Microbacterium lindanitolerans sp. nov., isolated from hexachlorocyclohexane-contaminated soil

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A Gram-stain-positive, aerobic, rod-shaped, yellow actinobacterium, designated MNA2^T, was isolated from a hexachlorocyclohexane-contaminated soil in North India. Strain MNA2^T showed 95 % 16S rRNA gene sequence similarity with *Microbacterium halotolerans* YIM 70130^T. Phylogenetic analysis of 16S rRNA gene sequences showed that strain MNA2^T belonged to a clade represented by the genus *Microbacterium* of the family *Microbacteriaceae*. Strain MNA2^T contained anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0} and iso-C_{15:0} as the predominant fatty acids and diphosphatidylglycerol, phosphatidylglycerol and two unknown polar lipids. The menaquinones were MK-12, MK-11, MK-13 and MK-10, in an approximate molar ratio of 45:40:13:3, respectively. The DNA G+C content was 65.3 mol%. The peptidoglycan was of the B type of cross-linkage with ornithine as the diagnostic diamino acid. The results of the phylogenetic, phenotypic and chemotaxonomic analysis indicate that strain MNA2^T belongs to a previously unrecognized species of the genus *Microbacterium*, for which the name *Microbacterium lindanitolerans* sp. nov. is proposed. The type strain is MNA2^T (=DSM 22422^T =CCM 7585^T).

The genus *Microbacterium* was proposed by Orla-Jensen (1919). The description was emended by Collins *et al.* (1983) and emended again by Takeuchi & Hatano (1998) to unite *Microbacterium* and *Aureobacterium*. The genus *Microbacterium* belongs to the family *Microbacteriaceae* in the order *Actinomycetales*. Members of the genus *Microbacterium* are Gram-positive and are characterized by the presence of *N*-glycolyl residues in the cell wall. The major isoprenoid quinones are menaquinones with 10–14 (mainly 11–13) unsaturated isoprenoid units. The DNA G+C content is 64–75 mol%. The presence of *L*-lysine or *D*-ornithine in the peptidoglycan is characteristic of species of this genus (Schumann *et al.*, 2009).

Strain MNA2^T was isolated from soil contaminated with hexachlorocyclohexane (HCH) in North India. For this purpose, 1 g soil was suspended in 9 ml saline and different dilutions were plated on Luria–Bertani agar (LB; 1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 1.5 % agar) and incubated at 28 °C. Yellow colonies were picked and purified on LB agar. Cultures were preserved in 40 % (v/v)

glycerol at –80 °C and routinely cultivated on LB agar or in LB broth at 28 °C.

16S rRNA gene sequence analysis was carried out as described by Gupta *et al.* (2008) using a 3100 Avant Genetic Analyzer sequencer (Applied Biosystems). The 16S rRNA gene sequence obtained was manually checked for quality and gaps. Similarity searches were conducted using Seqmatch (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and BLAST (<http://www.ncbi.nlm.nih.gov>). For the reconstruction of phylogenetic trees, sequences from the most closely related species of the genus *Microbacterium* were selected from the Ribosomal Database Project and NCBI databases, aligned using CLUSTAL X (Thompson *et al.*, 1997) and analysed using MEGA version 4.0 (Tamura *et al.*, 2007) and TREECON (Van de Peer & De Wachter, 1994). The evolutionary distance matrix was calculated according to the distance model of Jukes & Cantor (1969) and an evolutionary tree was reconstructed using the neighbour-joining method of Saitou & Nei (1987). The topology of the phylogenetic tree was statistically evaluated by bootstrapping with 1000 resamplings and a maximum-parsimony tree (Takahashi & Nei, 2000) was also reconstructed.

Strain MNA2^T showed 95 % 16S rRNA gene sequence similarity with *Microbacterium halotolerans* YIM 70130^T and 94.1–94.6 % similarity with the representatives of the genus *Microbacterium* included in the analysis: *M.*

Abbreviation: HCH, hexachlorocyclohexane.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MNA2^T is EU873539.

