Pseudomonas knackmussii sp. nov.

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The taxonomic position of *Pseudomonas* sp. B13^T, isolated as a 3-chlorobenzoate-degrading organism and used for several groundbreaking studies on the enzymology and genetics of the degradative pathway for haloaromatic compounds, was studied in detail. The previously performed physiological studies, the detection of ubiquinone Q-9, the polyamine pattern with putrescine and spermidine as major polyamines, a fatty acid profile with C_{18:1}ω7c, summed feature 3 and C_{16:0} as quantitatively the most important constituents and the 16S rRNA gene sequence demonstrated that *Pseudomonas* sp. B13^T indeed belongs to the genus *Pseudomonas*. The sequence of the *Pseudomonas* sp. B13^T 16S rRNA gene demonstrated a high degree of similarity with that of *Pseudomonas citronellolis* DSM 50332^T (98.9%), *Pseudomonas nitroreducens* DSM 14399^T (98.7%), *Pseudomonas jinjuensis* DSM 16612^T (98.1%) and *Pseudomonas multiresistorans* DSM 17553^T (98.7%). Thus it was shown that strain *Pseudomonas* sp. B13^T can be distinguished from related species by the ability/inability to assimilate *N*-acetylgalactosamine, D-galactose, putrescine, *trans*-aconitate and mesaconate and some differences in the fatty acid profile. The positioning of *Pseudomonas* sp. B13^T as a separate taxon was finally verified by DNA hybridization, which demonstrated less than 45% DNA–DNA similarity between strain *Pseudomonas* sp. B13^T and the reference strains. On the basis of these results, *Pseudomonas* sp. B13^T represents a novel species for which the name *Pseudomonas knackmussii* sp. nov. is proposed. The type strain is B13^T (=DSM 6978^T=LMG 23759^T).

Pseudomonas sp. B13^T was one of the first micro-organisms described that utilizes halogenated aromatic compounds as sole source of carbon and energy. The strain was isolated from a continuous enrichment in a chemostat with 3-chlorobenzoate. The original inoculum of the chemostat was obtained from a sewage treatment plant in Göttingen, Germany (Dorn *et al.*, 1974). The isolate was subsequently used to elaborate the degradative pathway of 3-chlorobenzoate and other halogenated aromatic compounds (e.g. Dorn & Knackmuss, 1978a, b; Reineke & Knackmuss, 1978a, b; Schmidt & Knackmuss, 1980a, b; Kaschabek & Reineke, 1992). Furthermore, *Pseudomonas* sp. B13^T was used for the first experiments that demonstrated the feasibility of *in vivo* and *in vitro* approaches for the construction of organisms with new metabolic capabilities (Reineke & Knackmuss, 1979, 1980; Rubio *et al.*, 1986; Ramos *et al.*, 1986; Rojo *et al.*, 1987). Very recently it was demonstrated that the ability to degrade 3- and 4-chlorocatechol is encoded on a self-transferable DNA element in *Pseudomonas* sp. B13^T (Gaillard *et al.*, 2006). Affiliation of *Pseudomonas* sp. B13^T

to the genus *Pseudomonas sensu stricto* has already been demonstrated by Busse *et al.* (1989) based on analysis of the quinone system and polyamine pattern.

Cells of *Pseudomonas* sp. B13^T are Gram-negative, polarly flagellated, motile short rods. The oxidase and catalase reactions were positive. Based on these results and several physiological tests which were performed in accordance with the study of Stanier *et al.* (1966) on the aerobic pseudomonads (see strain description), it was suggested that the strain belongs to the pseudomonads and was most closely related to *Pseudomonas fluorescens*, although no fluorescent pigments were observed during growth (Dorn *et al.*, 1974).

