Pseudomonas psychrotolerans sp. nov.

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Three yellow-pigmented, Gram-negative, rod-shaped, non-spore-forming bacterial strains, C36^T, C37 and C39, were isolated in the Medical Clinic for Small Animals and Ungulates at the University for Veterinary Medicine in Vienna, Austria. On the basis of 16S rRNA gene sequence similarity, strain C36^T was shown to belong to the genus *Pseudomonas*; *Pseudomonas oleovorans* DSM 1045^T was the nearest relative (99.5 % sequence similarity). Other *Pseudomonas* species shared <97 % sequence similarity with strain C36^T. The presence of Q-9 as the major ubiquinone, the predominance of putrescine and spermidine in its polyamine patterns and its fatty acid profile [i.e. the predominance of C_{16:0}, summed feature 3 (C_{16:1ω7c} and/or 2-OH C_{15:0} iso), C_{18:1ω7c} and the presence of 3-OH C_{10:0}, 3-OH C_{12:0} and 2-OH C_{12:0}] were in agreement with identification of this strain as a member of the genus *Pseudomonas*. Physiological and biochemical characteristics and the results of genomic fingerprinting clearly differentiated strain C36^T from its phylogenetic relative *P. oleovorans* DSM 1045^T. Results from DNA–DNA hybridization showed that strain C36^T represents a species that is distinct from *P. oleovorans* DSM 1045^T. These data demonstrate that strain C36^T represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas psychrotolerans* sp. nov. is proposed. The type strain is C36^T (=LMG 21977^T=DSM 15758^T). Additionally, physiological, biochemical, chemotaxonomic and genomic fingerprints indicate that *P. oleovorans* ATCC 29347 may not be a member of the species *P. oleovorans sensu stricto*.

During hygiene investigations at the Medical Clinic for Small Animals and Ungulates at the University for Veterinary Medicine in Vienna, Austria, numerous micro-organisms were isolated. To identify significant groups, isolates that showed obvious similarities in colony morphology and pigmentation were compared by analysis of their protein patterns using SDS-PAGE (Altenburger *et al.*, 1996). Protein similarity groups that consisted of at least three strains were characterized in more detail. Based on their preliminary classification, selected representatives of these protein similarity groups were identified as members of the yeast genus *Rhodotorula*, the bacterial

genera *Acinetobacter*, *Staphylococcus*, *Bacillus*, *Sphingomonas* and *Pseudomonas*, and coliforms.

Taxonomic characterization of three yellow-pigmented strains (C36^T, C37 and C39), of which the representative strain, C36^T, was preliminarily identified as a member of the genus *Pseudomonas*, is reported.

Strains C36^T and C37 were isolated from water under a dog's cage and strain C39 was isolated from a strip of metal under the treatment table after cultivation on PYE agar (pH 7.2), which contained (l⁻¹): 3.0 g peptone from casein, 3.0 g yeast extract and 15.0 g agar. The isolates were subcultivated on PYE agar at 28 °C for 48 h. *Pseudomonas oleovorans* DSM 1045^T and *P. oleovorans* ATCC 29347 were kindly provided by G. Schroll, Institute of Microbiology and Genetics, University of Vienna, Vienna, Austria.

The 16S rRNA gene was amplified and analysed as described previously (Zlamala *et al.*, 2002). The 16S rRNA gene sequence of strain C36^T was a continuous stretch of 1456 nt. Sequence comparisons (ungapped) by using FASTA3 (Pearson & Lipman, 1988) indicated that the closest

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Abbreviation: ERIC-PCR, enterobacterial repetitive intergenic consensus sequence-based PCR.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Pseudomonas psychrotolerans* C36^T is AJ575816.

A table giving fatty acid contents and figures showing two-dimensional TLC of polar lipids and SDS-PAGE analysis of strains C36^T, C37 and C39 and related strains are available as supplementary material in IJSEM Online.

relative of strain C36^T is *P. oleovorans* DSM 1045^T (99.5 % sequence similarity). Moderate sequence similarities (96.0–96.8 %) were found to other selected species of the genus *Pseudomonas*. Phylogenetic calculations supported the high degree of relatedness between strain C36^T and *P. oleovorans* DSM 1045^T (Fig. 1).

