Characterization of '*Pseudomonas azelaica*' DSM 9128, leading to emended descriptions of *Pseudomonas citronellolis* Seubert 1960 (Approved Lists 1980) and *Pseudomonas nitroreducens* Iizuka and Komagata 1964 (Approved Lists 1980), including *Pseudomonas multiresinivorans* as its later heterotypic synonym

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Polyphasic characterization of strain DSM 9128, described as '*Pseudomonas azelaica*' by Janota-Bassalik *et al.* [*Acta Microbiol Pol B* **3**, 143–153 (1971)], and four biochemically similar isolates was performed with the aim of validly publishing the name '*Pseudomonas azelaica*'. Based on 16S rRNA gene sequence analysis, DNA–DNA hybridization, fatty acid patterns and extensive biochemical testing, it was concluded that DSM 9128, two further strains and the type strains of *Pseudomonas nitroreducens* and *Pseudomonas multiresinivorans* form a highly related cluster. However, DNA–DNA binding did not conclusively resolve whether these strains should be regarded as members of one species. Based on results gained with the above-mentioned methods, two other isolates were assigned to the species *Pseudomonas citronellolis*, a species very close to *P. nitroreducens*. Based on genetic and biochemical similarities, it is suggested that *Pseudomonas multiresinivorans* should be considered as a later heterotypic synonym of *Pseudomonas nitroreducens*. The species descriptions of *P. nitroreducens* and *P. citronellolis* are emended.

Janota-Bassalik & Wright (1964) isolated a Gram-negative strain from garden soil when searching for bacteria that utilized azelaic acid as the sole source of carbon and energy. The isolate was described as '*Pseudomonas azelaica*' (Janota-Bassalik *et al.*, 1971), but the name was not included in the Approved Lists of Bacterial Names or validly published afterwards. The aim of this study was to determine whether the species name should be validly published. In previous DSMZ surveys of *Pseudomonas* strains, four strains physiologically very similar to '*P. azelaica*' were noticed and were included in this study, *Pseudomonas* sp. strains Ch1 (=DSM 6426; Blaschke *et al.*, 1991), AT (=DSM 12280), ADP (=DSM 11735; Mandelbaum *et al.*, 1995) and BG6903 (=DSM 18650). All these strains had been isolated with complex aliphatic or alicyclic hydrocarbons as the sole source of carbon and energy. During this study, a close relationship was detected between these strains and the species *Pseudomonas nitroreducens*, *Pseudomonas multiresinivorans* (Mohn *et al.*, 1999) and *Pseudomonas citronellolis*.

Strain BG6903 was isolated and supplied by A. Steinbüchel (University of Münster, Germany). The other strains were obtained from the DSMZ or the ATCC. Cultures were maintained on tryptic soy broth agar (TSBA; Difco, Becton Dickinson). Incubations were carried out at 28 °C.

DNA extraction, PCR amplification of the 16S rRNA gene and determination of the 16S rRNA gene sequence followed described methods (Rainey *et al.*, 1996). The 16S rRNA gene sequences were aligned with published sequences using the ae2 editor (Maidak *et al.*, 1997). Evolutionary distances were calculated by the method of Jukes & Cantor (1969). The sequence accession numbers are listed in Supplementary

Abbreviations: pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *P. nitroreducens* IAM 1439^T and DSM 14399^T, '*P. azelaica*' DSM 9128 and *Pseudomonas* sp. strains AT (=DSM 12280), Ch1 (=DSM 6426), ADP (=DSM 11735) and BG6903 (=DSM 18650) are AM088473–AM088478 and AM088480, respectively.

16S rRNA gene sequence similarities, whole-cell fatty acid compositions and physiological properties of '*P. azelaica*' DSM 9128 and related strains are detailed as supplementary material in IJSEM Online.

