Pseudomonas brassicacearum subsp. *neaurantiaca* subsp. nov., orange-pigmented bacteria isolated from soil and the rhizosphere of agricultural plants

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A large group of 38 strains of saprophytic bacteria was isolated from soil and the rhizosphere of agricultural plants. The novel organisms were Gram-negative, aerobic, rod-shaped bacteria that produced a green fluorescent pigment, a red–orange diffusible pigment and a complex mixture of phloroglucinol derivatives with antimicrobial activity. The latter have not been found in other bacteria, but are peculiar to ferns. The bacteria were vigorous denitrifiers that synthesized levan from sucrose and liquefied gelatin, but were found not to degrade aesculin, starch, agar, Tween 80 or DNA. Bacterial growth was found to occur at 4 °C but not at 40 °C. The predominant cellular fatty acids were 16 : 0, 16 : 1(n-7), 18 : 1(n-7) and 17 : 0 cyclo. The G + C content of the novel bacteria was 61.0–62.9 mol%. 16S rRNA gene sequence analysis indicated that the representative strain CIP 109457^T had a clear affiliation with *Pseudomonas sensu stricto* groups, with the nearest relatives being *Pseudomonas brassicacearum*, *P. thivervalensis*, *P. corrugata*, *P. mediterranea* and *P. kilonensis*. DNA–DNA hybridization experiments showed that the group of isolated strains exhibited high levels of genetic relatedness (81–100 %), confirming that they are representatives of the same species. At the same time, they bound at low levels (4–46 %) with DNA of the type strains of their nearest relatives with the exception of *P. brassicacearum*; DNA binding of 90 % with the DNA of *P. brassicacearum* CIP 107059^T suggested that the bacteria studied belong to this species. Analysis of taxonomic data indicated that the group of novel bacteria maintain a distinct phenotypic profile, allowing the description of novel subspecies within *P. brassicacearum*, for which the following names are proposed: *Pseudomonas brassicacearum* subsp. *brassicacearum* subsp. nov. (type strain DBK11^T = CFBP 11706^T = CIP 107059^T = DSM 13227^T = JCM 11938^T) and *Pseudomonas brassicacearum* subsp. *neaurantiaca* subsp. nov., with the type strain CIP 109457^T (=ATCC 49054^T = IMV 387^T = VKM B-1524^T).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CIP 109457^T is EU391388.

Details of strain isolation and DNA–DNA hybridization results are available as supplementary material with the online version of this paper.

Over the last few decades, the species of the genus *Pseudomonas* have undergone several reclassifications (Palleroni, 1984, 2005). Currently, the genus *Pseudomonas sensu stricto* consists of several intrageneric

clusters, including the *Pseudomonas syringae*, *P. chlororaphis*, *P. fluorescens*, *P. putida*, *P. stutzeri* and *P. aeruginosa* groups. Nonetheless, different hierarchical arrangements among the species of *Pseudomonas sensu stricto* have been reported repeatedly, indicating continuing difficulties associated with the taxonomic affiliation of pseudomonads (Moore *et al.*, 1996; Anzai *et al.*, 2000; Yamamoto *et al.*, 2000; Ait Tayeb *et al.*, 2005).

During a taxonomic investigation of *Pseudomonas*-like bacteria that produce antibacterial compounds (Kiprianova *et al.*, 1985), we isolated a large group of orange-pigmented bacteria from soil and the rhizosphere of agricultural plants that were found to be phenotypically similar to *Pseudomonas aurantiaca*. The latter species was originally described by Nakhimovskaya in 1948. Concurrently, it was shown that the DNA of the type strain of this species bound at high levels (82 %) with DNA of the type strain of *Pseudomonas aureofaciens* (Kiprianova *et al.*, 1985). Recently, the taxonomic status of these species was updated to create three subspecies within *P. chlororaphis*: *P. chlororaphis* subsp. *chlororaphis*, *P. chlororaphis* subsp. *aureofaciens* and *P. chlororaphis* subsp. *aurantiaca* (Peix *et al.*, 2007). The taxonomic status of our group of soil bacteria remained unclear. This study was aimed at clarifying the taxonomic affiliation of these bacteria. It was revealed that they differed genetically and phylogenetically from the subspecies of *P. chlororaphis* and other phylogenetic relatives such as *Pseudomonas thivervalensis*, *P. corrugata*, *P. mediterranea* and *P. kilonensis*, and that they are close to *Pseudomonas brassicacearum* on a genomospecies level. The characteristic phenotypic profile of the group of orange-pigmented bacteria allows them to be distinguished readily from close relatives including *P. brassicacearum*, thus justifying the proposal of a novel subspecies within *P. brassicacearum*.

