 and 8 within the plant-pathogenic *P. syringae* complex [11]. The ANIb between GEV388^T and the representative strains of phylogroup 7, *P. viridiflava* UASW0038, and phylogroup 8, *P. viridiflava* ICMP 2848^T, was 86.59 and 86.56 %, respectively. Both ANIb values are significantly lower than 95 %, which is considered as the standard ANI value for species differentiation [41, 43]. A strain of the recently proposed novel pathogenic species of the genus *Pseudomonas*, *P. asturiensis* LMG 26898^T, was identified as the next closest relative after *P. viridiflava*, with 86.11 % ANIb. *P. asturiesnsis* is a recently described plant pathogen isolated from soybean and weeds in Spain and is closely related to *P. viridiflava* based on DDH [4]. Along with ANIb, the *in silico* DDH estimated for GEV388^T and the *P. viridiflava* group of strains ranged between 31 and 64 %, significantly below the 70 % threshold value recommended for species description [44].

Considering the multiphasic analyses based on phenotypic characteristics, phylogenetic analyses, metabolic reactions, fatty acid composition and whole-genome sequence comparisons, these strains isolated from tomato in Florida represent a novel species, and the name *Pseudomonas floridensis* sp. nov. is proposed.

DESCRIPTION OF *PSEUDOMONAS FLORIDENSIS* SP. NOV.

Pseudomonas floridensis (flo.ri.den′sis. N.L. fem. adj. *floridensis* pertaining to Florida, the geographical location of first isolation).

Cells are Gram-reaction-negative, motile with multiple polar flagella, rod-shaped (1.75–3.6 µm long and ~0.75 µm wide), non-spore-forming, strictly aerobic and catalase-positive. The cells are fluorescent in King's B agar media and are oxidase-negative. The colonies are smooth and circular in shape, 0.5–1.0 mm diameter after incubation in 28 °C for 24 h on NA medium. Strains are levan-, oxidase- and arginine-dihydrolase-negative but produce a yellowish, mucoid biofilm on NA amended with 5 % sucrose. Positive for pectinolytic activity and induces a hypersensitive reaction on tobacco. Cell growth occurs between 4 and 37 °C, with optimum growth observed between 26 and 29 °C. The bacterium grows at pH 5–8 and with up to 4 % NaCl (w/v). The fatty acids are summed feature $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$, $C_{16:0}$, summed feature $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$, $C_{12:0}$, $C_{12:0}$

3-OH, $C_{10:0}$ 3-OH, $C_{12:0}$ 2-OH, $C_{18:1}\omega7c$ 11-methyl and $C_{18:0}$. Cells are positive for the utilization of α-D-glucose, D-mannose, D-fructose, D-galactose, D-sorbitol, glycerol, L-alanine, L-aspartic acid, L-glutamic acid, L-pyroglutamic acid, L-serine, α-ketoglutaric acid, L-lactic acid, γ-aminobutryric acid, acetic acid, D-mannitol, D-arabitol, *myo*-inositol, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, mucic acid, quinic acid, D-saccharic acid, D-malic acid, L-malic acid and citric acid. Cells are insensitive to troleandomycin, lincomycin, rifamycin sv, lincomycin, niaproof 4, vancomycin, tetrazolium violet and tetrazolium blue.

The type strain, GEV388^T (=LMG 30013^T=ATCC TSD-90^T), was isolated from symptomatic tomato in Florida. The DNA G+C content of the type strain is 59.17 %, based on a genome size of ~6.1 Mb.

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

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