Pseudoxanthomonas indica sp. nov., isolated from a hexachlorocyclohexane dumpsite

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A bacterial strain, designated P15^T, was isolated from the soil of an open hexachlorocyclohexane dumpsite. Comparative sequence analysis showed that strain P15^T displayed high 16S rRNA gene sequence similarities (94.4–97.2%) with members of the genus *Pseudoxanthomonas*. The isolate was most closely related to *Pseudoxanthomonas mexicana* AMX 26B^T (97.2% 16S rRNA gene sequence similarity) and *Pseudoxanthomonas japonensis* 12-3^T (97.2%). DNA–DNA relatedness studies showed unambiguously that strain P15^T represented a novel species that was separate from *P. mexicana* DSM 17121^T (7.7%) and *P. japonensis* DSM 17109^T (9.4%). The predominant cellular fatty acids of strain P15^T were iso-C_{16:0} (21.4%), iso-C_{15:0} (16.1%), summed feature 9 (comprising iso-C_{17:1ω9c} and/or 10-methyl C_{16:0}; 14.9%), iso-C_{11:0} 3-OH (8.3%) and iso-C_{14:0} (7.0%). The polar lipid profile of strain P15^T showed the presence of large amounts of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol in addition to unknown glycolipids, phospholipids and an amino-group-containing polar lipid. Ubiquinone 8 was found as the major quinone. The polyamine profile showed the presence of spermidine. The DNA G + C content was 62.9 ± 2 mol%. Strain P15^T is described as representing a new member of the genus *Pseudoxanthomonas*, for which the name *Pseudoxanthomonas indica* sp. nov. is proposed. The type strain is P15^T (=MTCC 8596^T=CCM 7430^T).

The genus *Pseudoxanthomonas* (Finkmann *et al.*, 2000) belongs to the family *Xanthomonadaceae* in the order *Xanthomonadales* (Garrity & Holt, 2001). Members of this genus comprise aerobic, Gram-negative, non-spore-forming rods and have been isolated from a variety of habitats such as biofilters (Finkmann *et al.*, 2000), hot springs (Chen *et al.*, 2002), compost (Weon *et al.*, 2006) and soil samples (Thierry *et al.*, 2004; Yang *et al.*, 2005; Chang *et al.*, 2005; Harada *et al.*, 2006). The genus is known for the presence of 3-OH fatty acids as the major hydroxylated components and ubiquinone 8 (Q-8) as the major respiratory quinone (Lee *et al.*, 2008), reduction of nitrite and lack of iso-C_{13:0} 3-OH (Thierry *et al.*, 2004), albeit with a few exceptions (Chang *et al.*, 2005; Young *et al.*, 2007; Lee *et al.*, 2008). The genus is currently represented by 13 recognized species: *Pseudoxanthomonas broegbernensis* (Finkmann *et al.*, 2000; Thierry *et al.*, 2004), *P. taiwanensis* (Chen *et al.*, 2002), *P. japonensis* and *P. mexicana* (Thierry *et al.*, 2004), *P. daejeonensis* and *P. koreensis* (Yang *et al.*, 2005), *P. kaohsiungensis* (Chang *et al.*, 2005), *P. suwonensis* (Weon *et al.*, 2006), *P. kalamensis* (Harada *et al.*, 2006), *P. dokdonensis* (Yoon *et al.*, 2006; Lee *et al.*, 2008),

P. yeongjuensis (Yoo *et al.*, 2007), *P. spadix* (Young *et al.*, 2007) and *P. sacheonensis* (Lee *et al.*, 2008).

A soil sample was collected from an open hexachlorocyclohexane dumpsite in north India (27° 00' 24.0" N 81° 09' 03.8" E), serially diluted and plated on nystatin-amended LB agar (Dadhwal *et al.*, 2009). A dull yellow-coloured colony, designated P15^T, appeared within 36 h of incubation at 28 °C and was purified by repeated streaking on LB agar.

The 16S rRNA gene sequence of strain P15^T was amplified using the 8F and 1492R universal primer set by colony PCR and sequenced as described elsewhere (Kumar *et al.*, 2008; Jit *et al.*, 2008). The sequence thus obtained was assembled manually using Clone Manager version 5. A continuous sequence of 1392 bp of the 16S rRNA gene was obtained and subjected to similarity searches using the sequence match tool of Ribosomal Database Project II (RDP; <http://rdp.cme.msu.edu/>) and BLAST program of National Center of Biotechnological Information (<http://www.ncbi.nlm.nih.gov/>). A non-redundant BLASTN search of full sequences in GenBank (Altschul *et al.*, 1990; Benson *et al.*, 1999) and RDP II (Maidak *et al.*, 2001) identified the closest relatives of the isolate. Analysis of the 16S rRNA gene sequence (1392 bp) revealed that strain P15^T showed highest sequence similarity with *P. mexicana* AMX 26B^T (97.2% 16S rRNA gene sequence similarity) and *P. japonensis* 12-3^T (97.2%). The nearly full-length 16S rRNA gene

Abbreviation: RDP, Ribosomal Database Project.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P15^T is EF424397.

