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ORIGINAL PAPER

Isoptericola rhizophila sp. nov., a novel actinobacterium isolated from rhizosphere soil

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Abstract A Gram-positive, yellow pigmented strain, BKS 3-46^T was isolated from a soil sample collected from the rhizosphere of *Ficus benghalensis* (banyan tree) in Bhitarkanika mangrove forest, in the Indian state of Odisha, and subjected to polyphasic taxonomic study. The 16S rRNA gene sequence of the strain was determined and the sequence analysis showed that strain BKS 3-46^T should be assigned to the genus *Isoptericola*. The chemotaxonomic data supported this taxonomic placement i.e. presence of menaquinone MK-9(H_4); major fatty acids anteiso $C_{15:0}$ and iso-$C_{15:0;}$ and phosphatidylglycerol, diphosphatidylglycerol and phosphatidylinositol (PI) as major polar lipids. Further phylogenetic analysis of the 16S rRNA gene sequence confirmed that the strain BKS 3-46^T belongs to the genus *Isoptericola* and is closely related to *Isoptericola halotolerans* MTCC 11265^T (98.6 %) followed by *Isoptericola nanjingensis* MTCC 11633^T (98.4 %) and *Isoptericola chiayiensis* MTCC 11634^T (98.1 %). However, the DNA–DNA hybridization values obtained between strain BKS 3-46^T and other related strains were well below the threshold that is required for the proposal of a novel species. The G+C content of the genomic DNA was determined to be 70.4 mol%. The phenotypic and genotypic data showed that the strain BKS 3-46^T merits the recognition as a representative of a novel species of the genus *Isoptericola*. It is proposed that the isolate should be classified in the genus *Isoptericola* as a novel species, *Isoptericola rhizophila* sp. nov. The type strain is BKS 3-46^T (=MTCC 11080^T=JCM 19252^T).

Keywords *Isoptericola rhizophila* · DNA–DNA hybridization · Menaquinone

Introduction

The genus *Isoptericola* was first proposed by Stackebrandt et al. (2004), by re-classification of *Cellulosimicrobium variabile* Bakalidou et al. 2002 as *Isoptericola variabilis* gen. nov., comb. nov. At present the genus *Isoptericola* comprises of seven species with validly published names (http://www.bacterio.cict.fr/a/isoptericola.html) and all these species have been isolated from different environmental sources: *Isoptericola chiayiensis* (soil, Tseng et al. 2011), *Isoptericola dokdonensis* (soil, Yoon et al. 2006), *Isoptericola halotolerans* (saline soil sample, Zhang et al. 2005), *Isoptericola hypogeus* (Roman catacomb of Domitilla, Groth et al. 2005), *Isoptericola*

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jiangsuensis (beach soil, Wu et al. 2010), *Isoptericola nanjingensis*, and *Isoptericola variabilis* (hind gut of termites, Stackebrandt et al. 2004). In the present study, an actinobacterial strain BKS 3-46^T, which was isolated from a soil sample collected from the rhizosphere of *Ficus benghalensis* (banyan tree), is reported for the first time and subjected to polyphasic taxonomy. 16S rRNA gene sequence comparison revealed that the isolate is *Isoptericola*-like organism. The aim of the present study was to determine the exact taxonomic position of the isolate.

Materials and methods

Strains, cultivation and phenotypic characterization

Strain BKS 3-46^T was isolated from a soil sample collected from the rhizosphere of *F. benghalensis* (banyan tree), in Bhitarkanika mangrove forest, in the Indian state of Odisha (86′45″–87′17″E longitude and 20′17″–20′47″N latitude), by dilution plate technique on Zobell Marine Agar (ZMA; HiMedia, India) at 30 °C. To study its phenotypic characters, the isolate was routinely cultivated on TSA medium at 30 °C and maintained as glycerol stocks at −70 °C. The reference type strains *I. halotolerans* MTCC 11265^T, *I. nanjingensis* MTCC 11633^T, *I. chiayiensis* MTCC 11634^T, *I. variabilis* MTCC 11260^T, *I. hypogeus* MTCC 11257^T, *I. dokdonensis* MTCC 11635^T and *I. jiangsuensis* MTCC 11262^T were obtained from Microbial Type Culture Collection & Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.

