Pandoraea oxalativorans sp. nov., *Pandoraea faecigallinarum* sp. nov. and *Pandoraea vervacti* sp. nov., isolated from oxalate-enriched culture

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Five isolates, designated TA2, TA4, TA25^T, KOx^T and NS15^T were isolated in previous studies by enrichment in mineral medium with potassium oxalate as the sole carbon source and were characterized using a polyphasic approach. The isolates were Gram-reaction-negative, aerobic, non-spore-forming rods. Phylogenetic analyses based on 16S rRNA and DNA gyrase B subunit (*gyrB*) gene sequences confirmed that the isolates belonged to the genus *Pandoraea* and were most closely related to *Pandoraea sputorum* and *Pandoraea pnomenusa* (97.2–99.7 % 16S rRNA gene sequence similarity). The isolates could be differentiated from their closest relatives on the basis of several phenotypic characteristics. The major cellular fatty acid profiles of the isolates comprised $C_{16:0}$, $C_{18:1}\omega7c$, $C_{17:0}$ cyclo and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH). On the basis of DNA–DNA hybridization studies and phylogenetic analyses, the isolates represent three novel species within the genus *Pandoraea*, for which the names *Pandoraea oxalativorans* sp. nov. (TA25^T =NBRC 106091^T =CCM 7677^T =DSM 23570^T), *Pandoraea faecigallinarum* sp. nov. (KOx^T =NBRC 106092^T =CCM 2766^T =DSM 23572^T) and *Pandoraea vervacti* sp. nov. (NS15^T =NBRC 106088^T =CCM 7667^T =DSM 23571^T) are proposed.

The genus *Pandoraea* was described by Coenye *et al.* (2000) with five named species (*P. apista*, *P. pulmonicola*, *P. pnomenusa*, *P. sputorum* and *P. norimbergensis*) and one unnamed genomospecies. Later, Daneshvar *et al.* (2001) described three additional unnamed genomospecies within the genus *Pandoraea*. Recently, the name *Pandoraea thiooxydans* was proposed by Anandham *et al.* (2010) for a facultatively chemolithoautotrophic, thiosulfate-oxidizing bacterial isolate. At present the genus consists of six species with validly published names and four unnamed genomospecies. Except *P. norimbergensis* (Wittke et al., 1997; Coenye *et al.*, 2000) and *P. thiooxydans*, strains of the genus *Pandoraea* have mainly been isolated from clinical specimens but have also been isolated from the environment, being associated with biodegradation of environmental pollutants (Okeke *et al.*, 2002; Siddique *et al.*, 2003; Ozaki *et al.*, 2007; Jiang *et al.*, 2009; Liz *et al.*, 2009) or being capable of heterotrophic sulfur oxidation (Graff & Stubner, 2003; Anandham *et al.*, 2010).

Several species of aerobic bacteria are known to be able to utilize oxalate as a sole carbon and energy source (oxalotrophic) (Sahin, 2003). A study was conducted with the purpose of isolating pure cultures of novel oxalate-oxidizing bacteria for characterization in order to further work in the field of oxalate-metabolizing bacteria and their taxonomy (Sahin, 2003). Among the strains, TA2, TA4 and TA25^T were isolated from soil litter close to oxalate-producing plants (Tamer & Aragno, 1980; Jenni *et al.* 1988), strain KOx^T was isolated from chicken dung (Chandra & Shethna, 1975) and strain NS15^T was isolated

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Abbreviation: FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strains TA25^T, KOx^T and NS15^T are AB469785, AB510956 and AB510957, and AB553287, AB553290 and AB553292, respectively.

One supplementary figure and two supplementary tables are available with the online version of this paper.

from a soil enrichment culture with 6 g potassium oxalate l^{-1} as the sole source of carbon and energy.

The present report focuses on the physiological properties and phylogenetic position of five oxalotrophic isolates. On the basis of 16S rRNA and *gyrB* gene sequence analyses, DNA–DNA hybridization studies and chemotaxonomic and phenotypic characteristics, three novel species are proposed within the genus *Pandoraea*.