The 16S rRNA gene sequence of *Pseudomonas* sp. B13^T containing a continuous stretch of 1528 bp has been deposited at the NCBI database under the accession number AF039489 (Wischnak *et al.*, 1998). Sequence searches at the NCBI database demonstrated that *Pseudomonas* sp. B13^T indeed belongs to the genus *Pseudomonas* as it is currently defined and the closest relatives of *Pseudomonas* sp. B13^T were found to be *Pseudomonas citronellolis* DSM 50332^T, *Pseudomonas nitroreducens* DSM 14399^T, *Pseudomonas*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Pseudomonas* sp. B13^T is AF039489.

jinjuensis DSM 16612^T and *Pseudomonas multiresinivorans* DSM 17553^T with 98.1–98.9 % sequence similarity. No other type strain of the genus showed sequence similarity values > 97 % to the 16S rRNA gene sequence from *Pseudomonas* sp. B13^T. This clearly demonstrated that *Pseudomonas* sp. B13^T belongs in the respective subcluster of pseudomonads which is part of the *Pseudomonas aeruginosa* subgroup as defined by 16S rRNA gene sequence comparisons (Anzai *et al.*, 2000). Since results from DNA–DNA hybridizations have demonstrated that *P. multiresinivorans* is a junior homonym of *P. nitroreducens* (E. Lang, unpublished), *P. multiresinivorans* DSM 17553^T was not included in all comparative analyses.

Analysis of the carbon substrate utilization for growth of *Pseudomonas* sp. B13^T, *P. citronellolis* DSM 50332^T, *P. nitroreducens* DSM 14399^T, *P. jinjuensis* DSM 16612^T and *P. multiresinivorans* DSM 17553^T demonstrated that all five strains converted a wide range of organic compounds and clearly resembled each other in their metabolic traits. Nevertheless, the five strains could be distinguished by their ability/inability to assimilate *N*-acetylglactosamine, D-galactose, putrescine, *trans*-aconitate and mesaconate (Table 1).

The G + C content of the genomic DNA of *Pseudomonas* sp. B13^T was determined to be 67.3 ± 0.5 mol%. The quinone system of *Pseudomonas* sp. B13^T was previously shown to consist of the major compound ubiquinone Q-9 and the polyamine pattern to show the major compounds putrescine and spermidine and minor amounts of 1,3-diaminopropane, cadaverine and spermine (Busse *et al.*, 1989). Re-examination of the quinone system by HPLC as described previously (Ventosa *et al.*, 1993; Altenburger *et al.*, 1996) but using an HPLC system consisting of a JASCO PU 2080 Plus Pump and JASCO UV-2075 Plus UV/VIS detector, and the polyamine pattern as described by Busse & Auling (1988) and Busse *et al.* (1997) but employing a JASCO PU 2080 Plus pump, confirmed previous results. The quinone system of *Pseudomonas* sp. B13^T consisted of the major compound Q-9 (94 %) and minor amounts of Q-8. A similar quinone system was detected for *P. citronellolis* DSM 50332^T, *P. nitroreducens* DSM 14399^T and *P. jinjuensis* DSM 16612^T, exhibiting 98, 97 and 98 % Q-9, respectively, and minor amounts of Q-8. The polyamine pattern of *Pseudomonas* sp. B13^T consisted of major amounts of putrescine [94.2 µmol (g dry weight)⁻¹], spermidine [13.5 µmol (g dry weight)⁻¹] and cadaverine [8.8 µmol (g dry weight)⁻¹]. Furthermore, small amounts of 1,3-diaminopropane [1.2 µmol (g dry weight)⁻¹] and spermine [0.3 µmol (g dry weight)⁻¹] were also detected. The reference strains exhibited similar polyamine patterns showing only some remarkable differences in the content of cadaverine. The patterns were as follows [amounts in µmol (g dry weight)⁻¹]: *P. citronellolis* DSM 50332^T: putrescine 95.7, spermidine 12.8, cadaverine 0.8, 1,3-diaminopropane 1.0, spermine 0.9; *P. nitroreducens* DSM 14399^T: putrescine 84.8, spermidine 14.4, cadaverine 10.1, 1,3-diaminopropane 7.9, spermine 1.3; and *P. jinjuensis*

Table 1. Physiological characteristics of the type strains of *Pseudomonas* species related to *Pseudomonas* sp. B13^T

