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Pseudomonas protegens sp. nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin☆

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ABSTRACT

Fluorescent *Pseudomonas* strains producing the antimicrobial secondary metabolite 2,4-diacetylphloroglucinol (Phl) play a prominent role in the biocontrol of plant diseases. A subset of Phl-producing fluorescent *Pseudomonas* strains, which can additionally synthesize the antimicrobial compound pyoluteorin (Plt), appears to cluster separately from other fluorescent *Pseudomonas* spp. based on 16S rRNA gene analysis and shares at most 98.4% 16S rRNA gene sequence identity with any other *Pseudomonas* species. In this study, a polyphasic approach based on molecular and phenotypic methods was used to clarify the taxonomy of representative Phl$^+$ Plt$^+$ strains isolated from tobacco, cotton or wheat on different continents. Phl$^+$ Plt$^+$ strains clustered separately from their nearest phylogenetic neighbors (i.e. species from the '*P. syringae*', '*P. fluorescens*' and '*P. chlororaphis*' species complexes) based on *rpoB*, *rpoD* or *gyrB* phylogenies. DNA-DNA hybridization experiments clarified that Phl$^+$ Plt$^+$ strains formed a tight genomospecies that was distinct from *P. syringae*, *P. fluorescens*, or *P. chlororaphis* type strains. Within Phl$^+$ strains, the Phl$^+$ Plt$^+$ strains were differentiated from other biocontrol fluorescent *Pseudomonas* strains that produced Phl but not Plt, based on phenotypic and molecular data. Discriminative phenotypic characters were also identified by numerical taxonomic analysis and siderotyping. Altogether, this polyphasic approach supported the conclusion that Phl$^+$ Plt$^+$ fluorescent *Pseudomonas* strains belonged to a novel species for which the name *Pseudomonas protegens* is proposed, with CHA0^T (=CFBP 6595^T, =DSM 19095^T) as the type strain.

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Introduction

Several fluorescent *Pseudomonas* strains contribute to the disease suppressiveness of soils [11,18,60] or protect plants from disease(s) caused by soil-borne fungal pathogens when used as inoculants [9,12,35,47]. Protection mechanisms include induced systemic resistance [34,46], competition [30,43] and, especially, antibiosis [8,23,47]. Indeed, many biocontrol *Pseudomonas* strains produce several antimicrobial secondary metabolites, such as 2,4-diacetylphloroglucinol (Phl), pyoluteorin (Plt), pyrrolnitrin, phenazine(s) or hydrogen cyanide (i.e. HCN) [23,24,47,53].

In addition to their biocontrol properties, Phl$^+$ Plt$^+$ *Pseudomonas* strains have also received research attention because (i) they are distributed on several continents [69], (ii) they have become models for understanding soil microbial ecology [33,40], and (iii) the genome sequence of one of them (strain Pf-5) is publicly available [44]. Yet, Phl$^+$ fluorescent *Pseudomonas* strains have often been wrongly classified as *P. fluorescens*, because of incomplete or superficial determination of the species status for many strains recovered from the environment [7,8,17,50].

In this study, a polyphasic approach was applied to characterize taxonomically representative Phl$^+$ Plt$^+$ *Pseudomonas* strains isolated from tobacco, cotton or wheat from three different continents. Phylogenetic (based on 16S rRNA and protein-coding

Abbreviations: Phl, 2,4-diacetylphloroglucinol; Plt, pyoluteorin.

☆ The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyrB*, *rpoD* and *rpoB* gene sequences of strain CFBP 6595^T are AJ278812, DQ458573, X84416 and DQ458638, respectively.

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genes), genomic (based on DNA-DNA hybridization experiments and whole-genome fingerprints), siderotyping and phenotypic analyses were used to compare strains to validly described *Pseudomonas* species, as well as to closely-related Phl$^+$ strains lacking Plt production ability. This polyphasic study led us to propose the species name *P. protegens* sp. nov. for Phl$^+$ Plt$^+$ fluorescent *Pseudomonas* strains.

Materials and methods

Pseudomonas strains

The *Pseudomonas* strains listed in Table 1 corresponded to 10 Phl$^+$ Plt$^+$ and 5 Phl$^+$ Plt$^-$ strains representative of the genetic diversity of Phl$^+$ fluorescent *Pseudomonas* [17,27,50,69]. These strains were routinely cultured at 27 °C on King's B agar [28] and stored at -80 °C in 40% glycerol. Additional strains used in this study were all type strains of *Pseudomonas* species (Table 2).

DNA sequence-based and phylogenetic analyses

Almost complete sequences of the 16S rRNA gene and partial sequences of the three housekeeping genes *rpoB*, *rpoD* and *gyrB* were obtained [17] for the four representative Phl$^+$ Plt$^+$ *Pseudomonas* strains CHA0, PGNR1, Pf-5 and PF, and the five Phl$^+$ Plt$^-$ reference strains CM1'A2, Q2-87, Q65c-80, PITR2 and F113. Phylogenetic analysis of 16S rRNA gene sequences was carried out using these nine strains and the *Pseudomonas* type strains with the most similar sequences retrieved from GenBank. Sequence alignment was carried out using the program CLUSTAL W [64] and corrected manually. Percentage sequence identity was calculated based on pairwise sequence alignment, using the Needleman-Wunsch global alignment algorithm in the EMBOSS package [54].