Colony morphology, cell morphology, motility and Gram's reaction of the strain were determined by using standard methods (Barrow and Feltham 1993; Murray et al. 1994). Phenotypic characterization was performed using TSA as basal medium and strains were incubated at their optimum growth temperature. Physiological tests such as growth at different temperatures, pH (using biological buffers), NaCl concentrations and acid production from various carbohydrates and other biochemical tests were performed as described (Barrow and Feltham 1993; Smibert and Krieg 1994; Stanier et al. 1966; Takeuchi and Hatano 1998). VITEK® 2-GP cards were used as per the instructions of the manufacturer (bioMérieux).

Chemotaxonomic characterization and DNA base composition

Freeze-dried cells for chemotaxonomic analysis (except for fatty acid analysis) were prepared by harvesting the bacterial cells in the late exponential phase following their growth in Tryptic Soy Broth (TSB; HiMedia, India) at 30 °C for 2 days. Isoprenoid quinones were extracted and purified as described by Saha et al. (2005). For the determination of cellular fatty acids, strain BKS 3-46^T and the reference strains were grown on TSB medium at 30 °C for 2 days; cellular fatty acids were extracted, methylated and analysed by using Gas Chromatography according to the instructions of the Sherlock Microbial Identification System (MIDI, USA Version 4.0) as described previously (Sasser 1990; Pandey et al. 2002). Extraction of polar lipids was performed based on the modified protocol of Bligh and Dyer (1959). Two-dimensional TLC was run for identification of polar lipids according to procedures described by Komagata and Suzuki (1987). Lipid spots were detected using the following spray reagents: molybdatophosphoric acid (5 % w/v) in absolute ethanol, molybdenum blue spray reagent (1.3 %, Sigma), ninhydrin (0.2 % w/v) in acetone and anisaldehyde reagent (Sigma) for detection of total lipids, phospholipids, aminolipids and glycolipids respectively. Standard procedures were used to determine the diagnostic cell wall sugars and amino acids (Staneck and Roberts 1974). The absence of mycolic acids was confirmed by TLC (Minnikin and Goodfellow 1976). Total hydrolysis (4.0 N HCl, 100 °C, 16 h) of peptidoglycan for amino acids and peptides were separated by two dimensional ascending TLC as method described by Schumann (2011).

Phylogenetic analysis and genomic relatedness

For 16S rRNA gene sequencing, the genomic DNA extraction and amplification was performed as described previously (Mayilraj et al. 2006). Identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (Kim et al. 2012) and aligned using Mega version 5.0 (Tamura et al. 2011). Phylogenetic trees were constructed using the neighbour-joining as well as maximum likelihood and maximum parsimony algorithms. Bootstrap analysis was performed to assess the confidence limits of the

Table 1 Differential phenotypic characteristics of strain BKS 3-46^T and members of the genus *Isoptericola*