A total of 38 strains of *Pseudomonas* were isolated from different sources in Kiev and western Ukraine in the period 1971–1989 (Supplementary Table S1, available in IJSEM Online) as described previously (Kiprianova *et al.*, 1985; Smirnov & Kiprianova, 1990). All strains were maintained on trypticase soy agar (TSA) plates and stored at –80 °C in a medium containing 4 % (w/v) tryptic soy broth (TSB; pH 7.2) and 20 % (v/v) glycerol.

Cell morphology was determined after growth for 24 h on TSA (Oxoid) at 28 °C. The motility of bacteria was determined by examining 18 h cultures in TSB. The ability of the bacteria to produce pigments was tested on King A and B medium (King *et al.*, 1954). The physiological and biochemical properties of the isolated bacteria were also examined. These included their glucose metabolism (Hugh & Leifson, 1953), catalase and oxidase activity, ability to denitrify and liquefy gelatin, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities, the ability to secrete extracellular hydrolytic enzymes (proteases, lecithinases, lipases, amylases) and the ability to produce levan from sucrose and to oxidize gluconate to 2-

ketogluconate. Unless indicated otherwise, all physiological and biochemical tests were carried out according to the methods described by Stanier *et al.* (1966).

The 38 strains isolated from soil and rhizosphere of plants exhibited all of the phenotypic characteristics of *Pseudomonas sensu stricto*; their characteristics are shown in the species description and Table 1. The bacteria were found to utilize a wide spectrum of organic compounds as sources of carbon and energy to enable growth. API ZYM tests were performed only for the type strain.

Most of the strains (about 90 %) grown on King A medium were found to produce an extracellular water- and alcohol-soluble red–orange pigment with λ_{max} 280 and 510 nm. A complex mixture of antimicrobial phloroglucinol derivatives was also synthesized concurrently with pigment production. The main component of this complex was found to be the antibacterial and antifungal agent 2,4-diacetylphloroglucinol. Minor components were identified as triacetylphloroglucinol, phloroacetophenone and biologically active phloroglucide di-(2,4-diacetylphloroglucinol) methane (Kiprianova *et al.*, 1985; Smirnov & Kiprianova, 1990). Compounds similar to the latter component have not been found in other bacteria, but are peculiar to ferns and are used as anthelmintics. All the compounds listed above were produced by all freshly isolated bacteria. However, the ability of these bacteria to produce antimicrobial compounds reduced significantly during prolonged preservation in the laboratory.

Analysis of fatty acid methyl ethers was performed by GLC as described previously (Svetashev *et al.*, 1995). The fatty acid profiles of five representative strains of the group were obtained. The bacteria were similar to other representatives of the genus *Pseudomonas sensu stricto* with regard to their fatty acid composition. Fatty acids with an even number of carbon atoms dominated: 16:0 (36.7 ± 6 %), 16:1(n-7) (32.4 ± 3 %) and 18:1(n-7) (16.9 ± 3 %). Cells also contained 17:0 cyclo (9.5 % ± 2 %). Among the minor components, 14:0, 15:0, 18:0 and 19:0 cyclo were found.