Taxa: 1, *Pseudomonas* sp. B13^T; 2, *P. citronellolis* DSM 50332^T; 3, *P. nitroreducens* DSM 14399^T; 4, *P. multiresinivorans* DSM 17553^T; 5, *P. jinjuensis* DSM 16612^T. +, Positive; –, negative; (+), weakly positive. All strains were positive for: acid formation from D-glucose^{bd}; utilization of D-gluconate^{cd}, D-glucose^{acd}, D-fructose, *cis*-aconitate, adipate, 4-aminobutyrate, azelate, citrate^{acd}, fumarate^a, glutarate, DL-3-hydroxybutyrate, itaconate, DL-lactate^{ac}, L-malate^a, pyruvate^{ab}, 2-oxoglutarate, suberate, L-alanine^{ac}, β-alanine, L-aspartate^c, L-histidine^a, L-leucine^c, L-ornithine, L-proline^{ac}, 4-hydroxybenzoate^a and phenylacetate^c; hydrolysis of bis-*p*NP phosphate, *p*NP phosphorylcholine, L-alanine *p*NA, L-glutamate-3-carboxy-*p*NA and L-proline *p*NA. All strains were negative for: acid formation from sucrose, D-mannitol, dulcitol, salicin, adonitol, inositol, D-sorbitol, L-arabinose, raffinose, L-rhamnose, maltose, D-xylose, trehalose, cellobiose, methyl α-D-glucoside, erythritol, melibiose, D-arabitol and D-mannose^a; utilization of *N*-acetyl-D-glucosamine^c, L-arabinose^{ac}, *p*-arbutin, D-cellobiose^a, D-trehalose, D-xylose^{ab}, adonitol, inositol^a, maltitol, D-mannitol^{ac}, sorbitol, acetate, propionate and L-tryptophan; hydrolysis of *p*NP β-D-galactopyranoside^c, *p*NP β-D-glucuronide, *p*NP α-D-glucopyranoside, *p*NP β-D-glucopyranoside and *p*NP β-D-xylopyranoside. *p*NP, *para*-nitrophenyl; *p*NA, *para*-nitroanilide.

Test	1	2	3	4	5
Assimilation of:					
<i>N</i> -Acetylglactosamine	–	–	+	–	–
D-Galactose	– ^a	+	–	– ^b	–
Putrescine	+	+	+	+	–
<i>trans</i> -Aconitate	–	+	+	+	+
Mesaconate	–	+	+	+	–

a–*d*, Tested using a different method in another study; results were congruent with those of this study. *a*, Dorn *et al.* (1974) with *Pseudomonas* sp. B13^T; *b*, Mohn *et al.* (1999) with *P. multiresinivorans*; *c*, Kwon *et al.* (2003) with *P. jinjuensis*; *d*, Palleroni (2005) with *P. nitroreducens* and *P. citronellolis*.

DSM 16612^T: putrescine 100.5, spermidine 18.5; cadaverine 1.2, 1,3-diaminopropane 0.6, spermine 3.2. The quinone system and polyamine patterns are in excellent agreement with those reported for other species of the genus *Pseudomonas sensu stricto* (Busse & Auling, 1988; Auling *et al.*, 1991).

Polar lipids were analysed as described by Tindall (1990). *Pseudomonas* sp. B13^T (Fig. 1) and the reference strains *P. citronellolis* DSM 50332^T, *P. nitroreducens* DSM 14399^T and *P. jinjuensis* DSM 16612^T (results not shown) exhibited almost identical profiles. Differences in the presence of trace amounts of some unknown lipids may occur but could not be unambiguously identified. The polar lipid profile of *Pseudomonas* sp. B13^T consisted of the major compounds phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol, moderate amounts of four

Fig. 1. Two-dimensional TLC of polar lipids of *Pseudomonas* sp. B13^T. APL1, unknown aminophospholipid; DPG, diphosphatidylglycerol; L1–7, unknown polar lipids; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL1–5, unknown phospholipids.