Characteristic	1	2	3	4	5	6	7	8
Oxidase	+	+	−	−	−	+	+	−
Growth at/with								
12 °C	+	+	+	+	+	+	+	−
42 °C	−	−	+	−	−	−	−	−
10 % NaCl (w/v)	+	−	+	−	+	−	+	+
Growth at pH 5.0	−	+	+	−	−	−	−	−
Hydrolysis of								
Casein	−	−	+	+	−	−	−	−
Gelatin	−	−	+	+	−	+	−	−
Hypoxanthine	+	+	+	+	+	+	−	+
Tyrosine	−	−	+	−	−	−	−	−
Urea	−	−	+	−	−	−	−	−
Xanthine	−	+	+	+	−	−	−	−
Nitrate reduction	−	−	+	+	+	+	−	−
Indole production	−	−	+	−	−	+	+	+
Methyl red	+	+	+	−	−	−	−	+
Utilization of								
D-Xylose	+	+	−	−	+	−	+	−
D-Galactose	−	+	+	+	+	+	+	+
D-Glucose	−	−	+	−	−	+	−	+
L-Arabinose	−	−	−	−	+	+	+	−
D-Mannose	−	+	+	+	+	−	+	+
Raffinose	−	+	−	−	−	+	−	−
Acid production from								
Inulin	−	+	−	+	+	−	+	−
Lactose	+	−	−	−	−	−	+	−
Melibiose	−	−	+	−	−	−	−	−
Trehalose	+	+	+	−	+	+	+	+
D-Xylose	+	−	−	−	−	+	−	+
Arginine dihydrolase 1	+	+	−	+	+	+	+	+
α-Glucosidase	+	+	+	−	+	+	+	−
Ala-Phe-Pro arylamidase	−	−	−	−	+	−	−	−
L-Aspartate arylamidase	−	−	−	+	−	−	−	−
α-Mannosidase	−	+	−	−	−	−	−	−
L-Pyrrolidonyl-arylamidase	+	−	−	−	+	−	−	+
Tyrosine arylamidase	−	+	+	−	−	−	−	+
D-Galactose	+	−	−	−	+	+	+	+
L-Lactate alkalinization	−	−	+	+	+	−	−	+
D-Maltose	−	−	−	−	+	−	+	−
D-Mannose	−	−	−	−	−	−	−	+
Salicin	−	−	−	−	−	+	−	−
Saccharose/sucrose	−	−	−	−	+	−	+	−
Arginine dihydrolase 2	+	+	+	+	+	+	−	+
Optochin resistance	−	−	−	−	+	−	−	−

Table 1 continued

Characteristic	1	2	3	4	5	6	7	8
Sensitivity to								
Ampicillin	S	R	R	R	R	R	R	R
Penicillin G	S	R	R	R	R	R	R	R
Novobiocin	S	S	R	S	S	S	S	S
Rifampicin	S	R	S	R	S	S	S	S
Cell wall sugars	Gl, X, R	Gl, X, M	Gl, G, R	Gl, G, R, M	Gl, R, X	Gl, G, R, Ri	Gl	Gl, G, R
Peptidoglycan type	@	@	@	#	@	@	@	$
Polar lipids	DPG, PG, PI	DPG, PG	DPG, PG, PI	DPG, PG, PI, PIM	DPG, PG, PI	DPG, PG, PI	DPG, PG, PI	DPG, PG, PI
DNA G+C (mol%)	70.4	72.4	70–72	73.8	70.3	74.1	72.8	72.8

All taxa are positive for Gram-staining, catalase, hydrolysis of aesculin and starch, utilization of glycerol, D-fructose, salicin, cellobiose, maltose, sucrose, starch, hydroxyproline, valine, cysteine, potassium nitrate, serine, histidine, threonine, methionine, arginine, phenylalanine. All taxa are negative for motility, utilization of citrate, erythritol, D-adonitol, L-sorbose, dulcitol, inositol, D-sorbitol, xylitol, D-fucose, potassium gluconate. In VITEK® 2-GP cards, all the taxa are positive for leucine arylamidase, l-proline arylamidase and alanine araylamidase; negative for D-amygdalin, phosphatidylinositol phospholipase C, β-galactosidase, cyclodextrin, β-galactopyranosidase, phosphatase, β-glucuronidase, α-galactosidase, D-ribose, D-sorbitol, polymyxin B resistance, L-lactate alkalinisation, lactose, *N*-acetyl-D-glucosamine, D-maltose, bacitracin resistance, novobiocin resistance, D-mannitol, methyl-β -D-glucopyranoside, pullulan, D-raffinose, O/129 resistance and D-trehalose