A supplementary table and two supplementary figures are available with the online version of this paper.

sequences of strains closely related to the isolate were retrieved for further analysis. The 16S rRNA gene sequence similarity between strain P15^T and members of the genus *Pseudoxanthomonas* was 94.4–97.2 %. Multiple alignment of these sequences was performed using CLUSTAL_X (Thompson *et al.*, 1997), common gaps were removed and the alignment was checked manually for quality. Pairwise evolutionary distances were calculated using the distance model of Jukes & Cantor (1969) within the TREECON version 1.3b (Van de Peer & De Wachter, 1994). A phylogenetic tree was constructed using the neighbour-joining method of Saitou & Nei (1987) and the resultant tree topology was evaluated by bootstrap analysis with 100 resamplings. Strain P15^T clustered with members of the genus *Pseudoxanthomonas* and formed a monophyletic clade within the cluster containing *P. mexicana* AMX 26B^T and *P. japonensis* 12-3^T (Fig. 1). It is notable that, although the genus *Pseudoxanthomonas* is not monophyletic and the taxonomic situation of this group is subject to future revision, strain P15^T fell within the cluster that exclusively contained members of the genus *Pseudoxanthomonas*. A similar tree topology was also observed using the

maximum-likelihood method in PHYLIP version 3.5 (Felsenstein, 1993) (data not shown).

DNA–DNA hybridization was performed between strain P15^T and *P. mexicana* DSM 17121^T and *P. japonensis* DSM 17109^T, which were obtained from the DSMZ. Extraction, purification and hybridization of total genomic DNA followed the protocol described by Prakash *et al.* (2007) and Kumar *et al.* (2008). The amount of bound probe DNA was calculated using a scintillation counter (1450 LSC Luminescence counter Wallac Microbeta Trilux; PerkinElmer). DNA–DNA relatedness was calculated on the basis of the means of four replicates and ranged from 7.7 to 9.4 %. Pooled standard deviations of all the hybridization experiments were found to vary by up to 12 %. Since all hybridization values were <70 %, the results confirmed that strain P15^T represented a novel species of the genus *Pseudoxanthomonas*, as recommended for the delineation of species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Fatty acid methyl ester analysis was performed as described by Prakash *et al.* (2007). The fatty acids of strain P15^T (>1.5 %) were iso-C_{16:0} (21.4 %), iso-C_{15:0} (16.1 %),

Fig. 1. Phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationship of strain P15^T to members of the genus *Pseudoxanthomonas* and related genera. Bootstrap values (>50 %) based on 100 resamplings are shown at branch nodes. *Luteimonas mephitis* B1953^T was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

summed feature 9 (comprising iso-C_{17:1}ω9c and/or 10-methyl C_{16:0}; 14.9%), iso-C_{11:0} 3-OH (8.3%), iso-C_{14:0} (7.0%), iso-C_{11:0} (5.0%), iso-C_{16:1} H (3.5%), iso-C_{10:0} (3.4%), iso-C_{12:0} 3-OH (3.4%), summed feature 3 (comprising C_{16:1}ω7c and/or C_{16:1}ω6c; 3%), anteiso-C_{15:0} (2.9%), iso-C_{15:1} F (2.4%) and C_{16:0} (1.6%). Strain P15^T contained large amounts of iso-C_{15:0} and iso-C_{16:0}, which are also predominant fatty acids in members of other species of the genus *Pseudoxanthomonas*. A comparison of the fatty acid profiles of strain P15^T with all members of the genus *Pseudoxanthomonas* is given in Supplementary Table S1 (available in IJSEM Online).

Polar lipid analysis of strain P15^T was performed by two-dimensional TLC using 1.0% Premulin as the spray reagent for detection of total lipids under UV (Gupta *et al.*, 2009; Sharma *et al.*, 2010; Nigam *et al.*, 2010). The major polar lipids were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol, in addition to small amounts of unknown glycolipids, phospholipids and an amino-group-containing polar lipid (Supplementary Fig. S1). The lipid profile of strain P15^T was found to be similar to those of *P. mexicana* and *P. sacheonensis* (Lee *et al.*, 2008) by having phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol as the major lipids; however, the unknown glycolipids and phospholipids found in strain P15^T were absent in *P. sacheonensis*.