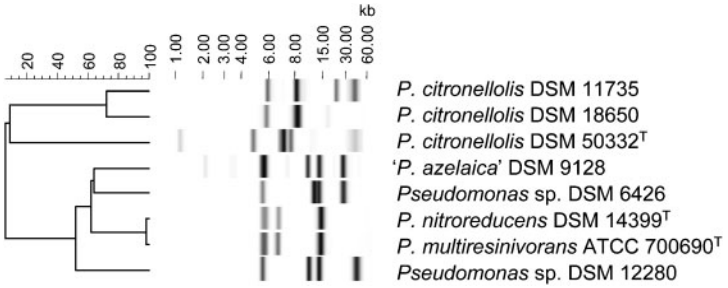

Fig. 1. RiboPrint patterns of strains of *P. citronellolis* and of the *P. nitroreducens*–*P. multiresinivorans*–'*P. azelaica*' group. The dendrogram was constructed using Pearson's correlation coefficient and UPGMA with the software package BioNumerics (Applied Maths).

Table S1 in IJSEM Online. The almost complete 16S rRNA gene sequences (about 97.5 % of the *Escherichia coli* sequence) of '*P. azelaica*' DSM 9128 and four biochemically similar strains were compared with the sequences of all known *Pseudomonas* species available in the EMBL database. Nearest neighbours were *P. multiresinivorans* ATCC 700690^T (99.8 % similarity), *P. nitroreducens* DSM 14399^T (99.5 %) and *P. citronellolis* DSM 50332^T (98.7 %) (Supplementary Table S1). The sequence of *P. nitroreducens* DSM 14399^T generated in this study (GenBank accession no. AM088474) differed from the sequence published for the IAM deposit of this type strain, IAM 1439^T (D84021). For this reason, the 16S rRNA gene of the type culture IAM 1439^T was resequenced (GenBank accession no. AM088473). The resulting sequence was 100 % identical to that of DSM 14399^T but differed from the data deposited as accession no. D84021. On the assumption that the sequence represented by AM088473 is the correct one for the *P. nitroreducens* type strain, it was confirmed that DSM 9128 is closely related to *P. nitroreducens*. Strains DSM 12280, DSM 6426, DSM 11735 and DSM 18650 also clustered within this sub-branch, within which two groups were discernible. One group comprised *P. citronellolis* DSM 50332^T and strains DSM 11735 and DSM 18650. The second group consisted of the type strains of *P. nitroreducens* and *P. multiresinivorans* and strains DSM 9128, DSM 6426 and DSM 12280, and will be referred to here as the *P. nitroreducens*–*P. multiresinivorans*–'*P. azelaica*' group. The similarities within the groups were higher than 99.5 %, whereas intergroup similarities were lower than 99.0 % (Supplementary Table S1). This grouping was in accordance with the similarity of some of the strains by riboprint patterns.

Ribotyping of strains was done with the automated RiboPrinter Microbial Characterization system (DuPont Qualicon). Riboprint analyses using *Eco*RI followed described methods (Allerberger & Fritschel, 1999). Riboprinting resulted in two unrelated patterns and three clusters each consisting of two strains (DSM 18650 and DSM 11375; DSM 6426 and DSM 9128; and *P. nitroreducens* DSM 14399^T and *P. multiresinivorans* ATCC 700690^T) (Fig. 1).

DNA–DNA hybridization was carried out at 69 °C as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983), using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992). The percentages of binding of '*P. azelaica*' DSM 9128 with *P. citronellolis* DSM 50322^T and *P. multiresinivorans* ATCC 700690^T were respectively 50 and 57 %. DNA–DNA relatedness of DSM 9128 with *P. nitroreducens* DSM 14399^T was 83 % (Table 1). The DNA–DNA relatedness values of DSM 9128 with DSM 6426, DSM 12280 and DSM 11735 were respectively 65, 65 and 54 %. The relatedness values of strain DSM 18650 and DSM 11735 with *P. citronellolis* DSM 50332^T were 87 and 76 %. The highest percentage of binding (88 %) was found between *P. nitroreducens* DSM 14399^T and *P. multiresinivorans* ATCC 700690^T. Following the 70 %

Table 1. DNA–DNA relatedness between '*P. azelaica*' DSM 9128, the type strains of *P. nitroreducens*, *P. multiresinivorans* and *P. citronellolis* and '*P. azelaica*'-like strains

Values are percentages of DNA–DNA binding at 69 °C. –, Not done.