Strains 1, BKS 3-46^T; 2, *I. nanjingensis* H17^T; 3, *I. variabilis* MX5^T; 4, *I. hypogeus* HKI 0342^T; 5, *I. jiangsuensis* CLG^T; 6, *I. dokdonensis* DS-3^T; 7, *I. halotolerans* YIM 70177^T; 8, *I. chiayiensis* 06182 M-1^T. +, positive; −, negative; @, L-Lys-D-Asp; #, L-Lys-D-Glu; $, L-Lys-L-Ala-L-Glu-D-Asp; S, sensitive; R, resistance; GL, glucose; X, xylose; G, galactose; R, rhamnose; Ri, ribose; M, mannose

branching. The G+C content of genomic DNA was determined spectrophotometrically (Lambda 35, Perkin Elmer, Waltham, MA, USA) using the thermal denaturation method (Mandel and Marmur 1968). DNA–DNA hybridization was performed each time with freshly isolated genomic DNA and was repeated three times by the membrane filter method (Tourova and Antonov 1987).

Results and discussion

Phenotypic characterization

The detailed differential phenotypic properties are shown in Table 1 and also given in the species description. Phenotypic data presented in Table 1 indicate that strain BKS 3-46^T differs from the closely related species at least by 28 characters, which includes acid production from carbohydrates, sensitivity to antibiotics, casein hydrolysis, nitrate reduction, hydrogen sulphide production, assimilation of different carbon sources and other tests. Growth of the strain on TSA produced yellow pigment after 2 days of incubation.

Chemotaxonomic characterization and DNA base composition

Most of the chemotaxonomic properties of strain BKS 3-46^T (presented in the species description) are typical of members of the genus *Isoptericola*. The major fatty acids were identified as anteiso-branched $C_{15:0}$ (56.2 %), iso-branched $C_{15:0}$ (16.8 %) and $C_{16:0}$ (14.8 %). The fatty acid compositions of the reference strains analyzed were qualitatively similar to but quantitatively different from those of the novel strain (Table 2). The major polar lipids were identified as phosphatidylglycerol, diphosphatidylglycerol, phosphatidylinositol, three unknown phospholipids, two unknown glycolipids and one phosphoglycolipid (Supplementary Fig. S1). The major menaquinone detected for strain BKS 3-46^T was MK-9 (H_4). The peptidoglycan type was A4α L-Lys-D-Asp (Supplementary Fig. S2), type A11.31 according to www.peptidoglycan-types.info, which was compared and confirmed with the data presented for *I. dokdonensis* strain DS-3^T (Schumann 2011). The valid proof for the proposed peptidoglycan structure with D-aspartic acid directly linked to L-lysine is the dipeptide D-Asp-L-Lys

Table 2 Percentage of total cellular fatty acids from strain BKS 3-46^T and members of the genus *Isoptericola*

Fatty acid	1	2	3	4	5	6	7	8
$C_{14:0}$	2.9	4.9	4.6	1.8	1.1	3.3	1.9	2.3
$C_{15:0}$	–	–	–	–	2.6	2.6	–	–
$C_{16:0}$	14.8	6.3	5.2	2.9	3.1	9.0	22.1	7.3
$C_{17:0}$	–	–	0.3	0.2	1.2	1.4	–	1.8
iso-$C_{13:0}$	–	–	–	–	3.9	1.1	–	–
iso-$C_{14:0}$	1.8	1..1	1.5	5.4	3.4	4.2	1.4	3.2
iso-$C_{15:0}$	16.8	21.6	17.4	13.1	14.2	11.5	6.4	11.4
iso-$C_{16:0}$	2.1	5.8	6.0	11.6	1.3	3.0	2.3	6.9
iso-$C_{17:0}$	–	1.4	1.9	0.4	1.0	–	1.1	2.1
anteiso-$C_{15:0}$	56.2	50.6	51.6	55.0	60.0	58.6	52.4	52.0
anteiso-$C_{17:0}$	3.6	4.6	7.0	8.1	5.4	4.1	9.6	7.6