For 16S rRNA gene sequencing, genomic DNA was obtained following lysis of bacterial cells by suspension in 200 µl water and boiling for 10 min at 100 °C. After centrifugation, an aliquot (5 µl) of the supernatant was used for PCR amplification of the 16S rRNA gene with primers A (5'-AGAGTTTGATCATGGCTCAG-3') and H (5'-AAGGAGGTGATCCAACCGCA-3') (Brosius *et al.*, 1978). The PCR conditions were as follows: after an initial denaturation step at 94 °C for 4 min, the reaction mixture was run through 25 cycles of denaturation at 94 °C for 1 min, an annealing step at 57 °C for 1 min and an extension step at 72 °C for 2 min. The amplification primers used in this study gave a 1.5 kb PCR product. PCR products were purified, using polyacrylamide P-100 gel (Bio-Gel P-100; Bio-Rad), by 96-well plate filtration (Millipore). Sequencing reactions were performed using an ABI PRISM BigDye Terminator cycle sequencing ready

Table 1. Characteristics that differentiate the novel strains from *P. brassicacearum* and some other phylogenetically related species

Taxa: 1, *P. brassicacearum* subsp. *neaurantiaca* subsp. nov.; 2, *P. brassicacearum* subsp. *brassicacearum* subsp. nov.; 3, *P. thivervalensis*; 4, *P. corrugata*; 5, *P. mediterranea*; 6, *P. kilonensis*. Data from this study and from Achouak *et al.* (2000), Sikorski *et al.* (2001) and Catara *et al.* (2002). Numbers in parentheses represent the number of novel strains that gave a positive reaction. ND, No data available; v, variable reaction.

Characteristic	1	2	3	4	5	6
Colony texture	Smooth	Mucoid	Mucoid	Wrinkled or smooth	Wrinkled or smooth	Smooth
Green fluorescent pigment	+	+	+	—	—	+
Other pigments	Orange pigment (aromatic), phloroglucinol derivatives	Brown–orange pigment	Brown–orange pigment	Yellow to brown pigment in some strains	Yellow to brown pigment in some strains	Dark-brown pigment after 2–3 weeks of incubation
Levan production	+	+	+	—	—	ND
Reduction of nitrate to nitrite	+	v	v	+	+	—
Denitrification	+	—	—	—	—	—
Arginine dihydrolase	+	+	+	v	v	—
DNase	—	+	+	—	—	ND
Assimilation of:						
D-Sorbitol	+	+	+	—	—	—
D-Mannitol	+	—	—	+	+	+
<i>meso</i> -Tartrate	—	—	—	—	+	—
L-Histidine	+	—	—	—	—	—
Benzoate	+	+	—	—	—	—
Ethanol	+	ND	ND	ND	ND	ND
D-Fucose	—	—	—	—	—	+

reaction kit and run on a 3700 Genetic Analyzer (Applied Biosystems) with eight sequencing primers.

The 16S rRNA gene sequence of a representative strain, CIP 109457^T, was compared by BLAST against a database of cultured species only (<http://bioinfo.unice.fr/blast>) and a database of type strains (EzTaxon; Chun *et al.*, 2007) (<http://147.47.212.35:8080>) in order to retrieve the 50 most similar sequences, that were then aligned with Clustal2 (Larkin *et al.*, 2007). Alignments were then checked manually with SeaView (Galtier *et al.*, 1996) and domains common to all sequences were used to derive a first phylogenetic tree using SeaView (new version). This tree allowed the new strains to be identified as strains of *Pseudomonas*. The sequences of all *Pseudomonas* type strains were then used for further studies. Alignments were checked again and a full phylogenetic analysis was undertaken using the BIONJ and maximum-likelihood algorithms, excluding positions containing indels and unknown nucleotides. For the neighbour-joining analysis, a distance matrix was calculated using Kimura's two-parameter correction. Bootstrap analysis was performed using 1000 replications. BIONJ was carried out according to Gascuel (1997) and maximum-likelihood was determined using PhyML (Guindon & Gascuel, 2003). Conflicting phylogenies in many parts of the tree and low bootstrap values confirmed the difficulty of resolving a phylogeny within the genus *Pseudomonas* using 16S rRNA gene

sequences (data not shown). Phylogenetic trees were drawn using TreeDyn (Chevenet *et al.*, 2006) and SeaView.

16S rRNA gene sequence analysis revealed that CIP 109457^T is a member of the *Gammaproteobacteria*, since its sequence grouped within the saprophytic, fluorescent pseudomonads (Fig. 1), and indicated clearly that this organism should be included in the genus *Pseudomonas*. The closest phylogenetic neighbour (99.2–99.5% 16S rRNA gene sequence similarity) of CIP 109457^T was the type strain of *P. brassicacearum*; other close relatives may be *P. thivervalensis*, *P. corrugata*, *P. kilonensis* and *P. mediterranea* (but the close relationships were not supported by all methods and bootstrap analysis; Fig. 1).

