Reclassification of *Pseudomonas aurantiaca* as a synonym of *Pseudomonas chlororaphis* and proposal of three subspecies, *P. chlororaphis* subsp. *chlororaphis* subsp. nov., *P. chlororaphis* subsp. *aureofaciens* subsp. nov., comb. nov. and *P. chlororaphis* subsp. *aurantiaca* subsp. nov., comb. nov.

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Pseudomonas chlororaphis, *Pseudomonas aureofaciens* and *Pseudomonas aurantiaca* were considered as separate species until 1989, when *P. aureofaciens* was proposed as a later heterotypic synonym of *P. chlororaphis* with *P. aurantiaca* remaining as a separate species. Nevertheless, analysis of the almost complete 16S rRNA gene sequences revealed that the type strain of *P. aurantiaca*, NCIMB 10068^T, shows gene sequence similarities close to 99.5 % with respect to *P. chlororaphis* DSM 50083^T and *P. aureofaciens* DSM 6698^T. DNA–DNA hybridization experiments among strains of *P. aurantiaca*, *P. chlororaphis* and *P. aureofaciens* showed values higher than 70 %, confirming that they represent members of the same species. The results of fatty acid analysis and phenotypic traits showed that these strains are closely related, although there are some differences among the strains belonging to *P. aurantiaca*, those from *P. chlororaphis* and those from *P. aureofaciens*. All these results confirm the previous reclassification of *P. aureofaciens* into *P. chlororaphis* and support the reclassification of *P. aurantiaca* as a synonym of *P. chlororaphis*. Phenotypic and molecular data permit the description of three novel subspecies within this last species, for which the following names are proposed: *P. chlororaphis* subsp. *chlororaphis* subsp. nov. [with the type strain DSM 50083^T (= ATCC 9446^T = NCIMB 9392^T)], *P. chlororaphis* subsp. *aureofaciens* subsp. nov., comb. nov. [with the type strain DSM 6698^T (= ATCC 13985^T = NCIMB 9030^T)] and *P. chlororaphis* subsp. *aurantiaca* subsp. nov., comb. nov. [with the type strain NCIMB 10068^T (= ATCC 33663^T = CIP 106718^T)].

Pseudomonas chlororaphis, *Pseudomonas aureofaciens* and *Pseudomonas aurantiaca* were included in the Approved Lists of bacterial names (Skerman *et al.*, 1980) and considered as separate species in the first edition of

Bergey's Manual of Systematic Bacteriology by Palleroni, who included the first two species in rRNA group I and *P. aurantiaca* in group V (Palleroni, 1984). Later, the species *P. aureofaciens* was proposed as a later heterotypic synonym of

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *P. aurantiaca* NCIMB 10068^T and *P. aureofaciens* DSM 6698^T are DQ682655 and AY509898, respectively.

Tables detailing the results of DNA–DNA hybridization studies and fatty acid analyses are available with the online version of this paper.

P. chlororaphis on the basis of DNA relatedness and phenotypic traits (Johnson & Palleroni, 1989). Recently, in the second edition of the *Bergey's Manual of Systematic Bacteriology*, Palleroni (2005) proposed that *P. aureofaciens* and *P. chlororaphis* may be considered as two subspecies of the same species and that *P. aurantiaca* remains a separate species. In 1985, a taxonomic study of *P. aurantiaca* was published outside of the *International Journal of Systematic Bacteriology* by Kiprianova *et al.* (1985) who proposed the selection of a neotype strain for this species. Their conclusion was based on phenotypic data from the type strain received from the VKM collection, which showed different characteristics from those recorded in the literature for *P. aurantiaca*. An analysis of the type strains of *P. aurantiaca* available from other culture collections was not performed, or at least was not recorded in the cited paper, and therefore the decision to designate a neotype strain was not strongly supported and was never officially approved.

