Pseudomonas cedrina subsp. fulgida subsp. nov., a fluorescent bacterium isolated from the phyllosphere of grasses; emended description of Pseudomonas cedrina and description of Pseudomonas cedrina subsp. cedrina subsp. nov.

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The taxonomic position of a group of four strains, isolated from the phyllosphere of grasses, within the species Pseudomonas cedrina was investigated. The isolates formed a separate cluster through ribotyping and MALDI-TOF MS, which could be clearly differentiated from the type strain of P. cedrina. The differences found between the patterns of the type strain of P. cedrina and the novel isolates were more distinct than those between the type strain and recognized species of the genus Pseudomonas, which were phylogenetically related by 16S rRNA gene sequence analysis. Physiological characterization also revealed significant differences between the novel grass isolates and the type strain of P. cedrina. Siderotyping of the pyoverdines revealed identical pyoverdine-isoelectrofocusing patterns for the novel isolates and the type strain of P. cedrina. However, pyoverdine-mediated 59Fe cross uptake studies indicated differences in the siderotype. In contrast, phylogenetic analysis based on 16S rRNA gene sequence analysis and DNA–DNA hybridization studies (reassociation value 76.4 %) supported the affiliation of the novel isolates to the species P. cedrina. As a consequence of these observations, the splitting of the species P. cedrina into two novel subspecies Pseudomonas cedrina subsp. cedrina subsp. nov. (type strain CFML 96-198T = CIP 105541T = DSM 17516T) and Pseudomonas cedrina subsp. fulgida subsp. nov. (type strain P 515/12T = DSM 14938T = LMG 21467T) is proposed.

The genus Pseudomonas is one of the genera harbouring the largest number of species and in which some taxonomic anomalies can be found. A multitude of genomovars and some species that should be considered synonymous have been described because the phenotypic characterizations did not reflect the results of the phylogenetic analyses (Sutra et al., 1997; Gardan et al., 1999; Sikorski et al., 2005). On the other hand, strains or species that should be affiliated to recognized species on the basis of DNA–DNA relatedness values have been investigated, but differences in the phenotypic and molecular data have led to the description of novel subspecies (Peix et al., 2007) or, in some cases, have led to the proposal of separate species (Sikorski et al., 2001).

In the context of studying the diversity of fluorescent pseudomonads associated with the phyllosphere of grasses, several genotypes determined by ribotyping were characterized (Behrendt et al., 2003). One strain group, designated genotype E1 was found to be highly related to Pseudomonas cedrina on the basis of comparisons of the 16S rRNA gene sequence. DNA–DNA hybridization studies revealed a similarity value of 76.4 % (replicate value 78.3 %) indicating
a clear affiliation to the species *P. cedrina* according to taxonomic rules (Wayne *et al.*, 1987). However, when compared to the strains of *P. cedrina* (originally published as *Pseudomonas cedrella* but corrected on validation) isolated from Lebanese spring water by Dabbousi *et al.* (1998, 1999), the isolates from grass showed differences in the phenotypic features that are normally used to distinguish species. For this reason, an extensive comparison of the novel grass isolates, strains P 515/12, P 515/10, P 517/04 and P 530/23, with the type strain of *P. cedrina* was performed to clarify their taxonomic position.

Analysis of 16S rRNA gene sequences of the novel grass isolate P 515/12 and *P. cedrina* CFML 96-198 revealed a close phylogenetic relationship with a sequence similarity value of 99.9% (Behrendt *et al.*, 2003). Ribotyping was performed in order to investigate the strains using a method that has potential for higher taxonomic resolution. As demonstrated by several studies (Gardan *et al.*, 1999; Sikorski *et al.*, 2001; Behrendt *et al.*, 2003; Clark *et al.*, 2006; Behrendt *et al.*, 2007), ribotyping is a highly discriminative technique that is effective in differentiating strains inter- and intra-specifically within the genus *Pseudomonas* depending on the restriction enzymes used. The grass isolates and the type strains of related species that formed an internal cluster through the analysis of 16S rRNA gene sequences (Behrendt *et al.*, 2003) were studied by ribotyping with the restriction enzyme EcoRI. The analysis was performed with an automated RiboPrinter microbial characterization system (Qualicon Du Pont) as described by Behrendt *et al.* (2008). As already shown by Behrendt *et al.* (2003), the riboprint patterns of the novel grass isolates were highly similar to one another and formed a tight cluster (see Supplementary Fig. S1 in IJSEM online). They could be clearly differentiated from the pattern produced by the *P. cedrina* type strain, which was most similar to that of *Pseudomonas jessenii* DSM 17150. These clear differences obtained through the use of EcoRI, which is generally used to discriminate between *Pseudomonas* species, indicated that the grass isolates formed a separate group within the species *P. cedrina*.

