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Description of a Novel Actinobacterium *Microbacterium assamensis* sp. nov., Isolated from Water Sample Collected from the River Brahmaputra, Assam, India

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Abstract A Gram-positive, yellow pigmented actinobacterium, strain S2-48^T was isolated from water sample collected from the river Brahmaputra, Assam, India and subjected to a polyphasic taxonomic study. Most of the physiological and biochemical properties, major fatty acids ($C_{15:0}$ Anteiso, iso $C_{16:0}$ and $C_{17:0}$ Anteiso), estimated DNA G+C content (70.2 mol%) and 16S rRNA gene sequence analysis showed that strain S2-48^T belonged to the genus *Microbacterium*. Strain S2-48^T exhibited highest 16S rRNA gene sequence similarity with *Microbacterium testaceum* (97.0%); however, the DNA–DNA relatedness value between strain S2-48^T and *M. testaceum* was 9.1%. On the basis of differential phenotypic characteristics and genotypic distinctiveness, strain S2-48^T should be classified within the genus *Microbacterium* as a novel species, for which the name *Microbacterium assamensis* is proposed. The type strain is S2-48^T (=MTCC 10486^T = DSM 23998^T).

The GenBank accession number for the 16S rRNA gene sequence of strain S2-48^T is HM146190

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Introduction

The genus *Microbacterium* was first described by Orla-Jensen, [9] and later on emended by Collins et al. [4] and Takeuchi and Hatano [18]. The genus *Microbacterium* was isolated from a wide range of environmental habitats like soil, insects, plants, clinical samples, water, fermented products and marine environments. At present the genus *Microbacterium* consists of 67 species with validly published names (http://www.bacterio.cict.fr/m/microbacterium.html). Here, we describe the taxonomic status of an actinobacterium strain S2-48^T isolated from surface water collected from the river Brahmaputra, Assam, India (93° 08′ E–93° 36′ E and 26° 30′ N–26° 45′ N) by using polyphasic taxonomy.

Materials and Methods

Strains, Cultivation and Phenotypic Characterization

The strain S2-48^T was isolated by dilution plate technique on tryptic soy agar medium (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 strain *M. testaceum* MTCC 10232^T (=DSM 20166^T) was obtained from Microbial Type Culture Collection & Gene Bank (MTCC), Chandigarh, India. Colony morphology, cell morphology, motility and Gram's reaction of the strain were determined by using standard methods [1, 8, 10]. 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 [1, 15, 17, 18]. The API ZYM and API 20NE micro test strips were used as per the instructions of the manufacturer (bioMérieux). Sensitivity of the strain to antibiotics was tested by using antibiotic susceptibility discs (HiMedia, India) after incubation of 48 h.

Chemotaxonomic Characterization and DNA Base Composition

Freeze-dried cells for chemotaxonomic analysis (except for fatty acid study) 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. [12]. Fatty acid methyl ester analysis was performed by using gas chromatography according to the instructions of the Sherlock Microbial Identification System (MIDI, Newark, DE, USA) as described previously [11]. Extraction of polar lipids was done based on the modified protocol of Bligh and Dyer [2]. Two-dimensional TLC was run for identification of polar lipids according to procedures described by Komagata and Suzuki [5]. 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. The peptidoglycan structure of cell wall of the strain S2-48^T was determined by using established procedures [13, 14].

Phylogenetic Analysis and Genomic Relatedness

For 16S rRNA gene sequencing the genomic DNA extraction and amplification was performed as described previously [7]. Identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (http://www.eztaxon.org/; Chun et al. [3]). The 16S rRNA gene sequence of S2-48^T and the closely related species were retrieved from Eztaxon server and aligned using the MEGA software version 4 [19]. Phylogenetic trees were constructed using the neighbour-joining as well as maximum parsimony algorithms. Bootstrap analysis was performed to assess the confidence limits of the branching. The G+C content of genomic DNA was determined spectrophotometrically (Lambda 35, Perkin Elmer, Waltham, MA, USA) using thermal denaturation method [6]. DNA–DNA hybridization was performed each time with freshly isolated genomic DNA and was repeated three times by the membrane filter method [20].

Results and Discussion

Phenotypic Characterization

The detailed differential phenotypic properties are shown in Table 1 and also mentioned in species description. Phenotypic data presented in the table indicated that strain S2-48^T differed from the closely related species at least by 29 characters, which includes acid production from carbohydrates, sensitivity to antibiotics, casein hydrolysis, nitrate reduction, hydrogen sulphide production, assimilation of different carbon sources and other mentioned tests. Growth of the strain on TSA produced yellow pigment after two days of incubation.