Physiological and biochemical tests were carried out at 28 °C. Conventional biochemical tests were performed according to standard methods (Smibert & Krieg, 1994). API 20 NE, API ZYM (bioMérieux) and Biolog GN MicroPlates were used according to the manufacturer's instructions and reactions were observed for 2 days. Susceptibility to antibiotics was determined using the disc diffusion method with commercial antibiotic-impregnated discs (OXOID) on Mueller–Hinton agar (OXOID). The following antibiotics were tested (μg per disc): streptomycin (10), colistin (10), penicillin (10), erythromycin (15), tetracycline (30) and chloramphenicol (30). Strains were considered susceptible when the inhibition zone was 12 mm or more in diameter. The isolates and reference cultures were grown routinely on Tryptic Soy agar (TSA) at 28 °C. Closely related type strains of species of the genus *Pandoraea* were obtained from the CCM (www.sci.muni.cz/ccm).

The template for 16S rRNA gene amplification was prepared from a single colony by using an InstaGene matrix (Bio-Rad) following the manufacturer's instructions. The 16S rRNA gene was amplified with universal primers 27f and 1492r (Lane, 1991) and PCR products were purified by elution from a 1.5 % agarose gel after electrophoresis. The purified products were sequenced with primers 518f and 800r by using a Big Dye terminator cycle sequencing kit and run on a 3730xl automated DNA sequencing system (Applied BioSystems). Related sequences were retrieved from GenBank for use in phylogenetic analysis and aligned by using the Ribosomal Database Project (RDP-II) Sequence Aligner program (Maidak *et al.*, 2001). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1980), excluding alignment gaps and unidentified base positions. Phylogenetic trees were reconstructed from the distance data by using the neighbour-joining method (Saitou & Nei, 1987). The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985). Pairwise nucleotide sequence similarity values were calculated by using the algorithm of Myers & Miller (1988) using the JPHYDIT program (Jeon *et al.*, 2005). Alignment gaps were not considered in the similarity calculation.

Genomic DNA was prepared as described by Marmur (1961). *gyrB* gene sequences were determined directly from PCR fragments using the method described by Tabacchioni *et al.* (2008). The primers used for *gyrB* amplification were gyr1 (5′-GGCAAGTTCGACCAGAACAGC-3′) and PC9r (5′-CGGATCCATSGTSGTTTCC-3′). The amplified portion corresponded to positions 340–2310 of the *gyrB* gene sequence of *Burkholderia xenovorans* (NC_007951). The resultant ~2.0 kb fragment was sequenced with primers gyr1, PC3, PC4, PC5r and PC8r. DNA sequencing was performed using a BigDye terminator sequencing kit version 1.1 and an ABI 3300 sequencer (Applied Biosystems). Sequences of ~1280 bp were obtained from each strain. *gyrB* gene sequences were aligned by using the CLUSTAL_X program version 2.0 (Larkin *et al.*, 2007). Genetic distances were obtained by using Kimura's two-parameter distance model (Kimura, 1980). Phylogenetic trees were reconstructed by using the neighbour-joining method (Saitou & Nei, 1987).

Fatty acid methyl ester (FAME) analysis was performed according to standardized procedures detailed in the Sherlock Microbial Identification System (version 6.0) operating manual. The physiological age of the biomass was standardized according to the MIDI protocol. Cells were grown on Tryptic Soy Broth agar (TSBA) for 48 h at 28 °C. After incubation, a loopful of well-grown cells was harvested from the third quadrant (or quadrant with most confluent growth) of the streaked plates. FAME peaks were automatically integrated and fatty acid names and percentages were determined using the Microbial Identification standard software package (MIDI; Sasser, 1990).