The cellular fatty acid composition and a transmission electron micrograph of cells of strain MNA2^T are available with the online version of this paper.

esteraromaticum DSM 8609^T (94.6%), *M. phyllosphaerae* GH06 (94.5%), *M. barkeri* DSM 20145^T (94.4%), *M. oxydans* B5 (94.3%), *M. foliorum* DSM 12966^T (94.2%), *M. testaceum* DSM 20166^T (94.2%), *M. resistens* DMMZ 1710^T (94.2%), *M. xylanilyticum* S3-E^T (94.1%) and *M. paraoxydans* CF36^T (94.1%). In the neighbour-joining tree (Fig. 1), strain MNA2^T occupied a separate lineage within a distinct cluster with *M. halotolerans* YIM 70130^T. This topology was confirmed in the maximum-parsimony tree.

To determine the fatty acid composition, cells of strain MNA2^T were grown on LB agar at 28 °C for 72 h. FAME analysis was carried out as described by Miller (1982) and Kuykendall *et al.* (1988), using a gas chromatograph (Agilent 6890) equipped with a flame-ionization detector and the Aerobe (TSBA50, version 5) database of the Sherlock Microbial Identification System (MIDI). Polar lipids were extracted from 100 mg lyophilized cells by the method of Bligh & Dyer (1959) and separated by two-dimensional TLC, using 10 × 10 cm silica-gel F₂₅₄ plates (Merck), chloroform/methanol/water (65:24:4, v/v) for the first dimension and chloroform/glacial acetic acid/methanol/water (80:12:15:4, v/v) for the second dimension. Aqueous premulin solution (1 %) and UV light were used to detect and visualize the lipids. For analysis of the amino acids and peptides, purified cell-wall hydrolysates were prepared after disrupting the cells with glass beads and subsequent trypsin digestion (Schleifer & Kandler,

1972) and analysed by two-dimensional ascending TLC on cellulose plates using the solvent systems described by Schleifer & Kandler (1972). The molar ratios of amino acids were determined by GC and GC-MS of *N*-heptafluorobutyl amino acid isobutyl esters, as described by MacKenzie (1987). The acyl type was determined as described by Uchida *et al.* (1999). Menaquinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC. For DNA isolation, cells were disrupted with a French pressure cell. DNA was purified on hydroxyapatite according to the procedure of Cashion *et al.* (1977) and degraded to nucleosides with P1 nuclease and bovine intestinal mucosa alkaline phosphatase (Mesbah *et al.*, 1989). The nucleosides were separated by reversed-phase HPLC as described by Tamaoka & Komagata (1984). The G + C content of the DNA was calculated from the ratio of deoxyguanosine to thymidine. Whole-cell sugars were determined as described by Lechevalier (1968).

Strain MNA2^T showed very good growth on LB agar and in LB broth at 28 °C and all phenotypic tests were performed at this temperature. Gram staining was performed with a Gram-staining kit (HiMedia). The morphology of colonies on LB agar was examined under a light microscope (Motic 200) after 3 days. The presence or absence of flagella in cells from an exponentially growing culture was determined by transmission electron microscopy (Morgagni 269D, Fei) after negative staining with 0.5 %

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences (1279 nt) showing the relationship of strain MNA2^T to members of the family *Microbacteriaceae*. Bootstrap values based on 1000 resamplings are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. *Arthrobacter globiformis* ATCC 8010^T was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

Table 1. Differential phenotypic characteristics of strain MNA2^T and other representatives of the genus *Microbacterium*

