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Pseudomonas gallaeciensis sp. nov., isolated from crude-oil-contaminated intertidal sand samples after the Prestige oil spill

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ABSTRACT

Strains V113^T, V92 and V120 have been isolated from sand samples taken at the Atlantic intertidal shore in Galicia, Spain, after the Prestige oil spill. A preliminary analysis of the 16S rRNA and the partial *rpoD* gene sequences indicated that these strains belonged to the *Pseudomonas* genus, but they were distinct from any known *Pseudomonas* species. They were extensively characterized by a polyphasic taxonomic approach and phylogenetic data that confirmed that these strains belonged to the *Pseudomonas pertucinogena* group. Phylogenetic analysis of 16S rRNA, *gyrB* and *rpoD* gene sequences showed that the three strains were 99% similar and were closely related to members of the *P. pertucinogena* group, with less than 94% similarity to strains of established species; *Pseudomonas pachastrellae* was the closest relative. The Average Nucleotide Index based on blast values was 89.0% between V113^T and the *P. pachastrellae* type strain, below the accepted species level (95%). The predominant cellular fatty acid contents and whole cell protein profiles determined by MALDI-TOF mass spectrometry also differentiated the studied strains from known *Pseudomonas* species. We therefore conclude that strains V113^T, V92 and V120 represent a novel species of *Pseudomonas*, for which the name *Pseudomonas gallaeciensis* is proposed; the type strain is V113^T (= CCUG 67583^T = LMG 29038^T).

Introduction

The genus *Pseudomonas* represents a group of Gram-negative bacteria within the *Gammaproteobacteria* that are non-spore forming rods that are motile by polar flagella [29]. The bacteria of the genus *Pseudomonas* are ubiquitous, metabolically versatile, and important for the recycling of elements in the environment. The genus was first described by Migula (1894) and currently comprises a large number of species, and new species are described continuously. At the time of this manuscript's composition, 167 species with valid names have been described (http://www.bacterio.cict.fr/p/pseudomonas.html) [8,30].

The characterization of *Pseudomonas* type strains by multilocus sequence analysis (MLSA), concatenating the sequences of the 16S rDNA, *gyrB*, *rpoD* and *rpoB* genes, permitted the establishment of two main lineages, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, which were divided into several groups (G) and subgroups (SG) [25,27]. In recent years, the *Pseudomonas pertucinogena* G has experienced a significant increase in the number of newly described species compared with the other groups. In 2004, this group was represented only by *P. pertucinogena* [16]; today, it contains 16 species, the following of which have been recently described: *Pseudomonas aestusnigri* [34,11], *Pseudomonas salegens* [3], *Pseudomonas salina* [39], *Pseudomonas oceani* [36,10], *Pseudomonas populi* [4] and '*Pseudomonas saudimassiliensis*' not yet validated [6].

Proteobacteria have played a key role in the bacterial community in the contaminated intertidal sand after the Prestige oil spill [1,2]. The characterization of the bacterial species present in this habitat is crucial to have a comprehensive knowledge of the microorganisms present in this environment. This paper is focused on the

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genus *Pseudomonas* and a putative new species in the genus. During a study of the *Pseudomonas* diversity in crude-oil contaminated intertidal sand samples after the Prestige oil spill (Galicia, Spain) in September 2004, several isolates were considered representatives of possible new species. These results were confirmed by the analysis of partial sequences of sigma 70 subunit of RNA polymerase, encoded by the *rpoD* gene [26]. Three of these strains, V113^T, V92 and V120, have been characterized taxonomically in the present study. A new *Pseudomonas* species is proposed in the *P. pertucinogena* G, with the strain V113^T as the type strain.

Materials and methods

Bacterial strains and growth conditions

Strains V113^T and V120 have been isolated from "Boca do Rio" beach (42°50′11.52″N, 9°6′11.52″W) from buried weathered fuel. Strain V92 was isolated from an unpolluted sand sample from "Praia de Seda" beach (42°46′29.27″N, 9°7′27.08″W). The strains were isolated after growth in mineral basal medium (MMB) [5] with naphthalene (V92 and V113^T) or hexadecane (V120) as energy and carbon sources [26]. A list of the bacterial strains studied as representatives of closely related species of the *P. pertucinogena* group is provided in Table S1 [13,18,20,21,31,37,38]. All bacteria were cultured at 30 °C on Luria-Bertani medium (LB) for 24–48 h.

DNA extraction, PCR amplification, DNA sequencing conditions

The DNA extraction, PCR amplification, primers used, purification of the amplified products and DNA sequencing conditions, as well as the sequence analysis procedures, were previously described [28].