Well-separated colonies on TSA agar plates (24 h at 30 °C) were used as a template for RiboPrinter analysis. Ribotyping with the restriction enzyme *Eco*RI was performed using a RiboPrinter microbial characterization system (DuPont Qualicon, USA) following the manufacturer's instructions. The ribotype patterns obtained were processed and automatically identified from searches performed in the DuPont Qualicon database DUP 2008 by using RiboExplorer version 2.1.4216.0 operating software (DuPont Qualicon). Cluster analysis of ribotype profiles was performed using BioNumerics version 6.0 software (Applied Maths). A dendrogram was reconstructed by calculating Pearson's correlation coefficients with the unweighted pair group method with arithmetic mean (UPGMA). An optimization value of 0.5 % was automatically determined by the BioNumerics software and allowed for the densitometric curves. The ribopatterns were imported into the BioNumerics software by using the load samples import script obtained from Applied Maths.

Genomic DNA was prepared using the protocol of Marmur (1961). DNA–DNA hybridizations were carried out at an optimal temperature of 45 °C in 2× SSC buffer and 50 % formamide using the fluorometric microplate method (Ezaki *et al.*, 1989). Reciprocal reactions were performed. The DNA–DNA relatedness values reported are the means of a minimum of three hybridizations. The DNA G+C content was determined by using the thermal denaturation (T_m) method according to Owen & Lapage (1976).

The phylogenetic tree based on 16S rRNA gene sequences (Fig. 1) indicated that all five isolates clustered with members of the genus *Pandoraea* and formed a coherent cluster with *Pandoraea sputorum* and *Pandoraea pnomenusa* (99.3–99.7 % sequence similarity). Previous studies

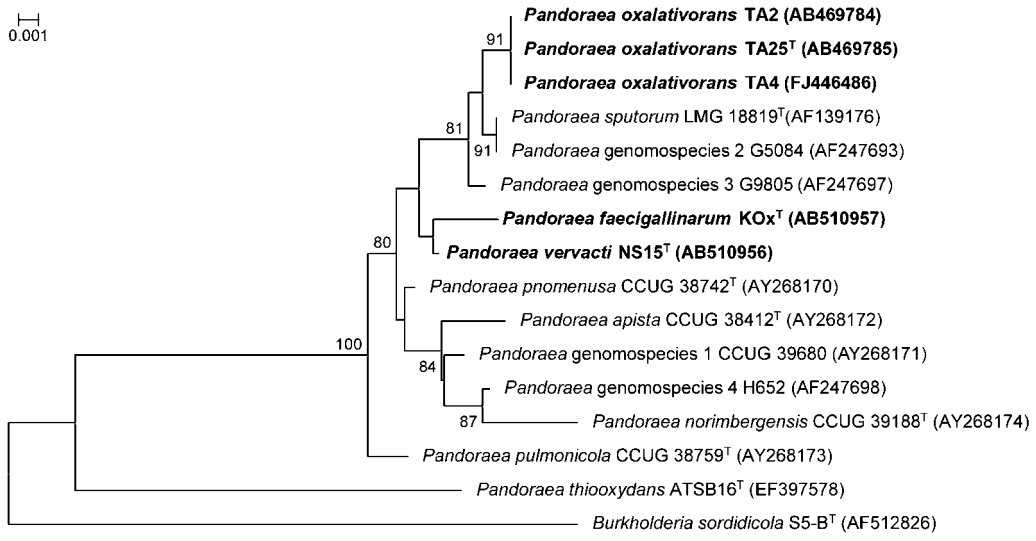

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences constructed after multiple alignment of data (1334 nt) and clustering using the neighbour-joining method. Bootstrap values >50 % (based on 1000 replications) are listed at the branching points. Bar, 0.001 substitutions per nucleotide position.

have indicated that species of the genus *Pandoraea* share high levels of 16S rRNA gene sequence similarity (Coenye *et al.*, 2000; Daneshvar *et al.*, 2001); therefore, the *gyrB* gene was used as an alternative marker and direct sequencing of the *gyrB* gene and phylogenetic analysis using *gyrB* gene sequences was used for the identification of strains at the species level (Coenye & LiPuma, 2002).