Physiological characterization was done as described previously (Kämpfer *et al.*, 1991). Growth at different temperatures was examined on PYE agar, NaCl tolerance was tested on PYE agar supplemented with NaCl and production of fluorescent pigment was assayed on King's B medium. Growth on TSA, R2A and MacConkey agar was also tested. Growth under microaerobic (95 % N₂, 5 % CO₂, with 1.5–2.0 % remaining O₂) and anaerobic (9–13 % CO₂, <1 % O₂ within 30 min) conditions was examined on PYE agar. Strains C36^T, C37 and C39 showed a high degree of similarity in their physiological characteristics. Out of 102 tests, the three strains reacted identically in 93 tests, which corresponds to 91 % similarity. In contrast, the similarities in reaction profiles between these isolates and their nearest phylogenetic relative, *P. oleovorans* DSM 1045^T, and also *P. oleovorans* ATCC 29347, as well as the similarity between these two strains of *P. oleovorans*, were

Fig. 1. Phylogenetic tree indicating the estimated relationship between strain C36^T (GenBank accession no. AJ575816) and species of the genus *Pseudomonas* that shared highest 16S rRNA gene sequence similarities. Sequences of reference species were obtained from GenBank/EMBL and *Acinetobacter calcoaceticus* ATCC 23055^T was selected as the outgroup. Sequences were aligned by using CLUSTAL X (Thompson *et al.*, 1997) and edited manually to remove ambiguous nucleotides and gaps. The tree was constructed based on a continuous stretch of 1389 nt by using the neighbour-joining method (Saitou & Nei, 1987) included in the PHYLIP package (Felsenstein, 1995). The tree was visualized by using TREEVIEW (Page, 1996). Bar, 10 % sequence dissimilarity.

<80 % (Table 1). The results are listed in Table 1 and in the species description.

Chemotaxonomic analyses were done as described previously: respiratory quinones and polar lipids (Tindall, 1990; Altenburger *et al.*, 1996); polyamines (Busse & Auling, 1988; Busse *et al.*, 1997); and fatty acids (Kämpfer *et al.*, 1997). The results are given in the species description.

The quinone system and polyamine pattern of strains C36^T and C39 were in excellent agreement with the characteristics of the genus *Pseudomonas sensu stricto* (Oyaizu & Komagata, 1983; Busse & Auling, 1988; Auling *et al.*, 1991). The fatty acid profile of strains C36^T, C37 and C39 showed the same major characteristics as those of *P. oleovorans* DSM 1045^T and *P. oleovorans* ATCC 29347 (see Supplementary Table, available in IJSEM Online). However, our isolates differed from *P. oleovorans* strains DSM 1045^T and ATCC 29347 in terms of quantitative differences of certain acids, such as summed feature 3 (C_{16:1}ω7c and/or 2-OH C_{15:0} iso), C_{16:0} and C_{18:1}ω7c. Differentiation between *P. oleovorans* DSM 1045^T and *P. oleovorans* ATCC 29347 was possible by examining differences in the relative amounts of 2-OH C_{12:0}, summed feature 3, C_{16:0} and C_{18:1}ω7c (see Supplementary Table in IJSEM Online).

Polar lipid profiles are relatively conserved characteristics with varying specificity, depending on the taxon. Sometimes, a polar lipid profile contains a genus- or family-specific characteristic, such as sphingoglycolipid in species of the family *Sphingomonadaceae*, but the complete profile is specific for a species or very closely related species (Busse *et al.*, 1999). The polar lipid profiles of strains C36^T (see Supplementary Fig. A in IJSEM Online) and C39 (results not shown) were identical. Their polar lipid profiles shared a high degree of similarity with that of *P. oleovorans* DSM 1045^T. However, strains C36^T and C39 could be distinguished clearly from *P. oleovorans* DSM 1045^T by the presence of two yellow pigment spots in their chromatograms. Differentiation between *P. oleovorans* DSM 1045^T and *P. oleovorans* ATCC 29347 was possible by the absence of phosphatidylcholine in *P. oleovorans* ATCC 29347 (see Supplementary Fig. A in IJSEM Online).

SDS-PAGE was performed with strains C36^T, C37, C39, *P. oleovorans* DSM 1045^T and *P. oleovorans* ATCC 29347 as described previously (Altenburger *et al.*, 1996). Comparisons of the protein patterns demonstrated that strains C36^T, C37 and C39 are almost indistinguishable and thus they can be considered to be members of a single species. No significant similarities were detected between the protein patterns of these three isolates and those of *P. oleovorans* DSM 1045^T and *P. oleovorans* ATCC 29347. However, the protein patterns could be used to distinguish between *P. oleovorans* strains DSM 1045^T and ATCC 29347 (see Supplementary Fig. B in IJSEM Online).