The type strain of *P. aurantiaca* available at the NCIMB and used in our study fulfils the characteristics described for *P. aurantiaca* as recorded in the different editions of *Bergey's Manual of Systematic Bacteriology*. The 16S rRNA gene sequence of *P. aurantiaca* NCIMB 10068^T obtained in this study is 100 % similar to that of strain ATCC 33663^T available at GenBank (Anzai *et al.*, 2000). This result is congruent with the fact that the ATCC received the strain from the NCIMB, from where it was also sent to the LMG and the CIP collections. Therefore, the original type strain of *P. aurantiaca* is currently available in several collections and the selection of a neotype strain is thus not necessary.

The 16S rRNA gene sequence of the type strain of *P. aurantiaca* showed a similarity greater than 99 % with respect to those of *P. chlororaphis* DSM 50083^T and *P. aureofaciens* DSM 6698^T as obtained in this study. Moreover, a recent study of *atpD*, *recA* and *carA* gene sequences from *Pseudomonas* species showed that the type strain of *P. aurantiaca* belongs to the same phylogenetic group as *P. chlororaphis* and *P. aureofaciens*, with gene sequence similarities greater than 96 % (Hilario *et al.*, 2004), suggesting that they belong to the same species. Although they show several phenotypic differences that have been pointed out by several authors (Palleroni, 1984, 2005; Kiprianova *et al.*, 1985; Johnson & Palleroni, 1989), the strains belonging to *P. aurantiaca*, *P. chlororaphis* and *P. aureofaciens* are phenotypically close. In this work, we therefore performed a polyphasic study in order to clarify the taxonomic status of *P. aurantiaca* in comparison with strains of *P. chlororaphis* and *P. aureofaciens*. From the results obtained, we conclude that *P. aurantiaca* is a subspecies of *Pseudomonas chlororaphis* and propose the name *Pseudomonas chlororaphis* subsp. *aurantiaca* subsp. nov., comb. nov. The names *P. chlororaphis* subsp. *chlororaphis* subsp. nov. and *P. chlororaphis* subsp. *aureofaciens* subsp. nov., comb. nov. are also proposed. The names of these novel subspecies were originally proposed by Palleroni (2005) but have not been validly published.

The 16S rRNA gene sequences of *P. aurantiaca* ATCC 33663^T and *P. aureofaciens* ATCC 12353^T were obtained by Anzai *et al.* (2000) and deposited in GenBank with accession numbers AB021412 and D84008, respectively. However, both sequences contain several undetermined nucleotides. Other sequences of the same strains held in public databases are not complete and so in this study, the 16S rRNA gene sequences of *P. aurantiaca* NCIMB 10068^T (GenBank accession no. DQ682655) and *P. aureofaciens* DSM 6698^T (AY509898) were determined according to a previously described method (Rivas *et al.*, 2003). The sequences were compared with those deposited in GenBank using the BLASTN programme (Altschul *et al.*, 1990) and were aligned using CLUSTAL_X software (Thompson *et al.*, 1997). Distances were calculated according to Kimura's method (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar *et al.*, 2001) was used for all analyses.

Fig. 1 shows a phylogenetic tree that includes representative species of the genus *Pseudomonas sensu stricto*. The 16S rRNA gene sequence of strain DSM 50083^T showed 99.5 and 99.4 % similarity to those of strains DSM 6698^T and NCIMB 10068^T, respectively. Strains DSM 6698^T and NCIMB 10068^T showed 99.7 % gene sequence similarity to each other. The three strains formed a separate group from other species of the genus *Pseudomonas sensu stricto*.

DNA–DNA hybridization experiments were carried out by using the method of Ezaki *et al.* (1989), following the recommendations of Willems *et al.* (2001). Besides the type strains, several strains of *P. chlororaphis*, *P. aurantiaca* and *P. aureofaciens* were also included (see Supplementary Table S1 in IJSEM Online). The DNA–DNA hybridization results showed that, in agreement with the 16S rRNA gene sequence similarities, the highest hybridization values were found between *P. aureofaciens* DSM 6698^T and *P. aurantiaca* NCIMB 10068^T with a mean similarity of 81 %. *P. chlororaphis* DSM 50083^T displayed 75 and 73 % DNA–DNA hybridization with respect to *P. aurantiaca* NCIMB 10068^T and *P. aureofaciens* DSM 6698^T, respectively. Values ranging between 60 and 87 % were obtained for other strains of the three species analysed (see Supplementary Table S1). Although some of these values are slightly lower than the threshold value of 70 % DNA–DNA similarity recommended for the delineation of species (Wayne *et al.*, 1987), the type strains of these species showed values higher than 75 %.