The *gyrB* gene sequence-based phylogenetic tree (Fig. 2) displayed a more highly discriminative topology. Strains TA2, TA4 and TA25^T and KOx^T and NS15^T were grouped in two clusters separate from the type strains of *P. sputorum* and *P. pnomenusa* with 90.7–97.3 % sequence similarity (Supplementary Table S1, available in IJSEM Online). The isolates showed *gyrB* gene sequence similarities <96.3 % with other members of the genus *Pandoraea*. The mean value of *gyrB* gene sequence similarities among available strains of the genus *Pandoraea*, including the novel isolates, was 91 %.

The five isolates were aerobic, Gram-reaction-negative motile rods. Colonies were 1–2 mm in diameter, cream, circular and convex with entire edges after 2 days incubation on TSA agar. All of the isolates were susceptible to tetracycline and chloramphenicol but resistant to all other antibiotics tested. Cells were oxidase- and catalase-positive and did not reduce nitrate to nitrite. Biochemical characteristics, carbon source utilization patterns and antibiotic susceptibilities were used to differentiate the isolates from their closest phylogenetic relatives. Isolates TA2, TA4 and TA25^T differed from the type strain of *P. sputorum* in their inability to utilize L-arabinose, cellobiose, D-gluconic acid, D-glucose-6-phosphate, gluconate, citrate or L-arabinose; their ability to utilize Tween 80, α-D-glucose, D-galacturonic acid, glucuronamide, L-proline, L-threonine and glycerol; and their susceptibility to tetracycline. On the other hand, strains KOx^T and NS15^T differed from the type strain of *P. pnomenusa* by their ability to use Tween 80, α-D-glucose, D-glucosaminic acid and glycerol. Other differential phenotypic characteristics between the five isolates and type strains of closely related species of the genus *Pandoraea* are given in Table 1.

The predominant cellular fatty acids in the novel isolates were $C_{16:0}$, $C_{18:1}\omega7c$ and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) (Table 2). The five isolates could be differentiated from the type strains of *P. sputorum* and *P. pnomenusa* by differences in the proportions of fatty acids $C_{16:0}$ and $C_{18:0}$.

Automated ribotyping generated bands ranging from 1.5 to 10 kbp (Supplementary Fig. S1) and separated strains TA02, TA04 and TA25^T into a cluster that was phylogenetically different from closely related species of the genus *Pandoraea*. The majority of bands in this cluster were in range of 5–10 kb with similarity values >90 %, which confirmed that these three isolates belonged to the same species. Isolates NS15^T and KOx^T were each clearly separated from the reference type strains also. Isolate KOx^T lacked bands in the range 5–10 kb. Species of the genus *Pandoraea* appeared to have great heterogeneity in their ribopatterns; as such, all three novel species could be clearly distinguished. Identification with RiboExplorer software did not assign the five novel isolates to any existing species of the genus *Pandoraea*.

To confirm the identification of these isolates as separate species based on the results of the phylogenetic analyses, DNA–DNA hybridization studies were performed, the results of which (Supplementary Table S2) revealed that