Chemotaxonomic Characterization and DNA Base Composition

Most of the chemotaxonomic properties of strain S2-48^T (presented in the species description) were typical of members of the genus *Microbacterium*. The major fatty acids ($C_{15:0}$ Anteiso, iso $C_{16:0}$ and $C_{17:0}$ Anteiso) detected in the novel strain (presented in the species description) were consistently found in members of the genus *Microbacterium*. The fatty acid compositions of the reference strain *M. testaceum* assayed were qualitatively similar to but quantitatively varied from those of the novel strain and particularly presence of $C_{16:0}$, iso$C_{17:0}$ and $C_{18:0}$ (Table 2). The major polar lipids were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), two unknown aminolipids (AL) and a mannose containing glycolipid (MGL). The major menaquinones detected for the strain S2-48^T were MK-12 and MK-13. The diamino acid in cell wall hydrolyzates was ornithine and the peptidoglycan type B2β. The DNA G+C content of strain S2-48^T was estimated to be 70.2 mol%, a value within the range (67–71.6 mol %) for the genus *Microbacterium* [18].

Phylogenetic Analysis and Genomic Relatedness

Almost complete sequence (1477 bp) of 16S rRNA gene of strain S2-48^T was determined (GenBank accession no. HM 146190) and compared with those of other closely related taxa retrieved from the GenBank database. Sequence analysis revealed that strain S2-48^T shared 16S rRNA gene sequence identity with *M. testaceum* (97.0%). Based on the 16S rRNA gene sequence identity, the strain could be assigned into the genus *Microbacterium*. The neighbour-joining phylogenetic tree as well as maximum parsimony algorithms (figure not shown) demonstrated that strain S2-48^T formed a separate lineage along with the closely related species (Fig. 1). However, the DNA–DNA relatedness values between strain S2-48^T and the closely related

Table 1 Characters that differentiate strain S2-48^T from the closest species *M. testaceum* (DSM 20166^T): 1, strain S2-48^T and 2, *M. testaceum*

Characteristics	1	2
Growth at pH 11	+	−
Growth at 12°C	−	+
Casein hydrolysis	−	+
Enzyme assayed for (API ZYM)		
Leucine arylamidase	−	+
Valine arylamidase	−	+
Cystine	−	+
Trypsin	−	+
α-galactosidase	−	+
α-glucosidase	−	+
β-glucosidase	−	+
N-acetyl-β-glucosaminidase	−	+
α-mannosidase	−	+
Assimilation of (API 20 NE)		
Arabinose	−	+
N-acetyl-glucosamine	−	+
Potassium gluconate	−	+
Malic acid	−	+
Sensitivity to antibiotics (μg disc^{-1})		
Nitrofurantion (300)	S	R
Norfloxacin (10)	S	R
Polymyxin B (300)	S	R
Cephalothin (30)	S	R
Oxacillin (5)	S	R
Hydrogen sulphide production	−	+
Nitrate reduction	−	+
Acid production from		
Adonitol	−	+
Raffinose	−	+
Rhamnose	−	+
Cellobiose	−	+
Arabinose	−	+
Xylose	−	+

Both the strains are positive growth at pH 8.0 to 10.0; NaCl concentration up to 7%; temperature between 20 and 37°C. Positive for citrate utilization, gelatin liquefaction; for enzyme activities of (using API ZYM) alkaline phosphatase, esterase (C4), esterase lipase (C8), α-chymotrypsin, acid phosphatase, napthol-AS-B1-phosphohydrolase, β-galactosidase and negative for lipase (C14), β-glucoronidase and α-fucosidase. Both the strains are positive (using API 20 NE) for hydrolysis esculin and gelatin, 4-nitrophenyl-β-D-galactopyranoside ONPG, nitrate reduction, urease, D-glucose fermentation, assimilation of glucose, citrate, D-mannitol, D-mannose and negative for indole production, MR-VP, growth at pH 5.0 and 12.0, NaCl; starch hydrolysis; arginine dihydrolase, assimilation of capric acid, adipic acid and phenylacetic acid. Both the strains are sensitive to antibiotics (μg disc^{-1}) novobiocin (30), streptomycin (10), ampicillin (10), methicillin (5), penicillin G (10 U), neomycin (30), triple sulphas (300), sulfonamide (300), kanamycin (30), trimethoprim (5), colistin (10), rifampicin (2), lincomycin (2), cepoxitin (30), gentamycin (10) and bacitracin (8) and resistance to optochin and oxytetracycline (30). Postive for acid production from carbohydrates sucrose, trehalose, salicin, dextrose, maltose, sorbitol, mannitol, mannose, fructose and inositol; negative for melibiose, dulcitol, lactose and galactose. All the data were obtained from the present study

Table 2 Cellular fatty acid composition of strain S2-48^T along with the closest species *M. testaceum* (DSM 20166^T): 1, strain S2-48^T and 2, *M. testaceum*

Fatty acid	1	2
Iso $C_{14:0}$	4.06	0.96
Iso $C_{15:0}$	10.48	7.04
Iso $C_{16:0}$	33.76	22.62
$C_{16:0}$	–	2.0
Iso $C_{17:0}$	–	3.06
$C_{15:0}$ ANTEISO	35.61	40.10
$C_{17:0}$ ANTEISO	16.09	23.47
$C_{18:0}$	–	0.75

Data from present study

taxa was 9.1% (±0.6%), which were well below the 70% threshold value recommended for the delineation of bacterial species [21]. DNA–DNA relatedness values between strain S2-48^T and the remaining type strains of the genus *Microbacterium* were not determined, since organisms with more than 3% 16S rRNA gene sequence dissimilarity belong to different genomic species [16].