Another powerful fingerprint technique that enables differentiation at the genus, species and sometimes, even strain level is matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Conway *et al.*, 2001; Fenselau & Demirev, 2001; Stackebrandt *et al.*, 2005; Valentine *et al.*, 2005). MALDI-TOF MS analysis of the novel grass isolates and their close phylogenetic relatives as determined by 16S rRNA gene sequence analysis (Behrendt *et al.*, 2003) was performed according to the method of Tóth *et al.* (2008). As shown in Fig. 1, the grass isolates formed a cluster that was separated from the type strain of *P. cedrina* and related *Pseudomonas* species by clear differences in spectral patterns. Comparable to the ribotyping results, the spectra of the type strain of *P. cedrina* were closer to those of representative strains of other *Pseudomonas* species than to those of the group of novel grass isolates. These results supported the assumption that the novel strains formed a separate group within the species *P. cedrina*.

Characterization of pyoverdines by siderotyping has shown that these molecules are efficient taxonomical markers for the classification of fluorescent pseudomonads at the species level (Meyer *et al.*, 2002, 2007; Meyer & Geoffroy, 2004). The siderotypes of the grass isolates were compared with those of the *P. cedrina* type strain. Cultures for pyoverdine production and the electrophoretic characterization of the pyoverdine isofoms that accumulated in the growth media were performed according to Meyer *et al.* (1998), with the exception of the isoelectric pH (pl) values, which were determined using an internal standard made from a mixture of pyoverdines with defined pl values, as described by Fuchs *et al.* (2001). The purification of pyoverdines with the XAD chromatographic procedure and their use in pyoverdine-mediated 59Fe uptake were performed as described previously (Meyer *et al.*, 1998). The novel strains displayed identical pyoverdine-isoelectro-focusing patterns with one isoform band at pl 7.3. In contrast, studies of pyoverdine-mediated iron cross uptake revealed differences in the siderotype (data not shown).

While the type strain of *P. cedrina* was able to use the pyoverdines of the novel grass isolates at a similar efficiency to its own pyoverdine, the grass isolates were unable to uptake the pyoverdine from the type strain. These results indicated that the type strain formed another siderovar with a different pyoverdine from the novel grass isolates, but had a supplementary pyoverdine-Fe receptor that recognized the pyoverdine of the grass isolates. Thus, the results of siderotyping confirmed the grass isolates separate group position in the species *P. cedrina*.

An extensive investigation of the physiological characteristics of the novel isolates and the type strain of *P. cedrina* was carried out using API 20NE test strips (BioMérieux) and Biolog GN MicroPlates (Biolog Inc.) according to the manufacturer’s instructions. Additional physiological and morphological features were determined by methods described previously (Behrendt *et al.*, 1999, 2008). Ice nucleation activity of the novel isolates was tested at −5 and −10 °C according to the method of Hildebrand et al. (1994). As shown in Table 1, the novel isolates displayed high conformity with each other as regards phenotypic features. Using Biolog GN MicroPlates, differences in the intensity of oxidation were noted for only a few carbohydrates. The utilization of L-ornithine differed between the strains. Whilst strain P 515/12 did not use this substrate, the other novel isolates showed a weak reaction. In contrast, comparisons between the novel isolates and the type strain of *P. cedrina* revealed several differences in physiological characteristics (Table 1). Particularly, the different reaction for arginine dihydrolase was remarkable as this feature is generally species-specific and is an important component of the levans, oxidase, potato rot, arginine dihydrolase, tobacco hypersensitivity (LOPAT) test that is used for the primary differentiation of saprophytic and phytopathogenic species of fluorescent pseudomonads (Rudolph *et al.*, 1990). Furthermore, the differences in substrate utilization found between *P. cedrina* and *P. cedrina*...
cedrina and the novel grass isolates with the Biolog GN MicroPlates were considerable and were more significant than the differences previously found between some recognized species of the genus *P. cedrina* (Behrendt et al., 2007).