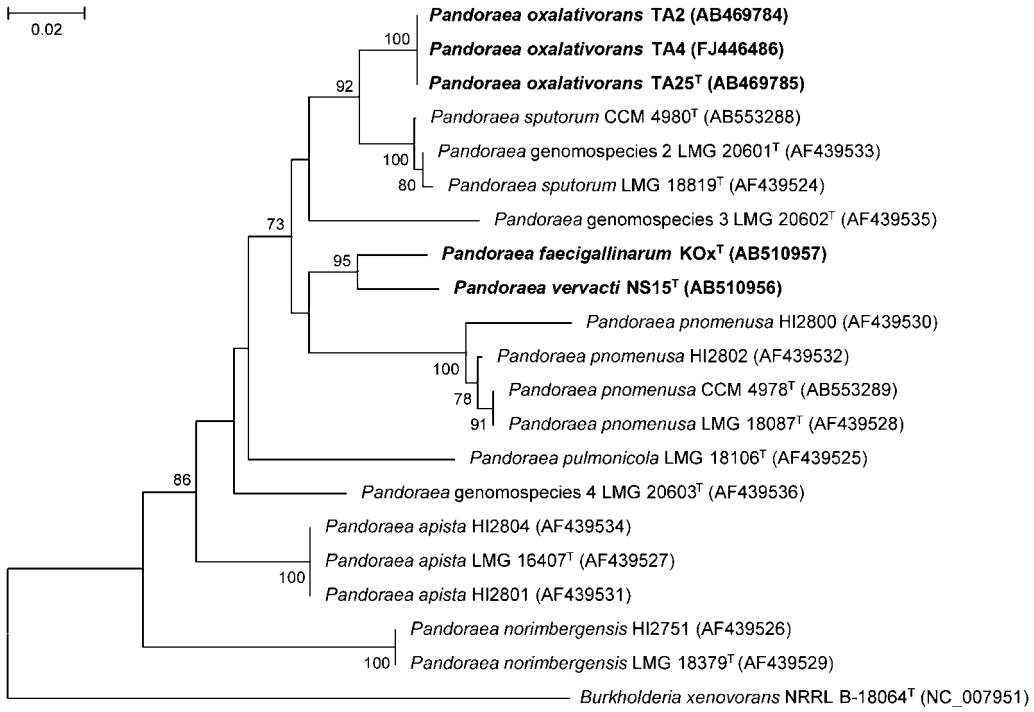

Fig. 2. Phylogenetic tree based on *gyrB* gene sequences constructed after multiple alignment of data (423 nt) by CLUSTAL_X version 2.0 and clustering using the neighbour-joining method. Bootstrap values >50 % (based on 1000 replications) are listed at the branching points. Bar, 0.02 substitutions per nucleotide position.

the five isolates could be assigned to three separate groups, representing novel species, that were distinct from previously described species of the genus *Pandoraea*. Based on 16S rRNA and *gyrB* gene sequences, strains TA25^T, TA2 and TA4 were closely related each other. DNA–DNA relatedness between strains TA25^T and TA2 was high (92.7 %) and DNA–DNA relatedness values between these strains and *P. sputorum* CCM 4980^T (52–60 %), well below the 70 % cut-off point recommended for the delineation of bacterial species (Wayne *et al.*, 1987), suggested that they belong to different species. Strain NS15^T showed 32.8 % and 37.3 % DNA–DNA relatedness to *P. sputorum* CCM 4980^T and *P. pnomenusa* CCM 4978^T, respectively. The newly proposed type strains TA25^T, NS15^T and KOx^T also showed low levels of DNA–DNA hybridization (27–35 %) with each other.

Based on the phylogenetic and chemotaxonomic evidence mentioned above, the five novel isolates represent three novel species of the genus *Pandoraea*, for which the names *Pandoraea oxalativorans* sp. nov., *Pandoraea faecigallinarum* sp. nov. and *Pandoraea vervacti* are proposed.

Description of *Pandoraea oxalativorans*

Pandoraea oxalativorans (o.xa.la.ti.vo′rans. N.L. n. *oxalas -atis* oxalate; L. part. adj. *vorans* eating; N.L. part. adj. *oxalativorans* oxalate-eating).

Cells are Gram-reaction-negative motile rods 0.7–0.9 × 2.3–2.8 μm. Poly-β-hydroxybutyric acid is accumulated. Forms round, cream-coloured, convex colonies, 1–2 mm in diameter on TSA plates after 2 days of incubation. Growth occurs at 30 and 37 °C but not at 42 °C. Grows in nutrient broth containing 3 % (w/v) NaCl. Oxidase- and catalase-positive. The specific growth rate (μ) of the type strain TA25^T at 28 °C with 6 g potassium oxalate l^{-1} as the sole source of carbon and energy is 0.113 h^{-1} (t_d=6 h). Oxidation of substrates, enzyme production, acid production and other phenotypic characteristics are given in Table 1. The major cellular fatty acids are $C_{16:0}$, $C_{18:1}\omega7c$ and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH).