Quinones were extracted according to the method described by Collins & Jones (1981). Q-8 was found to be the major respiratory quinone. Polyamines were extracted as described by Busse & Auling (1988) and analysed by TLC (Silica gel 60 F₂₅₄, 20 × 20 cm; Merck, Germany) and showed the presence of spermidine. The DNA G+C content was calculated by the method described by Gonzalez & Saiz-Jimenez (2002) using real-time PCR (7500; Applied Biosystems) and was found to be 62.9 mol%, which is in accordance with the range described for the genus *Pseudoxanthomonas* (Finkmann *et al.*, 2000; Thierry *et al.*, 2004).

Colony morphology of strain P15^T was studied on LB agar (pH 7.5) after 48 h of incubation at 28 °C. Colonies were dull yellow, circular with entire margins and about 1.5 mm in diameter (range 1–2 mm) after 2 days of incubation. The temperature range for growth was assessed at 10–41 °C. Oxidase activity was tested using oxidase discs from HiMedia. Catalase activity was tested by adding 3% (v/v) hydrogen peroxide solution to colonies grown on LB agar (McCarthy & Cross, 1984). Motility was checked on motility agar (Farmer, 1999). Hydrolysis of gelatin, casein, aesculin and Tween 20 was determined as described by Cowan & Steel (1965). Urease activity was tested in accordance with Christensen (1946). Indole production was tested as described by Smibert & Krieg (1994). β-Galactosidase activity was observed using ONPG discs (HiMedia), according to the manufacturer's instructions. Citrate utilization was tested on Simmons' citrate agar (Simmons, 1926). Nitrate reduction was tested as described by Smibert

& Krieg (1994). Assimilation of different carbohydrates was tested in basal media (Gordon *et al.*, 1974). Antibiotic sensitivity tests were performed on Muller-Hinton II medium using ready-made Sensi-Discs (HiMedia), containing (μg per disc, unless otherwise stated) amikacin (30), ampicillin (10), chloramphenicol (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), nalidixic acid (30), penicillin G (10 U), polymyxin B (300), rifampicin (5), tetracycline (30) and vancomycin (30). All tests were performed at least in duplicate with strain P15^T, *P. mexicana* DSM 17121^T and *P. japonensis* DSM 17109^T under similar conditions. The results are summarized in Table 1.

The Gram-stain test was performed using a Gram-staining kit (HiMedia). Cell morphology was examined by light and electron microscopy (TEM 269D; Morgagni, Fei) using cells from an exponentially growing culture on LB agar, negatively stained with 0.5% uranyl acetate and air dried (Supplementary Fig. S2).

Strain P15^T should be classified as a member of the genus *Pseudoxanthomonas* as it showed an absence of the fatty acid iso-C_{13:0} 3-OH and high 16S rRNA gene sequence similarity with members of the genus *Pseudoxanthomonas*. In addition, the polar lipid pattern, presence of spermidine and Q-8 as the major respiratory quinone further supported the phylogenetic findings. From the data presented here, it is evident that strain P15^T exhibits an overall chemotaxonomic profile consistent with those of members of the genus *Pseudoxanthomonas* and represents a novel species. The name *Pseudoxanthomonas indica* sp. nov. is proposed.

Description of *Pseudoxanthomonas indica* sp. nov.

Pseudoxanthomonas indica (in.di'ca. L. fem adj. *indica* of India).

Colonies are dull yellow, smooth and circular (1.5 mm on LB agar after 48 h at 28 °C). Cells are Gram-negative-staining, aerobic rods (1.5 × 0.5 μm) and bear a single polar flagellum. The optimum temperature for growth is 28 °C. Cells are catalase- and oxidase-positive. Hydrolyses ONPG (β-galactosidase), aesculin and Tween 20 but does not hydrolyse gelatin, casein or urea. Indole production is negative. Positive for nitrate reduction. Assimilates D-glucose, D-mannose, N-acetyl D-glucosamine, maltose, (+)-D-galactose, sucrose, D-fructose, lactose, L-histidine and L-proline, but not L-arabinose, D-mannitol, (+)-D-raffinose, L-alanine, L-serine or putrescine. Citrate is not utilized. Sensitive to amikacin, ampicillin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, polymyxin B, rifampicin, tetracycline and vancomycin, but resistant to penicillin G. The predominant cellular fatty acids are iso-C_{16:0}, iso-C_{15:0}, summed feature 9 (comprising iso-C_{17:1}ω9c and/or 10-methyl C_{16:0}), iso-C_{11:0} 3-OH and iso-C_{14:0}. The polyamine profile shows the presence of spermidine. Q-8 is the major respiratory quinone. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol, in addition to small amounts of glycolipids, phospholipids and an amino-group-containing polar lipid.