A neighbor-joining [55] tree was inferred from evolutionary distances calculated with the Kimura 2-parameter formula, using MEGA v4.1 [62], and confidence analysis was undertaken using 1000 bootstrap replicates [14]. Trees were also inferred using the maximum likelihood [13] and the maximum parsimony [16] methods, using PhyML v2.4.4 [22] and PAUP v4.0 [61], respectively. Phylogenetic analysis was also performed on individual housekeeping genes after sequence concatenation of *rpoB* (438 aligned nucleotides), *rpoD* (639 nucleotides), and *gyrB* (536 nucleotides). This was carried out with the same strains used for 16S rRNA gene phylogeny (whenever sequences were available from GenBank), and following the same methodology.

G + C content and DNA-DNA reassociation experiments

The G + C content of strain CHA0 was determined by thermal denaturation [32] and was calculated using the equation of Owen and Lapage [41]. Strain *Escherichia coli* K12 (DNA G + C content 50.6 mol%) was used as a control. The DNA G + C content of Phl$^+$ Plt$^+$ strain Pf-5 was deduced from its genome sequence [44].

Genomic DNA extractions and DNA-DNA hybridization experiments were carried out as described by Achouak et al. [1] for the ten Phl$^+$ Plt$^+$ *Pseudomonas* strains and eight type strains, including the nearest phylogenetic neighbors. DNA-DNA reassociation experiments were undertaken at 70 °C with the labelled DNA from strain CHA0. Each hybridization was conducted at least twice. DNA relatedness was reported as the average percentage reassociation relative to the reassociation of the DNA probe to itself. Variations between duplicates ranged between 1 and 10% and were the highest for hybridization values above 90%. In all other cases, variations never exceeded 4%.

Molecular profiling

ERIC-PCR was undertaken according to Rademaker et al. [48] and Ramette et al. [51], and RAPD analysis using primer D7, as previously described [27]. With both methods, there was no difference in the presence/absence of bands when analyses were repeated (not shown), as was also found for certain strains when compared with previous ERIC-PCR (in Fig. 3 of [51]) and RAPD analyses (in Fig. 3 of [27]). Pairwise similarities between electrophoretic patterns were calculated with the Dice coefficient and clustering was carried out by the unweighted pair group method with arithmetic means (UPGMA [58]), as previously described [51].

Siderotyping

Pyoverdine siderophores produced under iron deficiency were characterized by two siderotyping methods (i.e. isoelectrofocusing and pyoverdine-mediated ^{59}Fe uptake), as previously described [19]. For isoelectrofocusing experiments, bacteria were grown using Casamino acid (CAA) medium [36]. The pHi value of each fluorescent pyoverdine-isoform band separated during isoelectrophoresis on polyacrylamide gel was determined by automatic scanned comparison (Herolab System, Wiesloch, Germany) with an internal standard of known pyoverdines [19]. For pyoverdine-mediated ^{59}Fe uptake experiments, the bacteria were grown using succinate medium [36]. Purification of pyoverdines by the XAD chromatographic procedure and their use in iron uptake experiments were performed as described previously [37].

Phenotypic characterization

Numerical taxonomic analysis was performed with the ten Phl$^+$ Plt$^+$ *Pseudomonas* strains studied and 24 phylogenetic neighbors, which included strains representative of the '*P. syringae*', '*P. chlororaphis*', '*P. putida*' and '*P. fluorescens*' complexes (listed in Fig. 1 and in supplementary Table S1). The '*P. syringae*' complex was represented by type and pathotype strains of each genomospecies previously identified [21]. Twenty conventional tests were used, as described by Achouak et al. [1]. In addition, the assimilation of 99 carbon sources was determined by using Biotype 100 strips (BioMérieux, La Balme les Grottes, France), as recommended by the manufacturer. The distance matrix was calculated using the Jaccard coefficient and cluster analysis was undertaken by UPGMA. Discriminative tests were identified using a diagnostic ability coefficient deduced from numerical analysis [10].

Results and discussion

16S rRNA gene-based analyses

Sequence comparison among Phl$^+$ Plt$^+$ strains (based on 1521 aligned nucleotides) indicated that there was more than 99.5% identity between the 16S rRNA sequences of strains CHA0, PF and PGNR1 (Supplementary Table S1). The 16S rRNA gene sequence of strain Pf-5 differed slightly from that of the other three Phl$^+$ Plt$^+$ strains, but still displayed no more than 1% sequence difference with them. When 16S rRNA gene sequences of Phl$^+$ Plt$^+$ strains were compared to those of other *Pseudomonas* species, sequence identity values over 97% were obtained only with a few species within the '*P. syringae*', '*P. fluorescens*' and '*P. chlororaphis*' species complexes (sensu [3]), as follows: within the '*P. syringae*' species complex, the closest species were *P. tremae* (97.7–98.4% sequence identity with that of the Phl$^+$ Plt$^+$ strains), *P. congelans* (97.5–98.4%), *P. cannabina* (97.5–98.1%), and *P. ficuserectae* (96.9–97.1%). Within the '*P. fluorescens*' species complex, the highest sequence identities were found with *P. mandelii* (97.4–97.6%) and *P. rhodesiae*

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