The type strain, TA25^T (=NBRC 106091^T =CCM 7677^T =DSM 23570^T), and reference strains TA2 (=NBRC 106089 =CCM 7670 =DSM 23573) and TA4 (=NBRC 106090 =CCM 7671 =DSM 23574), were isolated from soil litter close to oxalate-producing plants of the genera *Oxalis*, *Rumex* and *Arum*, respectively, after enrichment with oxalate in mineral medium. The DNA G+C content of the type strain is 62–63 mol% (T_m).

Description of *Pandoraea faecigallinarum*

Pandoraea faecigallinarum (fa.e.ci.gal.li.na′rum. L. n. *faex*, *faecis* the dregs, faeces; L. gen. pl. n. *gallinarum* of hens; N.L. gen. pl. n. *faecigallinarum* isolated from faeces of hens).

Table 1. Morphological and physiological characteristics of strains TA2, TA4, TA25^T, NS15^T, KOx^T and closely related members of the genus *Pandoraea*

Strains: 1, TA2; 2, TA4; 3, TA25^T; 4, *Pandoraea sputorum* CCM 4980^T; 5, NS15^T; 6, KOx^T; 7, *Pandoraea pnomenusa* CCM 4978^T. In Biolog GN MicroPlates all strains were positive after 2 days for utilization of methyl pyruvate, mono-methyl succinate, acetic acid, cis-aconitic acid, citric acid, formic acid, D-glucuronic acid, α-, β- and γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, DL-lactic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, D- and L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-phenylalanine, L-pyroglutamic acid and L-serine. In API 20 NE tests, all strains were positive after 2 days for utilization of malate, citrate and phenyl acetate. In API ZYM tests all strains were positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities. All strains were negative for the utilization of other substrates included in the panels but not mentioned here. −, Negative; +, positive; (+), delayed positive reaction.

Characteristic	1	2	3	4	5	6	7
Growth at/in:							
42 °C	−	−	−	+	+	+	+
3 % (w/v) NaCl	+	+	+	+	+	−	+
4.5 % (w/v) NaCl	−	−	−	−	−	−	+
Biolog GN:							
Glycogen	−	−	−	+	−	−	+
Tween 80, glycerol	+	+	+	−	+	+	−
L-Arabinose, cellobiose	−	−	−	+	−	−	−
α-D-Glucose	+	+	+	−	+	+	−
D-Gluconic acid	−	−	−	+	+	+	+
D-Glucosaminic acid	−	−	−	−	+	+	−
α-Ketobutyric acid, α-ketovaleric acid	−	−	+	+	+	+	+
Malonic acid	−	−	+	−	−	−	−
D-Saccharic acid	−	−	−	−	−	+	+
Sebacic acid	+	−	−	−	−	−	−
Glucuronamide	+	+	+	−	−	+	−
Alaninamide	−	−	−	−	+	+	+
Glycyl L-aspartic acid	−	−	+	−	−	+	−
Glycyl L-glutamic acid	+	+	+	+	−	+	+
L-Proline, L-threonine	+	+	+	−	+	+	+
D-Serine	+	+	+	−	−	−	+
D-Glucose-6-phosphate	−	−	−	+	−	+	+
API 20 NE:							
Nitrate reductase	−	−	−	−	−	−	+
Urease	(+)	(+)	(+)	−	+	−	+
Glucose	−	−	+	−	+	−	−
Gluconate	−	−	−	+	+	+	+
Caprate	−	−	−	−	+	+	+
Adipate	−	−	−	+	−	−	−
Oxidase	+	+	+	+	+	+	−
API ZYM:							
Esterase (C4)	+	+	+	+	+	+	−
Esterase lipase (C8)	+	+	+	+	−	+	+
Valine arylamidase	−	+	−	−	−	−	−
Cystine arylamidase	−	−	−	−	−	−	+
Resistance to:							
Tetracycline (30 μg)	−	−	−	+	−	−	−
DNA G+C content (mol%)	62	63.3	62.1	61.9*	65.8	62.3	64.3*

*Data from Coenye *et al.* (2000).