Table 1. Differential morphological and physiological characteristics of strain P15^T and members of the genus *Pseudoxanthomonas*

Strains: 1, P15^T (data from this study); 2, *P. mexicana* DSM 17121^T (this study); 3, *P. japonensis* DSM 17109^T (this study); 4, *P. dokdonensis* DS-16^T (Yoon *et al.*, 2006; Lee *et al.*, 2008); 5, *P. kaohsiungensis* J36^T (Chang *et al.*, 2005); 6, *P. suwonensis* 4M1^T (Weon *et al.*, 2006); 7, *P. daejeonensis* TR6-08^T (Yang *et al.*, 2005); 8, *P. broegbernensis* B1616/1^T (Finkmann *et al.*, 2000; Weon *et al.*, 2006; Lee *et al.*, 2008); 9, *P. koreensis* T7-09^T (Yang *et al.*, 2005); 10, *P. kalamensis* JA40^T (Harada *et al.*, 2006); 11, *P. Sacheonensis* BD-c54^T (Lee *et al.*, 2008); 12, *P. yeongjuensis* GR12-1^T (Yoo *et al.*, 2007); 13, *P. spadix* IMMIB AFH-5^T (Young *et al.*, 2007); 14, *P. taiwanensis* CB-226^T (Chen *et al.*, 2002; Yoo *et al.*, 2007). All strains are Gram-negative-staining rods and, if motile, possess a single polar flagellum. CY, Cream yellow; DY, dull yellow; DYDBP, deep yellow, producing diffusible brown pigment; GY, greenish yellow; LY, light yellow; PY, pale yellow; W, white; Y, yellow; YDBP, yellow, producing date-brown pigment; +, Positive; (+), weakly positive; v, variable reaction between duplicates; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Colony colour	DY	PY	DYDBP	CY	LY	Y	Y	Y	W	PY	Y	GY	YDBP	LY
Motility and flagellation	+	+	+	–	+	+	+	+	–	–	–	+	+	–
Assimilation of:														
D-Glucose	+	+	+	+	+	+	+	+	–	+	+	+	+	+
L-Arabinose	–	+	+	–	–	+	+	+	–	+	–	–	–	–
D-Mannose	+	+	+	+	–	–	–	+	–	–	+	–	–	–
D-Mannitol	–	+	+	–	–	–	–	–	–	–	–	–	–	ND
N-Acetyl D-glucosamine	+	+	–	+	+	+	+	+	–	+	+	+	–	–
Maltose	+	+	+	+	+	+	+	+	–	+	+	+	–	–
Citrate	–	–	–	–	–	–	–	+	–	–	–	–	–	–
(+)-D-Raffinose	–	+	–	ND	–	ND	ND	–*	ND	+	+	ND	–	–
Sucrose	+	+	–	–	–	ND	–	+	–	+	ND	ND	–	–
Lactose	+	+	–	–	–	ND	–	+	–	ND	+	ND	–	–
L-Histidine	+	v	+	ND	–	ND	–	ND	–	ND	ND	ND	ND	ND
L-Serine	–	+	+	ND	+	v	–	–*	–	+	ND	ND	ND	–
Putrescine	–	v	+	ND	–	ND	ND	–*	ND	ND	ND	ND	ND	ND
Enzyme activity														
Oxidase	+	+	+	+	+	+	+	ND	+	+	+	+	+	+
Catalase	+	(+)	–	+	–	+	+	+	+	+	+	+	+	+
β-Galactosidase	+	–	(+)	–	+	+	+	+	–	–	+	+	–	+
Casein hydrolysis	–	+	(+)	–	ND	–	+	–	+	ND	+	+	ND	–
Urease	–	–	–	–	–	–	–	–	+	–	+	–	–	ND
Gelatin hydrolysis	–	–	–	+	–	+	–	–	–	–	+	+	+	–

*Data from Thierry *et al.* (2004).

The type strain, P15^T (=MTCC 8596^T=CCM 7430^T), was isolated from the soil of an open hexachlorocyclohexane dumpsite situated in north India. The DNA G+C content of the type strain is 62.9±2 mol%.

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