Thus, the results of the phenotypic characterization performed in this study substantiated the conclusion by Behrendt et al. (2003) that the novel isolates form a separate phenotypic group in the species *P. cedrina*.

In summary, the novel isolates from grass could be clearly discriminated from the type strain of *P. cedrina* by ribotyping, MALDI-TOF MS analysis, siderotyping and physiological characterization, although the phylogenetic analysis of Behrendt et al. (2003) demonstrated an affiliation to the species *P. cedrina*. On this basis, the novel isolates merit a separate taxonomic status at the subspecies level and thus the name *Pseudomonas cedrina* subsp. *fulgida* subsp. nov. is proposed. According to Rule 40b of the Bacteriological Code (Lapage et al., 1992), the description of a novel subspecies necessitates the proposal of the subspecies *Pseudomonas cedrina* subsp. *cedrina* subsp. nov. The description of *P. cedrina* (Dabboussi et al., 1999) is therefore emended. As shown in Supplementary Table S1 (available in IJSEM online), both subspecies of *P. cedrina* can be differentiated from their phylogenetic neighbours by physiological characteristics despite the emendation of the species description.

**Emended description of Pseudomonas cedrina corrig. Dabboussi et al. 2002**

Possesses the following characteristics in addition to those described by Dabboussi et al. (1999). Cells are motile by...
means of one or two polar flagella. All strains are positive for the utilization of N-acetyl-D-glucosamine, L-arabinose, D-arabitol, caprate, citrate, D-fructose, D-galactose, \(\alpha\)-D-glucose, glucionate, D-mannitol, D-mannose, methyl pyruvate, monomethyl succinate, acetic acid, \(cis\)-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-gluconsaminic acid, D-glucuronic acid, \(\alpha\)-hydroxybutyric acid, \(\beta\)-hydroxybutyric acid, \(\alpha\)-ketobutyric acid, \(\alpha\)-ketoglutaric acid, DL-lactic acid, malleate, malonic acid, propionic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinic acid, glucoronamide, D-alanine, L-alanine, L-alanyl glycin, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, hydroxy L-proline, L-leucine, L-proline, L-pyroglycemic acid, L-serine, l-threonine, \(\gamma\)-aminobutyric acid, urocanic acid, inosine, 2-aminoethanol and glycerol. None of the strains uses adipate, butyric acid, itaconic acid, sebacic acid, glycyl L-aspartic acid, N-acetyl-D-glucosamine, L-arabinose, 2,3-butanediol, glucose 1-phosphate or glucose 6-phosphate. Oxidase and catalase reactions are positive whereas DNase, \(\beta\)-galactosidase and urease activities are negative. The strains do not produce indole from tryptophan. Hydrolysis of ascuisolin and tyrosine is negative. All strains are able to grow on cetrimide agar. \(\beta\)-Haemolysis of sheep blood does not occur.

**Description of Pseudomonas cedrina subsp. fulgida subsp. nov.**

*Pseudomonas cedrina* subsp. fulgida (ful’gi.da. L. fem. adj. fulgida flashing, glittering, shining, pertaining to the bright fluorescence).

Cells are Gram-negative, non-spore-forming rods of about 0.8 \(\mu\)m in diameter and 2.5–4.2 \(\mu\)m in length that occur singly. They are motile by one or two polar flagella. The white–yellowish colonies on King B medium are smooth and shiny. They produce a pigment that gives off a light yellow–green fluorescence when irradiated with UV-light. Metabolism is aerobic. Does not hydrolyse H\(_2\)S from sodium thiosulphate are negative. Optimal growth can be observed at 4 °C. Further physiological features are presented in Table 1.

The type strain, P 515/12\(^T\) (≡DSM 14938\(^T\) = LMG 21467\(^T\)), was isolated from the phyllosphere of grasses in Paulinenrae (Germany).

**Description of Pseudomonas cedrina subsp. cedrina subsp. nov.**

*Pseudomonas cedrina* subsp. cedrina (ce’dr.i.na. L. fem. adj. cedrina of a cedar tree, the characteristic tree of Lebanon, the country from which isolates were taken).

The description is essentially as given by Dabboussi et al. (1999). Additional physiological features determined for the type strain are shown in Table 1.

The type strain, CFML 96-198\(^T\) (≡CIP 105541\(^T\) = DSM 17516\(^T\)), was isolated from Lebanese spring waters.

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**References**