unknown phospholipids (PL2–5), an unknown aminolipid (APL1) and two unknown polar lipids (L2, 3) and minor to trace amounts of one phospholipid (PL1) and five unknown polar lipids (L1, 4–7). Two highly hydrophobic lipids were also detected (Fig. 1). These polar lipid profiles contain the same major compounds reported for two other species of the genus, *Pseudomonas psychrotolerans* DSM 15758^T and *Pseudomonas oleovorans* DSM 1045^T, and the misnamed strain *Pseudomonas oleovorans* ATCC 29347, but they lack phosphatidylcholine, detected in major to moderate amounts in *P. psychrotolerans* DSM 15758^T and *P. oleovorans* DSM 1045^T (Hauser *et al.*, 2004). Also differences in the presence/absence of minor compounds characteristic for representatives of one or the other group were found. These observations indicate that polar lipid profiles may be useful for characterizing subgroups within the genus *Pseudomonas sensu stricto* and hence their analysis for classification of novel species can be strongly recommended.

The fatty acid profiles of strain *Pseudomonas* sp. B13^T, *P. citronellolis* DSM 50332^T, *P. nitroreducens* DSM 14399^T and *P. jinjuensis* DSM 16612^T were determined as described previously by Kämpfer *et al.* (1997). All four strains had rather similar fatty acid profiles, with C_{18:1}ω7c (29.8–41.8 %), summed feature 3 (16.5–24.8 %) and C_{16:0} (19.4–21.2 %) as quantitatively the most important constituents (Table 2).

DNA–DNA hybridizations were performed between *Pseudomonas* sp. B13^T and the four reference strains as described by Kämpfer *et al.* (2003). The similarity values (mean values of two individual analyses) determined between the genomic DNA of *Pseudomonas* sp. B13^T and that of the reference strains were as follows: *P. citronellolis* DSM 50332^T, 44.5 %; *P. nitroreducens* DSM 14399^T, 35.2 %;

Table 2. Major fatty acid composition (%) of type strains of species of the genus *Pseudomonas*

Strains: 1, *Pseudomonas* sp. B13^T; 2, *P. citronellolis* DSM 50332^T; 3, *P. nitroreducens* DSM 14399^T; 4, *P. jinjuensis* DSM 16612^T. All strains were grown on trypticase soy broth agar at 28 °C for 48 h prior to fatty acid analysis.

Fatty acid	1	2	3	4
Saturated fatty acids				
C _{10:0}		0.7		0.3
C _{12:0}	3.7	3.3	1.5	6.3
C _{14:0}	0.8	0.8	0.4	0.5
C _{15:0}	0.3	0.3		1.0
C _{16:0}	19.4	21.0	21.2	19.6
C _{17:0}				0.8
C _{18:0}	0.3	0.3	0.7	0.4
Unsaturated fatty acids*				
C _{17:1} ω8c				1.2
C _{18:1} ω7c	36.6	35.7	41.8	29.8
Hydroxy fatty acids				
C _{8:0} 3-OH		0.4		
C _{10:0} 3-OH	3.5	6.2	3.8	3.7
C _{12:0} 2-OH	3.5	3.6	5.0	1.5
C _{12:0} 3-OH	4.0	3.5	3.9	3.2
Summed feature 3†	24.8	17.8	16.5	24.5
Cyclopropane acids				
C _{17:0} cyclo	2.7	2.7	1.6	4.7
C _{19:0} cyclo ω8c	0.4	3.2	0.9	2.6

*For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. cis isomers are indicated by the suffix c.

†Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains one or more of the fatty acids C_{16:1}ω7c and C_{15:0} iso 2-OH.

P. jinjuensis DSM 16612^T, 38.7 %; and *P. multiresinivorans* DSM 17553^T, 38.0 %.

Organisms sharing a 16S rRNA gene sequence similarity lower than 97 % are usually regarded as belonging to different bacterial species (Stackebrandt & Goebel, 1994). In the genus *Pseudomonas*, several recently reported *Pseudomonas* species have shown 16S rRNA gene sequence similarities of more than 99 % to other established species (Achouak *et al.*, 2000; Andersen *et al.*, 2000; Hauser *et al.*, 2004; Kwon *et al.*, 2003; Sikorski *et al.*, 2001). This situation also seems to be relevant for the subgroup of pseudomonads analysed in the present study. Thus from the DNA–DNA hybridization studies and the physiological tests it is evident that *Pseudomonas* sp. B13^T represents a distinct, previously undescribed species within the genus *Pseudomonas*. In conclusion, *Pseudomonas* sp. B13^T is phylogenetically and physiologically unique and represents a novel species within

the genus *Pseudomonas*, for which we propose the name *Pseudomonas knackmussii* sp. nov.