DNA from type strains of *Pseudomonas* species initially selected on the basis of their phenotypic similarity was isolated according to the method described by Marmur (1961). DNA from *P. brassicacearum* CIP 107059^T was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). The G+C content of the DNA of the newly isolated strains ranged from 61.0 to 62.9 mol%, as determined by the thermal denaturation method of Marmur & Doty (1962). Since the group of 38 strains exhibited nearly identical phenotypic characteristics, only five representative strains were selected for DNA–DNA hybridization (listed in Supplementary Table S2). The reference strains obtained for DNA–DNA hybridiza-

Fig. 1. Phylogenetic position of *Pseudomonas brassicacearum* subsp. *neaurantiaca* CIP 109457^T within the genus *Pseudomonas* based on 16S rRNA gene sequences. The topology is that of the BIONJ analysis, with branches also found by maximum-likelihood shown by asterisks; numbers indicate percentages of bootstrap replication ($\geq 50\%$). Bar, 0.002 substitutions per nucleotide position.

tion experiments (Supplementary Table S2) were routinely cultured on TSA plates. DNA–DNA hybridization was performed spectrophotometrically and initial renaturation rates were recorded according to the method described by Marmur & Doty (1962) and De Ley *et al.* (1970). The reassociation temperature was 70 °C. The ΔT_m (difference between the T_m of the homoduplex and the heteroduplex) was 3–4 °C. The DNA–DNA relatedness of the strains representing the group ranged from 81 to 100 %, indicating that the strains belong to the same genomospecies. An extended DNA–DNA hybridization study with strain CIP 109457^T and the type strains of the *P. chlororaphis* group, some other phenotypically similar species (mentioned above) including *P. fluorescens* and the closest phylogenetic neighbour *P. brassicacearum* showed that its DNA bound at low levels (4–46 %) with the DNA of all type strains except *P. brassicacearum* (90 %) (Supplementary Table S2). According to the generally accepted criterion for the definition of genomic species of Wayne *et al.* (1987) (i.e. based on DNA–DNA hybridization of 70 % or greater with ΔT_m less than 5 °C), the group of studied strains belong to *P. brassicacearum* (Achouak *et al.*, 2000).

Detailed comparative taxonomic analysis revealed that, although the studied strains are similar to *P. brassicacearum* and *P. thivervalensis* in their ecology and some phenotypic properties, the new isolates maintain a different phenotype. Interestingly, although *P. brassicacearum* and *P. thivervalensis* are genetically and phylogenetically distinct, they appeared very similar phenotypically (Table 1; Achouak *et al.*, 2000). In contrast, the group of studied strains maintain a clearly defined phenotype and can be distinguished easily from their phylogenetic relatives, including *P. brassicacearum*; for example, they produce characteristic pigments and antibiotics, are vigorous denitrifiers, do not have DNase and differ in the spectrum

of organic compounds that they assimilate, particularly alcohols. *P. corrugata* and *P. mediterranea* do not produce fluorescent pigment or levan and *P. kilonensis* does not have arginine dihydrolase. Other differential characteristics of these bacteria are presented in Table 1. This analysis allows us to conclude that the group of studied strains merit the status of a subspecies. Hence, we propose to include the group of studied bacteria in *P. brassicacearum* and establish two novel subspecies within this species, *Pseudomonas brassicacearum* subsp. *brassicacearum* subsp. nov. and *Pseudomonas brassicacearum* subsp. *neaurantiaca* subsp. nov.

Emended description of *Pseudomonas brassicacearum* Achouak *et al.* 2000

In addition to the characteristics reported in the original description by Achouak *et al.* (2000), the following properties are observed. Main non-polar fatty acids are C_{16:0}, C_{16:1}(n-7) and C_{18:1}(n-7), comprising up to 80 % of the total fatty acids. Nitrate reduction, denitrification and DNase production are variable. Assimilation of D-mannitol, L-histidine and ethanol is variable. The DNA G+C content ranges from 60.8 to 62.9 mol%.