Genomic fingerprints of strains C36^T, C37 and C39 obtained after enterobacterial repetitive intergenic

Table 1. Physiological characteristics of *P. psychrotolerans*, *P. oleovorans* DSM 1045^T and *P. oleovorans* ATCC 29347

Species/strains: 1, *P. psychrotolerans* ($n=3$); 2, *P. oleovorans* DSM 1045^T; 3, *P. oleovorans* ATCC 29347. +, Positive; (+) weakly positive; –, negative; v, variable. Abbreviations: pNP, *para*-nitrophenyl; pNA, *para*-nitroanilide. Test results were read after 72 h incubation at 30 °C. All strains were able to grow under microaerobic, but not anaerobic, conditions. They all grew on R2A, TSA, MacConkey agar, PYE agar supplemented with 1 or 5 % NaCl and at temperatures of 15, 28 and 37 °C. None of the strains was able to grow on PYE agar supplemented with 7.5 or 10 % NaCl. All strains were negative for acid production from lactose, sucrose, dulcitol, salicin, adonitol, raffinose, cellobiose, methyl D-glucoside and erythritol. All strains were negative for hydrolysis of aesculin, pNP- β -D-galactopyranoside, pNP- β -D-glucuronide, pNP- α -D-glucopyranoside, pNP- β -D-glucopyranoside, pNP- β -D-xylopyranoside, pNP-phosphorylcholine and L-glutamate- γ -3-carboxy-pNA. All strains were positive for hydrolysis of L-alanine-pNA. All strains were negative for assimilation of N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, *p*-arbutin, D-cellobiose, α -D-melibiose, sucrose, salicin, adonitol, maltitol, adipate, azelate, L-tryptophan, 3-hydroxybenzoate and suberate. All strains were positive for assimilation of D-glucose, putrescine, fumarate, glutarate, DL-3-hydroxybutyrate, 4-aminobutyrate, DL-lactate, L-malate, oxoglutarate, pyruvate, 4-hydroxybenzoate, L-alanine, L-proline and L-serine.

Characteristic	1	2	3
Yellow pigment	+	–	–
Fluorescent pigment	–	–	+
Growth at:			
4 °C	+	–	–
42 °C	–	+	–
Acid production from:			
Glucose, L-arabinose, D-xylose, melibiose, D-mannose	+	–	+
D-Mannitol, inositol, trehalose, D-arabitol	+	–	–
Sorbitol, rhamnose, maltose	v*	–	–
Hydrolysis of:			
Bis-pNP-phosphate, L-proline-pNA	+	–	–
pNP-Phenyl-phosphonate, 2-deoxy thymidine-5'-pNP-phosphate	v†	–	–
Assimilation of:			
L-Rhamnose	v†	–	–
D-Trehalose, mesaconate	+	+	–
D-Fructose	+	(+)	+
L-Arabinose, D-galactose, D-mannose, D-mannitol, D-xylose, <i>i</i> -inositol, D-sorbitol	+	–	–
Gluconate, D-maltose, D-ribose, <i>cis</i> -aconitate, <i>trans</i> -aconitate, L-aspartate, citrate	+	–	+
Acetate	(+)	(+)	+
Propionate	(+)	–	+
Itaconate	+	(+)	–
β -Alanine	–	(+)	+
L-Histidine, L-leucine	v†	–	+
L-Ornithine, phenylacetate	–	–	+
L-Phenylalanine	–	–	(+)

*The type strain reacts negatively.

†The type strain reacts positively.

consensus sequence-based PCR (ERIC-PCR) (Wieser & Busse, 2000) revealed that they shared at least two of the three major bands, indicating that the three strains are highly related. No similarities were observed between the fingerprints of our isolates and those of *P. oleovorans* DSM 1045^T and *P. oleovorans* ATCC 29347 (Fig. 2). Thus, the ERIC-PCR-generated fingerprints are in agreement with our other findings and indicate that our three isolates are members of a novel, so far unrecognized, single species. DNA–DNA hybridizations between strain C36^T and *P. oleovorans* DSM 1045^T, which were performed according to Ziemke *et al.* (1998) and Kämpfer *et al.* (2003), showed 6 and 8 % (reciprocal values) relatedness. These values

demonstrate unambiguously that the two strains belong to different species.

Results from analysis of the 16S rRNA gene, polyamine patterns, the quinone system, polar lipids and fatty acids indicate that strain C36^T is a member of the genus *Pseudomonas* and that it is related closely to *P. oleovorans* DSM 1045^T.