Data from present study. Fatty acids amounting to <0.5 % of the total fatty acids in strains are not shown

Strains 1, BKS 3-46^T; 2, *I. nanjingensis* H17^T; 3, *I. variabilis* MX5^T; 4, *I. hypogeus* HKI 0342^T; 5, *I. jiangsuensis* CLG^T; 6, *I. dokdonensis* DS-3^T; 7, *I. halotolerans* YIM 70177^T; 8, *I. chiayiensis* 06182 M-1^T; –, not detected

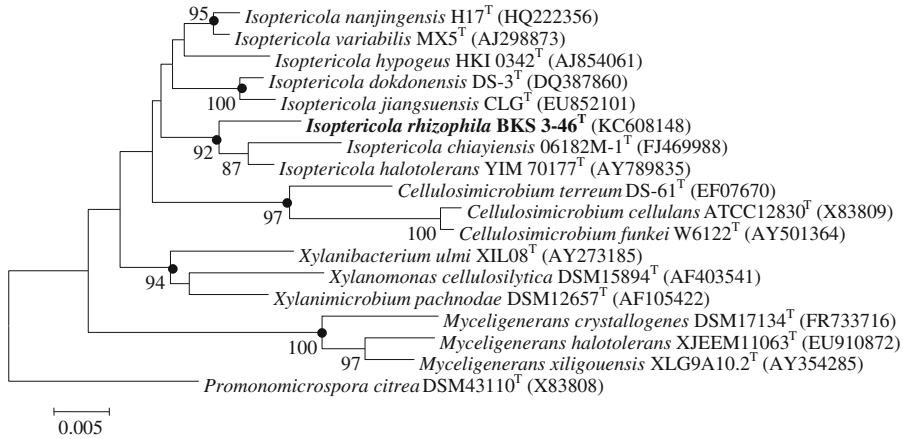

Fig. 1 Phylogenetic neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between *Isoptericola rhizophila* BKS 3-46^T and related taxa. *Promonomicrospora citrea* DSM 43110^T (X83808) was used as an outgroup. Bootstrap values (expressed as percentages of 100 replications) greater than 70 % are given at nodes. Branches recovered in the maximum-parsimony and maximum likelihood algorithms are indicated by filled circles. *Bar* 0.005 % sequence variation. GenBank accession numbers are given in *parentheses*

which is presented at the position labeled as "1". This peptide D-Asp-L-Lys is resistant towards hydrolysis and is therefore one of the rare exceptions that a peptide can be found in the total hydrolysate, which usually contains only amino acids and no peptides. The DNA G+C content of strain BKS 3-46^T was estimated to be 70.4 mol%, a value within the range (70–74.1 mol%) for members of the genus *Isoptericola*.

Phylogenetic analysis and genomic relatedness

Almost complete sequence (1410 bp) of 16S rRNA gene of strain BKS 3-46^T was determined (GenBank accession no. KC 608148) and compared with those of other closely related taxa retrieved from the GenBank database. Based on the 16S rRNA gene sequence identity, the strain could be assigned to the genus *Isoptericola.* Sequence analysis revealed that strain