Primer sequences corresponding to enterobacterial repetitive intergenic consensus (ERIC) elements (ERIC 1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and ERIC2: 5′-AAGTAAGTGACTGGGGTGAGCG-3′) [35] and BOX elements (BoxA1R: 5′-CTACGGCAAGGCGACGCTGACG-3′) [17] were used for DNA fingerprinting.

Phylogenetic analysis

Individual trees based on the partial sequences of the 16S rRNA (1300 nucleotides) and *gyrB* (801 nucleotides) genes have been included in the analysis, together with the *rpoD* (737 nucleotides) gene sequence. An analysis of these three concatenated gene sequences was also performed with a total of 2838 nt [25]. An update (until 2018) of the type strains of all species in the *P. pertucinogena* phylogenetic group, as defined by Mulet and collaborators [27], was included and compared in the present study (Table S1). The Jukes–Cantor (JC) [14], maximum likelihood (ML) [9] and maximum parsimony (MP) [22] algorithms were used for the comparisons.

Genome sequencing and analysis

Genomic DNA was isolated from strain V113^T, using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer instructions. The Illumina HiSeq 2000 obtained paired-end library reads were *de novo* assembled using the Newbler Assembler v2.7 program (Roche). The draft genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). The Whole Genome Shotgun project for strain V113^T has been deposited in DDBJ/ENA/GenBank under the accession number LMAZ00000000. The version described in this paper is the first version, LMAZ01000000. Analysis and comparison of the functional annotation was done using the Kyoto Encyclopedia of genes and Genomes (KEGG Automatic Annotation Server [KAAS]) [24]. The presence of plasmids has been assessed manually and also with the Plasmid Finder *in silico* web tool [7].

The relatedness of the novel species *Pseudomonas gallaeciensis* V113^T genome to whole genome shotgun sequences of all species type strains in the *P. pertucinogena* G available in public databases was determined based on the tetranucleotide frequency correlation coefficients (TETRA), average nucleotide identity (ANI) using the BLASTN algorithm (ANIb), and the MUMMER ultra-rapid aligning tool (ANIm) as well as genome-to-genome distance (GGDC) methods. ANIb and ANIm were calculated using the JSpecies software tool available at the webpage http://www.imedea.uib.es/jspecies. The recommended species cut-off was 95% for the ANIb and ANIm indices [32]. GGDC was calculated using the web service http://ggdc.dsmz.de [23] and using the recommended BLAST method. The GGDC results shown are based on the recommended formula 2 which is independent of the genome length and is thus robust against the use of incomplete draft genomes.

Cell morphology and physiological tests

Cell size, morphology and flagellar insertion were determined by transmission electron microscopy of cells from the exponential growth phase in LB. A Hitachi model H600 electron microscope was used at 75 kV. The samples were negatively stained with phosphotungstic acid (1%, pH 7.0) as previously described [19].

The production of fluorescent pigments was tested on King B medium (*Pseudomonas* agar F, Difco), and pyocyanin production was tested on King A medium (*Pseudomonas* agar P, Difco). The strains V113^T, V92 and V120 were characterized phenotypically using API 20 NE strips (bioMérieux), Biolog GN2 and GENIII MicroPlates (Biolog, Hayward, CA). Growth temperatures (4, 6, 10, 15, 18, 25, 30, 37 and 42 °C) were determined in LB medium and growth in the presence of NaCl (0–15% w/v) and pH ranges (4–11) were observed in Nutrient Broth (Merck).

Chemotaxonomic analysis

The chemotaxonomic data obtained with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) for strains V113^T, V92 and V120 and their closely related species type strains were obtained at the Scientific-Technical Services (University of Balearic Islands, Spain) and analysed as previously described [34].

Whole cell fatty acid methyl esters (FAME) analysis was performed at the Spanish Type Culture Collection, CECT, Valencia, Spain (http://cect.org/identificaciones) under high standardized conditions. Fatty acids were extracted, prepared and analysed as described in Ref. [34].

Results and discussion

Phylogenetic analysis

A preliminary analysis of the partial sequences of the *rpoD* gene compared among all of the *Pseudomonas* type strains revealed that strains V113^T, V92 and V120 could be representatives of a new species [26]. A complete phylogenetic analysis has now been accomplished.

In all individual and concatenated gene sequence trees studied, strains V113^T, V92 and V120 were located in the same branch, independent from the other type strains in the *P. pertucinogena* group, with *Pseudomonas pachastrellae* CCUG 46540^T being the closest type strain to the group (Figs. 1 and 2). High bootstrap values supported the following JC tree branches: concatenated and *rpoD* gene Trees 100%, 16S rRNA gene Tree 90%, and *gyrB* gene Tree

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