Description of *Pseudomonas knackmussii* sp. nov.

Pseudomonas knackmussii (knack.muss'i.i. N.L. masc. gen. n. *knackmussii* of Knackmuss, in honour of Hans-Joachim Knackmuss, who initiated the biochemical studies about the degradation of chlorinated aromatics and other xenobiotic compounds by strain B13^T and various other micro-organisms).

Cells are Gram-negative, polarly flagellated, motile short rods. Oxidase- and catalase-positive. On solid 3-chlorobenzoate-mineral agar the strain forms round, smooth, colourless, opaque colonies of 1–2 mm diameter when grown at 28 °C for 3 days. Growth is observed at 20–41 °C but not at 4 °C. Good growth is found at pH 6.0 and 8.3; there is no growth at pH 5 or pH 9. No fluorescent or phenazine pigments are formed. Nitrate is not reduced under aerobic or anaerobic conditions. No liquefaction of gelatin, degradation of starch or cellulose, or formation of ethanol from acetate is detected. The strain is positive for: acid formation from D-glucose; utilization of D-gluconate, D-glucose, D-fructose, *cis*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, fumarate, glutarate, DL-3-hydroxybutyrate, itaconate, DL-lactate, L-malate, pyruvate, 2-oxoglutarate, suberate, L-alanine, β -alanine, L-aspartate, L-histidine, L-leucine, L-ornithine, L-proline, 4-hydroxybenzoate, phenylacetate and putrescine; hydrolysis of bis-*p*NP phosphate, *p*NP phosphorylcholine, L-alanine *p*NA, L-glutamate-3-carboxy *p*NA and L-proline *p*NA (*p*NP, *para*-nitrophenyl; *p*NA, *para*-nitroanilide). Acids are not produced from sucrose, D-mannitol, dulcitol, salicin, adonitol, inositol, D-sorbitol, L-arabinose, raffinose, L-rhamnose, maltose, D-xylose, trehalose, cellobiose, methyl α -D-glucoside, erythritol, melibiose, D-arabitol, D-mannose, *N*-acetyl-galactosamine, D-galactose, *trans*-aconitate or mesaconate. The following glycosides are not hydrolysed: *p*NP β -D-galactopyranoside, *p*NP β -D-glucuronide, *p*NP α -D-glucopyranoside, *p*NP β -D-glucopyranoside and *p*NP β -D-xylopyranoside. *N*-Acetyl-D-glucosamine, L-arabinose, *p*-arbutin, D-cellobiose, D-trehalose, D-xylose, adonitol, inositol, maltitol, D-mannitol, sorbitol, acetate, propionate and L-tryptophan are not utilized. The quinone system consists predominantly of ubiquinone Q-9. Putrescine and spermidine are the major polyamines. The polar lipid profile consists of the major compounds phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol, moderate amounts of four unknown phospholipids, an unknown aminolipid and two unknown polar lipids, and minor to trace amounts of one phospholipid and five unknown polar lipids. Two highly hydrophobic lipids are also detectable. Fatty acid composition is as follows: C_{18:1} ω 7c (36.6 %), summed feature 3 (24.8 %) and C_{16:0} (19.4 %), C_{12:0} 3-OH (4.0 %), C_{12:0} (3.7 %), C_{12:0} 2-OH (3.5 %), C_{10:0} 3-OH (3.5 %) and C_{17:0} cyclo (2.7 %).

The type strain is B13^T (=DSM 6978^T=LMG 23759^T), isolated from a sewage treatment plant in Göttingen (Germany) after continuous enrichment with 3-chlorobenzoate.

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