Conclusion

Based on the phenotypic and genotypic results, strain S2-48^T has to be regarded as a new species of *Microbacterium*. Table 1 shows the main features that distinguish strain S2-48^T from the closely related taxa *M. testaceum*. Therefore, the polyphasic evidences gathered in this study conclude that strain S2-48^T represents a novel species of the genus *Microbacterium*, for which the name *Microbacterium assamensis* sp. nov. is proposed.

Description of *Microbacterium assamensis* sp. nov

Microbacterium assamensis (as.sam.en'sis. N.L. masc. adj. *assamensis*, pertaining to Assam, a north-eastern state in India, where the type strain was isolated).

Cells are Gram-positive, non-spore forming, aerobic, motile, catalase-positive, oxidase-positive, short rods (0.8–1.0 × 2.0–2.5 μm). The colonies are yellow pigmented, circular, opaque and convex, with smooth margin on TSA. Growth occurs at 25–37°C (optimal temperature is 30°C), at a pH of 6.5–11.0 (optimal pH is 7.0) and at 2–7% (w/v) NaCl. The strain does not grow on Simmon's citrate and MacConkey agar and do not reduce nitrate to nitrite. Detailed phenotypic characteristics are given in Table 1. The predominant fatty acids are ($C_{15:0}$ Anteiso, iso $C_{16:0}$ and $C_{17:0}$ Anteiso). The major polar lipids are phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), two

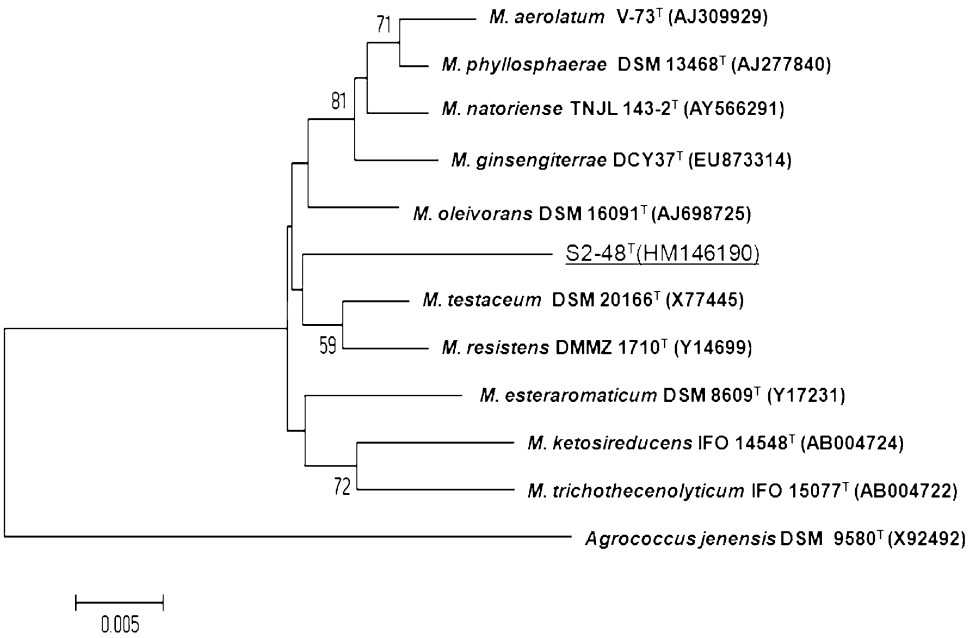

Fig. 1 Phylogenetic neighbour-joining tree based on 16S rRNA gene sequences showing the relationship between *Microbacterium assamensis* S2-48^T and related members of the genus *Microbacterium*. *Agrococcus jenensis* DSM 9580^T (X92492) was used as an out-group. Numbers at nodes indicate levels of bootstrap support $\geq$50% based on a neighbour-joining analysis of 100 re-sampled datasets. GenBank accession numbers are given in parentheses. *Bar*, 5% sequence variation

unknown aminolipids (AL) and a mannose containing glycolipid (MGL). The major menaquinones detected for the strain S2-48^T are MK-12 and MK-13. The diamino acid in cell wall hydrolyzates was ornithine and the peptidoglycan type B2β. The DNA G+C content is 70.2 mol%. The type strain S2-48^T (MTCC 10486^T = DSM 23998^T) was isolated from water sample collected from the river Brahmaputra, Assam, India.

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