Fatty acid analyses were performed with cultures grown for 24 h in TSA medium (Merck) at 28 °C as already described (Peix *et al.*, 2003). The main non-polar fatty acids detected were C_{16:0} and those summed in summed feature 3 (C_{16:1ω7c} and C_{15:0} iso 2-OH). The amounts of fatty acid C_{16:0} and summed feature 3 were, respectively, 33.4 % and 34.8 % in strain DSM 50083^T, 26.3 % and 34.8 % in strain DSM 6698^T and 30.3 % and 25.3 % in strain NCIMB 10068^T. The full fatty acid contents of the strains are shown in

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of the subspecies of *Pseudomonas chlororaphis* and other related organisms of the genus *Pseudomonas*. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 5 nucleotide substitutions per 1000 nucleotides.

Supplementary Table S2 in IJSEM Online. Small differences in the amounts of some other fatty acids were found between the three strains. For example, strains DSM 50083^T and DSM 6698^T differ in the amount of C_{12:0}, C_{16:0}, C_{10:0} 3-OH and C_{12:1} 3-OH; strains DSM 50083^T and NCIMB 10068^T differ in C_{10:0} 3-OH, C_{12:1} 3-OH, C_{12:0} 3-OH, C_{17:0} cyclo and summed feature 3, and strains DSM 6698^T and NCIMB 10068^T differ in C_{10:0} 3-OH, C_{12:0} 3-OH, C_{17:0} cyclo and summed feature 3.

The same strains that were included in the DNA–DNA hybridization experiments were also studied phenotypically by using the API 20NE and API 50CH systems as recommended by the manufacturer. The results of the phenotypic characterization concurred with those reported by Johnson & Palleroni (1989), who proposed the reclassification of *P. aureofaciens* as *P. chlororaphis*, and with those reported by Doudoroff & Palleroni (1974) and Palleroni (1984, 2005).

According to these results, strains of *P. aureofaciens* use L-arabinose as a carbon source in both API 20NE and API 50CH tests while those strains belonging to *P. chlororaphis* do not. Nitrate reduction was positive for *P. chlororaphis* strains, negative in strains of *P. aurantiaca* and variable for strains of *P. aureofaciens*. *P. aurantiaca* strains differed from *P. aureofaciens* in the use of 5-ketogluconate and from *P. chlororaphis* strains in the use of both 5-ketogluconate and L-arabinose (Table 1).

The results of fatty acid analysis, phenotypic characterization, 16S rRNA gene sequencing, DNA–DNA relatedness, as well as the results obtained by Hilario *et al.* (2004) on the phylogenetic analysis of several housekeeping genes, support the reclassification of *P. aurantiaca* as a later heterotypic synonym of *P. chlororaphis*. The results also revealed that strains of *P. aurantiaca*, *P. aureofaciens* and *P. chlororaphis* form three clearly distinguishable groups within *P.*

Table 1. Phenotypic differences among the subspecies of *P. chlororaphis*

Data are from Palleroni (1984, 2005), Johnson & Palleroni (1989) and this study. +, Positive; –, negative; V, variable.

	<i>P. chlororaphis</i> subsp. <i>chlororaphis</i>	<i>P. chlororaphis</i> subsp. <i>aureofaciens</i>	<i>P. chlororaphis</i> subsp. <i>aurantiaca</i>
Non-fluorescent pigments:			
Green (chlororaphin)	+	–	+
Orange (phenazine-1-carboxylate)	–	+	+
Denitrification	+	V	–
Assimilation as carbon source:			
L-Arabinose	–	+	+
5-Ketogluconate	+	+	–

chlororaphis that merit the status of subspecies. Therefore, we propose the establishment of three novel subspecies within *P. chlororaphis*; *P. chlororaphis* subsp. *chlororaphis* subsp. nov., *P. chlororaphis* subsp. *aureofaciens* subsp. nov., comb. nov. and *P. chlororaphis* subsp. *aurantiaca* subsp. nov., comb. nov.