Description of *Pseudomonas brassicacearum* subsp. *brassicacearum* subsp. nov.

The description is identical to the original description of *Pseudomonas brassicacearum* reported by Achouak *et al.* (2000). The type strain is DBK11^T (=CFBP 11706^T =CIP 107059^T =DSM 13227^T =JCM 11938^T).

Description of *Pseudomonas brassicacearum* subsp. *neaurantiaca* subsp. nov.

Pseudomonas brassicacearum subsp. *neaurantiaca* (ne.o.au.ran'ti.a'ca. Gr. adj. *neos* new; N.L. fem. adj.

aurantiaca orange-coloured and also a bacterial specific epithet; N.L. fem. adj. *neaurantiaca* new *aurantiaca*, referring to the phenotypic similarity to *Pseudomonas aurantiaca*).

Rod-shaped cells, single, 1.0–1.5 µm long and about 0.3–0.8 µm in diameter. Gram-negative. Motile, with three to five polar flagella. Aerobic. Chemorganotroph with respiratory metabolism. Produces a water-soluble yellow–green fluorescent pigment, a water- and alcohol-soluble red–orange pigment and a complex of antimicrobial phloroglucinol derivatives. The temperature range for growth is 4–37 °C, with optimum growth at 25 °C. No growth at 40 °C. The pH for growth is pH 6.0–10.0, with optimum growth at pH 7.0. Does not accumulate poly-β-hydroxybutyrate as an intracellular reserve product. Positive for arginine dihydrolase; negative for lysine and ornithine decarboxylases. Forms levan from sucrose. Vigorous denitrifier. Does not oxidize gluconate to 2-ketogluconate. Liquefies gelatin and does not hydrolyse starch, agar, aesculin, chitin or DNA. Lipase production (hydrolysis of Tween 80) is variable. Only activities for alkaline phosphatase, esterase (C4), esterase lipase (C8), trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are detected in API ZYM tests. The following compounds are utilized as single sources of carbon and energy: D-glucose, D-xylose, L-arabinose, D-mannose, D-galactose, D-fructose, sucrose, trehalose, gluconate, acetate, propionate, butyrate, valerate, caproate, heptanoate, caprylate, pelargonate, malate, lactate, succinate, fumarate, glutarate, citrate, isocitrate, α-ketoglutarate, pyruvate, *cis*-aconitate, mannitol, sorbitol, inositol, glycerol, 2,3-butylene glycol, ethanol, n-propanol, n-butanol, isobutanol, benzoate, *p*-hydroxybenzoate, quinate, α-alanine, β-alanine, L-serine, L-leucine, L-isoleucine, L-valine, L-lysine, L-arginine, L-aspartate, L-glutamate, L-ornithine, γ-aminobutyrate, L-histidine, L-proline, L-tyrosine, L-phenylalanine, betaine and sarcosine. Strains isolated from oil-saturated soils utilize C₆–C₁₀ hydrocarbons and produce phloroglucinol derivatives when grown in synthetic medium in a C₆–C₁₀ atmosphere with aeration (Smirnov & Kiprianova, 1990). D-Fucose, L-rhamnose, maltose, cellobiose, lactose, starch, inulin, salicin, oxalate, maleate, adipate, pimelate, *meso*-tartrate, glycolate, itaconate, dulcitol, adonitol, mandelate, *o*-hydroxybenzoate, *m*-hydroxybenzoate, phthalate, phenol, phenylacetate, glycine, citrulline, L- and D-tryptophan, anthranilate, *p*-amino-benzoate, creatine, hippurate, pantothenate, acetamide and nicotinate are not utilized. The G+C content of the DNA is 61.7–62.9 mol%.

The type strain is CIP 109457^T (=ATCC 49054^T =IMV 387^T =VKM B-1524^T). Strains have been isolated from soils and the rhizosphere of plants in temperate areas.

Acknowledgements

This study was partly supported by the Australian Research Council (ARC) and in part by grants (State Contracts) from the Federal

Agency for Science and Innovations of the Ministry for Education and Science of the Russian Federation 'KMM' and 'Scientific Schools'.

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