BKS 3-46^T shared 16S rRNA gene sequence identity with *I. halotolerans* (98.6 %), followed by *I. nanjingensis* (98.4 %), *I. chiayiensis* (98.1 %), *I. variabilis* (98.0 %), *I. hypogeus* (97.7 %), *I. dokdonensis* (97.5 %) and *I. jiangsuensis* (97.3 %). The neighbour-joining phylogenetic tree, demonstrated that strain BKS 3-46^T forms a separate lineage along with the closely related species, this was also evident in the phylogenetic tree constructed using the maximum-parsimony and maximum likelihood algorithms, shown as closed circles at the nodes (Fig. 1). The DNA–DNA relatedness values between strain BKS 3-46^T and the closely related taxa were found to be *I. halotolerans* MTCC 11265^T (54.6 ± 0.8 %), followed by *I. nanjingensis* MTCC 11633^T (50.4 ± 0.6 %), *I. chiayiensis* MTCC 11634^T (46.0 ± 0.2 %), *I. variabilis* MTCC 11260^T (48.1 ± 0.6 %), *I. hypogeus* MTCC 11257^T (46.8 ± 0.2 %), *I. dokdonensis* MTCC 11635^T (39.4 ± 0.8 %) and *I. jiangsuensis* MTCC 11262^T (42.7 ± 0.8 %). These values are below the standard threshold value for bacterial species delineation (Wayne et al. 1987).

Conclusion

Based on the differential phenotypic characteristics such as oxidase, hydrolysis of hypoxanthine, utilization of D-xylose, acid production from lactose to trehalose, sensitivity to antibiotics such as ampicillin, penicillin G, novobiocin and rifampicin and genotypic results, strain BKS 3-46^T should be regarded as a new species of the genus *Isoptericola*. Table 1 shows the main features that distinguish strain BKS 3-46^T from all the validly named members of the genus. For the first time a species belonging to the genus *Isoptericola* isolated from rhizosphere of *F. benghalensis* (banyan tree) is reported. The polyphasic evidence gathered in this study allow the conclusion that strain BKS 3-46^T represents a novel species of the genus *Isoptericola*, for which the name *Isoptericola rhizophila* sp. nov. is proposed.

Description of *Isoptericola rhizophila* sp. nov.

Isoptericola rhizophila (rhi.zo'phi.la.Gr. n. *rhiza* a root; Gr. adj. *philos* on loving; N.L. fem. adj. *rhizophila* root-loving).

Gram-positive, non-acid-fast, aerobic bacterium. Forms lemon-orange coloured, circular, glistening, opaque colonies with an entire margin on TSA medium. Colony sizes are 0.5–2.0 mm. Cells are irregularly spherical or rods, occurring in pairs and clusters. Cells vary in size (0.6–1.0 μm wide by 1.0–1.5 μm long). Does not form endospores. Non-motile. No mycelial growth phase is observed. Catalase-positive and oxidase-negative. Tolerates up to 7.0 % NaCl and grows at temperatures between 25 and 37 °C, with optimum growth 30 °C. Growth occurs at between pH 6 and 10, with optimum growth at pH 8.0. Positive for decomposition of casein, starch and gelatin. Negative for indole, urease and hydrogen sulfide production, methyl red and Voges–Proskauer reactions, utilization of citrate, decomposition of aesculin and tyrosine, and for nitrate reduction. Acid is produced from L-rhamnose, D-fructose and trehalose. Acid is produced weakly from L-arabinose but not from D-cellobiose, *myo*-inositol, D-mannitol, D-maltose, D-galactose, glycerol, salicin, sucrose or D-xylose. Utilization of various compounds as sole carbon sources is detailed in Table 1. The dipeptide in cell wall hydrolyzates was A4α L-Lys-D-Asp. The whole-cell sugars are galactose, xylose and mannose. The predominant lipids are diphosphatidylglycerol, phosphatidylglycerol, one unknown glycolipid and other unidentified lipids. No mycolic acids are present. Contains major amounts of anteiso-branched $C_{15:0}$, iso-branched $C_{15:0}$ and $C_{16:0}$ fatty acids. The major menaquinone is MK-9 (H_4). The DNA G+C content of the type strain is 70.4 mol%.

The type strain BKS 3-46^T (=MTCC 11080^T=JCM 19252^T) was isolated from a soil sample collected from rhizosphere of *F. benghalensis* (banyan tree), Bhitarkanika mangrove forest, in the Indian state of Odisha. The GenBank accession number for the 16S rDNA sequence of strain BKS 3-46^T is KC 608148.

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