Taxa: 1, *Microbacterium lindanitolerans* sp. nov. MNA2^T; 2, *M. halotolerans*; 3, *M. oxydans*; 4, *M. testaceum*; 5, *M. resistens*; 6, *M. foliorum*; 7, *M. phyllosphaerae*; 8, *M. xylanilyticum*; 9, *M. esteraromaticum*; 10, *M. barkeri*; 11, *M. paraoxydans*. Data were taken from this study and Behrendt *et al.* (2001), Kim *et al.* (2005) and Li *et al.* (2005). All strains were positive for acid production from D-mannose and utilization of D-glucose, sucrose and D-mannose. +, Positive; +^w, weakly positive; d, differs among strains; –, negative; O, orange; w, white; y, yellow; Gal, galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Colony colour	y	w	y	O–YO	y	y	y	y	YW–Y	W–Y	y
Growth at 37 °C	+	+	+	–	+	d	d	+	d	+	+
H ₂ S production	–	–	+	+	–	–	–	–	+	+	–
Voges–Proskauer test	–	–	–	–	–	–	–	+	–	–	+
Hydrolysis of:											
Gelatin	–	–	+	+	+	+	+	–	–	+	+
Starch	+	–	–	–	–	+ ^w	+ ^w	+	+	+	–
Aesculin	+	+	+	+	–	–	–	–	+	+	+ ^w
Acid production from:											
L-Arabinose	+	+	–	+	–	+	+	+	+	+	–
D-Glucose	+	+	+	–	+	+	+	+	–	–	+
Lactose	–	–	–	+	+	–	–	+	–	+	–
Raffinose	+	–	–	–	–	+	+	+	–	+	–
D-Ribose	–	+	+	–	+	+	+	+	+	+	+
D-Sorbitol	–	–	–	–	–	–	–	–	–	+	–
Sucrose	+	–	+	+	+	+	+	+	+	+	+
D-Xylose	–	+	–	+	–	+	+	+	–	+	–
myo-Inositol	–	–	–	–	–	–	–	+	–	+	–
D-Mannitol	+	+	+	+	+	+	+	+	–	+	+
Utilization of:											
L-Arabinose	–	+	–	+	–	+	+	+	+	+	–
Citrate	–	–	+ ^w	–	–	–	–	–	–	+	–
Lactose	+	–	+	+	+	–	–	+	+	+	–
Raffinose	+	–	+	+	–	+	+	+	–	+	+
D-Ribose	–	+	+	+	+	+	+	+	+	+	+
D-Sorbitol	+	+	+	–	+	–	+	–	–	+	–
D-Xylose	–	+	+	+	–	+	+	+	–	+	–
myo-Inositol	–	–	–	–	+	–	–	+	–	+	–
D-Mannitol	–	+	+	+	+	+	+	+	–	+	+
Major menaquinones	MK-11, MK-12	MK-10, MK-11	MK-11, MK-12	MK-10, MK-11	MK-11, MK-12	MK-10, MK-11, MK-12	MK-10, MK-11, MK-12	MK-11, MK-12, MK-13	MK-12, MK-13	MK-11, MK-12	MK-11, MK-12
Peptidoglycan type	B type, based on ornithine*	B2β	B2β	B2β	B2α	B2β	B2β	B2β	B2β	B2β	B2β
Major cell-wall sugars	Gal, Rib	Rib, Gal, Glc, Xyl	Rha, Man, Gal, Glc	Rha, Man, Gal	Rha, Man, Gal	Rha, Gal, Man	Rha, Gal	Gal, Glc	Gal, Glc	Rha, Gal, Glc, Gal	Rha, Gal, Glc, Gal
DNA G + C content (mol%)	65.3	66.5	70–71	67.7	71.6	67	64	69.7	68.8	68.7	69.9

*Homoserine absent.

uranyl acetate and air drying. The analysis suggested that the cells were non-flagellated (Supplementary Fig. S1, available in IJSEM Online).

Growth at pH 2–12 (adjusted with 1.0 M HCl or 1.0 M NaOH) and with 0, 1, 2, 3, 4 and 5 % (w/v) NaCl was tested in LB broth as described by Arden-Jones *et al.*

(1979). Growth at 4, 10, 20, 25, 28, 37 and 42 °C was also tested in LB broth. Tests for indole production and nitrate reduction and the Voges–Proskauer reaction were performed according to Smibert & Krieg (1994). Oxidase activity was tested as described by McCarthy & Cross (1984) and catalase activity was determined by bubble production in 3 % (v/v) hydrogen peroxide solution.

Hydrolysis of casein, gelatin, starch, aesculin, xanthine and hypoxanthine was determined as described by Cowan & Steel (1965). Urease activity was tested according to Christensen (1946). Acid production from carbohydrates was determined according to Gordon *et al.* (1974). Three litres of the basal medium was adjusted to pH 7.0 before the addition of 15 ml 0.04 % bromocresol purple solution l^{-1} . After dispensing 15 ml basal medium into tubes and autoclaving, 0.5 ml of 10 % filter-sterilized carbohydrate solution was added aseptically and tubes were inoculated with culture and observed for 7–28 days. H_2S production was tested as described by Bruns *et al.* (2001). Lecithinase activity was performed as described by Kushner (1957). DNase activity was tested with DNase agar (HiMedia) supplemented with 0.005 % methyl green. The ability to degrade HCH was tested according to Sharma *et al.* (2006).