Strain	DSM 18650	DSM 11735	'*P. azelaica*' DSM 9128	*P. nitroreducens* DSM 14399^T	DSM 6426
P. citronellolis DSM 50332^T	87	76	50	–	–
P. nitroreducens DSM 14399^T	–	–	83	–	–
P. multiresinivorans ATCC 700690^T	–	–	57	88	–
DSM 6426	–	54	65	–	–
DSM 12280	–	48	65	–	60
DSM 11735	–	–	54	–	–

value as the threshold to indicate the membership of two strains in one species (Wayne *et al.*, 1987), the DNA–DNA hybridization results suggested that strains DSM 11735 and DSM 18650 belong to the species *P. citronellolis*.

The G+C content of the DNA of '*P. azelaica*', *P. nitroreducens*, *P. citronellolis* and *P. multiresinivorans* has not been determined previously. The G+C contents, determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989), of '*P. azelaica*' DSM 9128, *P. multiresinivorans* ATCC 700690^T and strain DSM 18650 were respectively 65.8, 64.5 and 66.4 mol%.

For analysis of fatty acids, cells were grown on TSBA for 24 h. Fatty acid methyl esters were obtained as described previously (Kämpfer & Kroppenstedt, 1996) and separated by gas chromatography (model 5898A; Hewlett Packard). Peaks were computed automatically using the Microbial Identification standard software package (Sasser, 1990). Strains DSM 9128, DSM 6426, *P. nitroreducens* DSM 14399^T, *P. multiresinivorans* ATCC 700690^T, *P. citronellolis* DSM 50332^T and DSM 11735 demonstrated fatty acid patterns typical of the *Pseudomonas aeruginosa* group; the octadecenoic acid content was greater than 30 % (Vancanneyt *et al.*, 1996) (Supplementary Table S2).

For morphological and physiological characterizations, standard methods were used. Utilization of carbon sources was tested in a mineral medium (Stanier *et al.*, 1966) with 2 g of the respective carbon source per litre. The tubes were read after incubation for up to 21 days. API 50CH galleries (bioMérieux) were inoculated with bacteria suspended in AUX medium for testing the utilization of substrates. API 20NE galleries were read after 1 and 2 days and API 50CH after 1, 2 and 5 days of incubation at 28 °C. Reduction of the tetrazolium salt indicator in Biolog GN plates (Oxoid) was read after 24 and 48 h of incubation. The results obtained after 48 h were chosen for analysis. Cavities showing a photometric value above 300 or of 150–300 were scored as positive or weak, respectively. Growth at different temperatures was tested in tryptic soy broth (Becton Dickinson). All strains preferentially metabolized organic acids, short-chain alcohols, isoprenoids and amino acids, but metabolized carbohydrates and polyalcohols poorly (Supplementary Table S3). Of 48 substrates tested in the API 50 CH galleries, only glycerol, D-glucose, D-fructose and gluconate were utilized as carbon sources. *P. citronellolis* DSM 50332^T, DSM 18650 and DSM 11735 additionally utilized 2-ketogluconate. Substrate utilization tests with differential results for the strains are listed in Table 2. The species *P. citronellolis*, as represented by the three strains DSM 50332^T, DSM 11375 and DSM 18650, may be differentiated biochemically from the *P. nitroreducens*–*P. multiresinivorans*–'*P. azelaica*' group by the ability to utilize malonate, 2-ketogluconate, benzylformate and *o*-aminobenzoate.