Strains C36^T, C37 and C39 are strikingly homogeneous in terms of their protein patterns, ERIC-PCR-generated genomic fingerprints and physiological traits and thus they can be considered to be members of a single species.

Fig. 2. ERIC-PCR-generated genomic fingerprinting. Lanes: 1, λ BstEII digest; 2, C39; 3, C37; 4, C36^T; 5, *P. oleovorans* DSM 1045^T; 6, *P. oleovorans* ATCC 29347.

They can be distinguished from the two strains of *P. oleovorans* by genomic fingerprints, protein patterns, fatty acid composition, polar lipid profiles and numerous physiological characteristics. Data from DNA–DNA hybridization between strain C36^T and *P. oleovorans* DSM 1045^T demonstrate that they belong to different species. Based on these results, it is concluded that our isolates represent a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas psychrotolerans* sp. nov. is proposed. The species description is given below.

Based on clearly differing protein patterns after SDS-PAGE, ERIC-PCR-generated fingerprints and significant differences in physiological characteristics, as well as in fatty acid and polar lipid profiles, it is assumed that *P. oleovorans* DSM 1045^T and *P. oleovorans* ATCC 29347 do not belong to a single species. Our assumption is supported by comparison of the 16S rRNA gene sequence of *P. oleovorans* ATCC 29347 (GenBank accession no. AJ249825), which has been published recently (van Beilen *et al.*, 2001). Similarity values demonstrate that it is related closely to the 16S rRNA gene sequence of *Pseudomonas plecoglossicida* FPC 951^T (99.9% sequence similarity) and to sequences of other members of the *Pseudomonas putida* lineage (Moore *et al.*, 1996), including *Pseudomonas monteilii* CIP 104883^T (99.7%), *Pseudomonas mosselii* CIP 105259^T (99.4%) and *P. putida* DSM 291^T (98.9%). In contrast, *P. oleovorans* ATCC 29347 shares only 97.5% 16S rRNA gene sequence similarity with *P. oleovorans* DSM 1045^T. Thus, it is suggested that *P. oleovorans* ATCC 29347 might be a strain of *P. plecoglossicida*, although DNA–DNA

hybridization studies or comparison of genomic fingerprints will be necessary to clarify its taxonomic status.

Description of *Pseudomonas psychrotolerans* sp. nov.

Pseudomonas psychrotolerans (psy.chro.to'ler.ans. Gr. adj. *psychros* cold; L. pres. part. *tolerans* tolerating; N.L. part. adj. *psychrotolerans* cold-tolerating).

Cells are Gram-negative, non-spore-forming rods with slightly exaggerated poles, 0.5–0.7 × 1.5–1.8 µm. Cells generally occur singly, but sometimes in pairs. Oxidase-negative and catalase-positive. Growth occurs under aerobic and microaerobic, but not anaerobic, conditions. Motility is observed by light microscopy. Good growth occurs on PYE agar, TSA, R2A and MacConkey agar, on PYE agar supplemented with 1–5% (w/v) NaCl and at 4–37 °C on PYE agar. No growth occurs at 42 °C. On PYE agar, yellow, irregular, leathery, dry, wrinkled colonies form within 48 h, with a diameter of approximately 1–5 mm. After 3–5 days, colonies can be up to 8 mm in diameter. The quinone system of strain C36^T consists of Q-9 (96%) and Q-8 (4%). Polyamine pattern consists of the major compounds putrescine and spermidine [98.8 and 18.5 µmol (g dry wt)⁻¹, respectively] and minor amounts of spermine and diaminopropane [0.7 and 0.3 µmol (g dry wt)⁻¹, respectively]. Predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine. Additionally, moderate amounts of an unidentified phospholipid and an unknown lipid are present, as well as small amounts of an unidentified aminophospholipid and an unknown lipid. Fatty acid composition of strain C36^T is as follows: C_{18:1}ω7c (49.1%), C_{16:0} (22.6%), summed feature 3 (C_{16:1}ω7c and/or 2-OH C_{15:0} iso; 16.9%), C_{12:0} (4.5%), 3-OH C_{12:0} (3.0%), 3-OH C_{10:0} (2.6%) and 2-OH C_{12:0} (1.8%). Other characteristics are listed in Table 1.

The type strain is C36^T (=LMG 21977^T=DSM 15758^T). Reference strains are C37 and C39. Strains C36^T and C37 were isolated from water under a dog's cage and C39 was isolated from a strip of metal under the treatment table in the Medical Clinic for Small Animals, University for Veterinary Medicine, Vienna, Austria.

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