The morphological, physiological, biochemical and chemotaxonomic features of strain MNA2^T are given in the species description and Table 1. Although isolated from a highly HCH-contaminated environment, strain MNA2^T was unable to degrade HCH. The peptidoglycan analysis revealed the occurrence of the amino acids ornithine, glutamic acid, alanine and glycine in a molar ratio of 1.0:2.5:1.0:2.6, respectively. Homoserine and hydroxyglutamic acid were not found. The peptides Gly-D-Glu, Orn-D-Ala and Glu-Ala were detected. These data did not correspond to any of the published peptidoglycan structures for the B type of cross-linkage (http://www.dsmz.de/microorganisms/main.php?content_id=35). The absence of homoserine as well as the high content of glutamic acid in the peptidoglycan differentiates strain MNA2^T from all of the type strains of species of the genus *Microbacterium* that display the peptidoglycan type B2 β . The peptidoglycan of strain MNA2^T contained glycolyl residues. The menaquinones were MK-12, MK-11, MK-13 and MK-10, in an approximate molar ratio of 45:40:13:3, respectively. The major cellular fatty acids were anteiso-C_{15:0} (37.6 %), anteiso-C_{17:0} (22.0 %), iso-C_{16:0} (21.0 %) and iso-C_{15:0} (10.1 %).

The complete fatty acid profile is shown in Supplementary Table S1. The polar lipids comprise diphosphatidylglycerol, phosphatidylglycerol and two unknown polar lipids (Fig. 2). The G+C content of the genomic DNA was 65.3 mol%. The cell-wall analysis and the utilization and hydrolysis patterns demonstrated that strain MNA2^T differed from the other type strains of the genus *Microbacterium* (Table 1).

Therefore, on the basis of the phylogenetic, chemotaxonomic and phenotypic data, we conclude that strain MNA2^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium lindanitolerans* sp. nov. is proposed.

Description of *Microbacterium lindanitolerans* sp. nov.

Microbacterium lindanitolerans (lin.da.ni.to.le'rans. N.L. n. *lindanum* lindane; L. part. adj. *tolerans* tolerating; N.L. part. adj. *lindanitolerans* lindane-tolerating).

Colonies are smooth, circular, 1.0–1.5 mm in diameter and yellow after incubation on LB agar at 28 °C for 3 days. Cells are non-flagellated. Growth occurs at 25–37 °C (optimum 28–37 °C). Optimum growth occurs with 0–2 % NaCl; growth is not observed with 5 % NaCl. Growth occurs at pH 6–11 (optimum pH 7–8); growth is not observed at pH 5. Positive for catalase and nitrate reductase; negative for urease, oxidase and DNase. Citrate utilization and indole production are negative. Aesculin and starch are hydrolysed, but gelatin is not; hydrolysis of xanthine and hypoxanthine is observed after 3 weeks. H_2S is not produced. No precipitate is formed on egg yolk agar. Voges–Proskauer reaction is negative. Acid production is not observed from adonitol, lactose, *myo*-inositol, D-ribose, D-sorbitol and D-xylose. As sole carbon sources, cellobiose, D-fructose, D-galactose, D-glucose, lactose, D-mannose, raffinose, sucrose, D-sorbitol and trehalose are utilized, but adonitol, L-arabinose, D-mannitol, *myo*-inositol, D-ribose and D-xylose are not. The peptidoglycan is of the B type of cross-linkage and contains ornithine as the diagnostic diamino acid and glycolyl residues, but does not contain homoserine or hydroxyglutamic acid. The menaquinones are MK-12, MK-11, MK-13 and MK-10, in an approximate molar ratio of 45:40:13:3, respectively. The major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0} and iso-C_{15:0}. The polar lipids comprise diphosphatidylglycerol, phosphatidylglycerol and two unknown polar lipids. The whole-cell sugars include ribose and galactose. The DNA G+C content of the type strain is 65.3 mol%.

The type strain, MNA2^T (=DSM 22422^T =CCM 7585^T), was isolated from HCH-contaminated soil in North India.

Fig. 2. Two-dimensional thin-layer chromatogram showing the polar lipids of strain MNA2^T, visualized under UV light with 1 % aqueous premulin solution. DPG, Diphosphatidylglycerol; PG, phosphatidylglycerol; UPL, unknown polar lipid.

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