DNA–DNA hybridization results suggested that '*P. azelaica*' DSM 9128 may be a member of the species *P. nitroreducens* (DNA–DNA binding of 83 % with DSM 14399^T; Table 1).

Table 2. Differential physiological properties among the strains studied

Strains: 1, *P. nitroreducens* DSM 14399^T; 2, *P. multiresinivorans* ATCC 700690^T; 3, '*P. azelaica*' DSM 9128; 4, DSM 12280; 5, DSM 6426; 6, *P. citronellolis* DSM 50332^T; 7, DSM 18650; 8, DSM 11735. W, Weak reaction; V, delayed reaction (negative after 8 days, positive or weak after 22 days). All results were obtained in this study. Utilization of substrates was tested in mineral medium (Stanier *et al.*, 1966) except adipate, which was tested in API 20NE strips. None of the strains showed growth at 4 °C.

Property	1	2	3	4	5	6	7	8
Colonies after storage	Butyrous	Butyrous	Tough, sticks to agar	Butyrous	Tough	Butyrous	Butyrous	Butyrous
Utilization of:								
L-Serine	−	+	W	W	−	+	+	+
Hydroxy-L-proline	+	+	+	+	+	−	−	+
Malonate	−	−	−	−	−	+	+	+
2-Ketogluconate	−	−	−	−	−	+	+	+
Kynurenate	−	−	+	−	−	+	+	−
Glycolate	−	−	V	+	+	+	+	+
Benzylformate	−	−	−	−	−	+	+	+
Mucate	V	−	+	V	+	−	−	−
o-Aminobenzoate	−	−	−	−	−	+	+	+
Hippurate	−	−	V	−	−	+	+	+
2-Butanol	V	−	V	V	−	−	−	−
Acetamide	−	−	−	−	+	−	−	−
Adipate	+	+	+	+	+	−	+	+
Growth at 41 °C	W	W	−	−	−	+	+	+

As a consequence, '*P. azelaica*' should not be regarded as a distinct species, which rules out the valid publication of the name.

The DNA–DNA binding percentage of 88 % between DSM 14399^T and ATCC 700690^T strongly suggests that the two strains belong to the same species. Taking into account the similarities of the biochemical properties and riboprint patterns of DSM 14399^T and ATCC 700690^T, we suggest that *P. nitroreducens* and *P. multiresinivorans* should be considered as synonyms for the same species. Since the name *Pseudomonas nitroreducens* has priority, *Pseudomonas multiresinivorans* should be regarded as a later heterotypic synonym of *Pseudomonas nitroreducens*.

Considering the high hybridization rates of DSM 14399^T with DSM 9128 and with ATCC 700690^T, we expected that DSM 9128 and ATCC 700690^T would also show binding rates greater than 70 %. However, the percentage of DNA–DNA relatedness was lower, at 57 %. DNA–DNA binding of the other strains of the *P. nitroreducens*–*P. multiresinivorans*–'*P. azelaica*' group also resulted in percentages of 48–65 %, indicating a degree of relatedness somewhat below species membership. High physiological similarity on the one hand and some variability within the group on the other hand leave open the door to two interpretations, the incorporation of these strains into one species or the separation into several species, as soon as the respective arguments are provided. The cluster would be a good example to study the explanatory power of molecular methodologies such as multilocus sequence analysis, which have been suggested recently for the clarification of the relatedness of strains and for the distinction of species (Stackebrandt *et al.*, 2002; Zeigler, 2003; Santos & Ochmann, 2004).