Cells are Gram-reaction-negative motile rods 0.7 × 1.5 μm. Poly-β-hydroxybutyric acid is accumulated. Forms cream-coloured, smooth, low convex colonies 1–2 mm in diameter on TSA plates after 2 days of incubation. Growth occurs at 30 and 42 °C. Grows in nutrient broth containing 3 % (w/v) NaCl. Oxidase- and catalase-positive. Oxidation of substrates, enzyme production, acid production and other physiological characteristics are given in

Table 2. Whole-cell fatty acid composition (%) of strains TA2, TA4, TA25^T, NS15^T, KOx^T and closely related members of the genus *Pandoraea*

Strains: 1, TA2; 2, TA4; 3, TA25^T; 4, *Pandoraea sputorum* CCM 4980^T; 5, NS15^T; 6, KOx^T; 7, *Pandoraea pnomenusa* CCM 4978^T. Values are percentages of total fatty acids. −, not detected.

Fatty acid	ECL*	1	2	3	4	5	6	7
$C_{12:0}$	12.00	3.0	2.5	2.9	2.6	2.4	−	2.0
$C_{14:0}$	14.00	−	1.0	0.6	−	−	1.3	0.9
$C_{14:0}$ 3-OH†	15.49	2.8	4.7	3.7	5.7	5.9	6.0	3.4
$C_{16:1}\omega7c$‡	15.82	29.2	26.9	30.2	18.8	21.9	12.8	25.0
$C_{16:0}$	15.99	25.9	30.1	24.6	21.8	27.7	34.3	24.9
$C_{17:0}$ cyclo	16.89	3.2	3.0	2.4	6.2	4.7	18.6	4.8
$C_{16:1}$ 2-OH	17.05	−	1.2	−	−	−	−	−
$C_{16:0}$ 3-OH	17.52	2.8	4.6	3.1	5.4	5.7	6.2	2.9
$C_{18:1}\omega7c$	17.82	31.4	24.2	30.3	34.1	31.6	14.8	32.0
$C_{18:0}$	18.00	−	−	0.9	1.9	−	−	1.5
$C_{19:0}$ cyclo ω8c	18.90	1.8	1.8	1.4	3.5	−	6.0	2.0
$C_{18:1}$ 2-OH	19.09	−	−	−	−	−	−	0.6

*ECL, Equivalent chain-length.
† As part of summed feature 2 comprising $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I. Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system.
‡As part of summed feature 3 comprising $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

Table 1. The major cellular fatty acids are $C_{16:0}$, $C_{18:1}\omega7c$, $C_{17:0}$ cyclo and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH).

The type strain, KOx^T (=NBRC 106092^T =CCM 2766^T =DSM 23572^T) was isolated from chicken dung after enrichment with oxalate in mineral medium. The DNA G+C content of the type strain is 62.3 mol% (T_m).

Description of *Pandoraea vervacti*

Pandoraea vervacti (ver.vac′ti. L. gen. n. *vervacti* of/from a fallow field, the source of the type strain).

Cells are Gram-reaction-negative motile rods 0.9 × 2.8 μm. Poly-β-hydroxybutyric acid is accumulated. Forms cream-coloured, smooth, convex colonies 1–2 mm in diameter on TSA plates after 2 days of incubation. Growth occurs at 30 and 42 °C. Grows in nutrient broth containing 3 % (w/v) NaCl. Oxidase- and catalase-positive. Oxidation of substrates, enzyme production, acid production and other physiological characteristics are given in Table 1. The major cellular fatty acids are $C_{16:0}$, $C_{18:1}\omega7c$ and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH).

The type strain, NS15^T (=NBRC 106088^T =CCM 7667^T =DSM 23571^T) was isolated from field soil after enrichment with oxalate in mineral medium. The DNA G+C content of the type strain is 65.8 mol% (T_m).

Acknowledgements

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