Emended description of *Pseudomonas chlororaphis* (ex Guignard and Sauvageau 1894) Bergey et al. 1930^{AL}

Characteristics in addition to those reported in the original description recorded in *Bergey's Manual of Determinative Bacteriology* (Doudoroff & Palleroni, 1974) are as follows. The main non-polar fatty acids detected are C_{16:0} and summed feature 3, comprising around 30 % of total fatty acids, followed by C_{18:1ω7c}, with amounts of approximately 10 %. A more detailed breakdown of fatty acid content is presented in Supplementary Table S2. Negative result in tests for urease, β-galactosidase, indole production and aesculin hydrolysis. Nitrate reduction is variable. *N*-acetylglucosamine, trehalose, raffinose and D-arabitol are used as carbon sources. Assimilation of L-arabinose, phenylacetate and 5-ketogluconate is variable. The use of L-xylose, sorbose, amygdalin, arbutin, salicin, melibiose, melezitose, starch, glycogen, gentiobiose, turanose, lyxose, tagatose, L-fucose, L-arabitol, xylitol, dulcitol, methyl α-D-glucoside, methyl α-D-mannoside and methyl β-D-xyloside is negative. The DNA G + C content ranges from 63.5 to 63.6 mol%.

Description of *Pseudomonas chlororaphis* subsp. *chlororaphis* subsp. nov.

Pseudomonas chlororaphis subsp. *chlororaphis* (chlo.ro.ra'phis. Gr. adj. *chlorus* green; Gr. n. *raphis* a needle; N.L. fem. n. *chlororaphis* a green needle).

Displays characteristics typical for the species *P. chlororaphis* as described above. Chlororaphin, a green insoluble phenazine pigment, is produced. Nitrate reduction is positive. Utilization of L-arabinose is negative. Utilization of 5-ketogluconate is positive. Phylogeny based on 16S rRNA, *atpD*, *recA* and *carA* gene sequences separates this subspecies from the other subspecies of *P. chlororaphis*.

The type strain is DSM 50083^T (= ATCC 9446^T = NCIMB 9392^T).

Description of *Pseudomonas chlororaphis* subsp. *aureofaciens* subsp. nov., comb. nov.

Pseudomonas chlororaphis subsp. *aureofaciens* (au.re.o.fa'ci.ens. L. adj. *aureus* golden; L. part. adj. *faciens* producing; N.L. part. adj. *aureofaciens* golden-producing, referring to the pigment produced).

Displays characteristics typical for the species *P. chlororaphis* as described above. Produces a diffusible yellow–orange phenazine pigment. Nitrate reduction is variable. Utilization of L-arabinose is positive. Utilization of 5-ketogluconate is

positive or weakly positive. Phylogeny based on 16S rRNA, *atpD*, *recA* and *carA* gene sequences separates this subspecies from the other subspecies of *P. chlororaphis*.

The type strain is DSM 6698^T (= ATCC 13985^T = NCIMB 9030^T).

Description of *Pseudomonas chlororaphis* subsp. *aurantiaca* subsp. nov., comb. nov.

Pseudomonas chlororaphis subsp. *aurantiaca* (au.ran.ti.a'ca. N.L. fem. adj. *aurantiaca* orange-coloured).

Displays characteristics typical for the species *P. chlororaphis* as described above. Green and orange pigments are produced. Nitrate reduction is negative. Utilization of L-arabinose is positive. Utilization of 5-ketogluconate is negative. Phylogeny based on 16S rRNA, *atpD*, *recA* and *carA* gene sequences separates this subspecies from the other subspecies of *P. chlororaphis*.

The type strain is NCIMB 10068^T (= ATCC 33663^T = CIP 106718^T).

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