Emended description of *Pseudomonas nitroreducens* Iizuka and Komagata 1964 (Approved Lists 1980)

Cell morphology and colonies are as described by Iizuka & Komagata (1964). Fluorescence may be present in early stages of isolation but was absent in all cultures studied here. Positive reactions for denitrification of nitrate to dinitrogen, arginine dihydrolase and utilization of hydroxyl-L-proline. Grows at 4 °C according to Prakash *et al.* (2007), but no growth was observed at 4 °C in this study; weak or no growth at 41 °C. Negative reactions for ornithine decarboxylase, indole production from tryptophan, hydrolysis of gelatin and aesculin, urease, β-galactosidase and lecithinase. No utilization of malonate, 2-ketogluconate, *o*-aminobenzoate or benzylformate. Utilizes a wide range of organic acids and amino acids but a limited spectrum of carbohydrates (Table 2 and Supplementary Table S3). Main fatty acids are octadecenoic acid (35–37 %), summed feature 3 (including hexadecenoic acid; 21–27 %) and hexadecanoic acid (21–24 %). The hydroxylated fatty acids 3-OH $C_{10:0}$, 2-OH $C_{12:0}$ and 3-OH $C_{12:0}$ are present in amounts of 3–6 %. According to Prakash *et al.* (2007), shows positive reactions for growth in the presence of 5 % NaCl, hydrolysis of Tween 80 and hypoxanthine, urease and acid production from fructose and glycerol and negative reactions for degradation of citronellol and acid production from adonitol, dulcitol, glucose, mannitol, ribose, xylose, galactose and arabinose. According to Stolz *et al.* (2007), the species is positive for assimilation of *N*-acetylgalactosamine, *trans*-aconitate and mesaconate, utilization of fumarate, glutarate, pyruvate and 4-hydroxybenzoate, acid formation from glucose and hydrolysis of bis-*p*-nitrophenyl (pNP) phosphate, pNP phosphorylcholine, L-alanine *p*-nitroanilide (pNA), L-glutamate-3-carboxy pNA and L-proline pNA and negative for assimilation of D-galactose and utilization of acetate and propionate.

The type strain, P-6^T (=DSM 14399^T=IAM 1439^T=CIP 106747^T), was isolated from an oil brine in Japan by enrichment with kerosene and crude oil as the sole source of carbon and energy.

Emended description of *Pseudomonas citronellolis* Seubert 1960 (Approved Lists 1980)

Cell morphology, colony appearance and physiological properties are as described by Seubert (1960). Positive reactions for denitrification of nitrate to dinitrogen and arginine dihydrolase. Besides the substrates described by Seubert (1960), the substrates listed in Table 2 and Supplementary Table S3 are utilized. The ability to utilize malonate, 2-ketogluconate, benzylformate and *o*-aminobenzoate distinguishes the species from the nearest 16S rRNA gene sequence-based neighbours, *P. multiresinivorans* and *P. nitroreducens*. Does not grow at 4 °C, but grows at 41 °C. Main fatty acids are octadecenoic acid (36–38 %), summed feature 3 (including hexadecenoic acid; 21–25 %) and hexadecanoic acid (21–22 %). The hydroxylated fatty acids 3-OH $C_{10:0}$, 2-OH $C_{12:0}$ and 3-OH $C_{12:0}$ are present in amounts of 3–4 %. According to Prakash *et al.* (2007), the species shows positive reactions for growth at 4 °C and in the presence of 5 % NaCl, hydrolysis of aesculin and hypoxanthine and acid production from fructose and glycerol and negative reactions for hydrolysis of Tween 80 and acid production from adonitol, dulcitol, glucose, mannitol, ribose, xylose, galactose and arabinose. According to Stolz *et al.* (2007), the species is positive for assimilation of D-galactose, *trans*-aconitate and mesaconate, utilization of fumarate, glutarate, pyruvate and 4-hydroxybenzoate, acid formation from glucose and hydrolysis of bis-pNP phosphate, pNP phosphorylcholine, L-alanine pNA, L-glutamate-3-carboxy pNA and L-proline pNA and negative for assimilation of *N*-acetylgalactosamine and utilization of acetate and propionate.

The type strain, ATCC 13674^T (=DSM 50332^T=IAM 15129^T=CIP 104381^T), was isolated from soil collected under pine trees with citronellol as the sole source of carbon.

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