

Myroides gitamensis sp.nov., L-asparaginase Producing Bacteria Isolated from Slaughter House Soil Sample in Visakhapatnam, India

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Abstract

A novel strain designated as BSH-3^T was isolated from the slaughter house soil sample in Visakhapatnam, India. Cells of this strain are Gram-negative, aerobic, yellowish, rod shaped, non-motile and non-spore forming bacteria. This isolate was observed to grow optimally at 30°C and pH 7.0. It requires 1- 6% (w/v) NaCl for its growth. Phylogenetic analysis based on 16S rRNA gene sequence revealed that the strain BSH-3^T belongs to the genus *Myroides* and is closely related to *Myroides odoratimimus* (98.4 %). However, DNA-DNA hybridization with *M. odoratimimus* JCM 7460^T showed a relatedness of 48.2% with respect to strain BSH-3^T. The major cellular fatty acids identified were iso-C_{15:0} and iso-C_{17:0} 3-OH and summed feature 3 (comprising iso-C_{15:0} 2OH and/or C_{16:1} ω7c). The polar lipids of the strain BSH-3^T were phospholipids, aminolipids and phosphatidyl ethanolamine. The G + C content of the genomic DNA was determined to be 33.8 mol%. The enzyme production was carried out by submerged fermentation and the maximum enzyme activity was 85.7 IU showed by 24 hrs BSH-3^T culture at 37°C. Based on the morphological, phenotypic characteristics, phylogenetic inference and chemotaxonomic studies, strain BSH-3^T is proposed as a novel species *Myroides gitamensis* sp.nov. in the genus *Myroides*. The type strain is BSH-3^T (= MTCC 11601^T = BCC 64301^T = MCC 2182^T). The GenBank/EMBL / DDBJ accession number for the 16S rRNA gene sequence of strain BSH-3^T is HF571338.

Keywords: BSH-3^T; *Myroides gitamensis*; 16S rRNA gene; L-asparaginase

Introduction

In the course of screening L-asparaginase producing bacteria from different soil samples in Visakhapatnam, India, many bacterial strains have been isolated and characterized taxonomically. In the present investigation, one of the isolates was found to be novel and designated as BSH-3^T. 16S rRNA gene sequence analysis indicated that the novel strain BSH-3^T belongs to family Flavobacteriaceae and genus *Myroides*. This family was first mentioned by Reichmbach in the first edition of Berger's manual of systematic bacteriology in 1989. At present, the classification of Flavobacteriaceae is still in development stage. The genus *Myroides* was established by the reclassification of *Flavobacterium odoratum* as *Myroides odoratus*, the type species, on the basis of phenotypic, genomic and chemotaxonomic studies [1]. *M. odoratum*, *M. odoratimimus* [2], *M. pelagicus* [3], *M. profundus* [4], *M. marinus* [5] are the species present in the *Myroides* family. The main source for *Myroides* is clinical specimens, wet environment [6], insect gut [7], and marine sample [3]. *Myroides* strains show susceptibility to β-lactam antibiotics [8]. Nabeel et al. [9] have reported on the production of L-asparaginase from different microbial species. This is the first available report of L-asparaginase production from the *Myroides* species.

In this study, taxonomic characteristics of novel strain BSH-3^T isolated from the slaughter house soil sample in Visakhapatnam, India is being reported. On the basis of phylogenetic analysis, phenotypic characteristics, chemotaxonomic data, the isolate is considered to represent a novel species in the genus *Myroides*, for which the name *Myroides gitamensis* sp.nov is proposed.

Materials and Methods

Isolation and screening

Strain BSH-3^T was isolated from slaughter house (17°68'N

83°21'E) soil sample of Visakhapatnam, India. The collected soil sample was serially diluted upto 10⁻¹ to 10⁻⁶ and plated on modified Czapek Dox's agar medium which has the composition of glucose-2.0, L-asparagine-10.0, KH₂PO₄-1.52, KCl-0.52, MgSO₄·7H₂O-0.52, CuNO₃·3H₂O-trace, ZnSO₄·7H₂O-trace, FeSO₄·7H₂O-trace (grams per liter of distilled water) at pH 6.2 and 0.09 % (v/v) phenol red was added as an indicator to the medium [10]. The plates were incubated at 37°C for 48 hr. To avoid the fungal contamination, nystatin (50 µg/mL) was added to the medium. Control plates were without L-asparagine (NaNO₃ was added as nitrogen source instead of L-asparagine) and phenol red dye. Strains with pink color zones around the colonies were considered as L-asparaginase producing strains (Figure 4).

Estimation of L-Asparaginase activity

L-asparaginase activity was estimated according to Mashburn and Wriston [11]. In this assay, the reaction mixture consisting of 100 µL enzyme extract, 200 µL 0.05 M Tris-HCl buffer (pH 8.6) and 1.7 mL of 0.01 M L-asparagine was incubated for 10 min at 37°C. The rate of hydrolysis of L-asparagine was determined by measuring the released ammonia using Nessler's reagent.

The reaction was stopped by the addition of 500 µL of 1.5 M TCA.

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Received February 23, 2014; Accepted March 30, 2014; Published March 25, 2014

Citation: Prasad Talluri VSSL, Bhavana M, Siva Kumar K, Anil Kumar P, Rajagopal SV (2014) *Myroides gitamensis* sp.nov., L-asparaginase Producing Bacteria Isolated from Slaughter House Soil Sample in Visakhapatnam, India. J Microb Biochem Technol 6: 144-147. doi:10.4172/1948-5948.1000135

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Then the reaction mixture was centrifuged at 3000 rpm at 4°C for 10 min. The supernatant of 0.5 mL was diluted to 7 mL with distilled water and 1 mL of Nessler's reagent was added. The reaction mixture was incubated on boiling water bath for 10 min and then cooled. The absorbance (A500) was recorded using UV-Vis spectrophotometer (Shimadzu U-800). One Unit (U) of L-asparaginase is defined as the amount of enzyme required to liberate one μmol of ammonia per min at 37°C

Morphological, phenotypic and biochemical characterization

Cell morphology was observed under Nikon inverted microscope (Eclipse TE200-U) and Scanning electron microscope (JEOL JM – 5600). Fully grown 24 hr culture of BSH-3^T was taken and fixed for 2hr in 2% formalin [12]. After washing with saline solution, the culture was dehydrated in 30–100% water - ethanol series. The air-dried bacterium was coated with thin layer of platinum in Gatancryostage (Hitachi S-900 FESEM) and was observed using JEOL JM – 5600 electron microscope at 20 kV accelerating voltage with a 5–6 nm electron beam.

Growth of strain BSH-3^T was assessed (i) on nutrient agar (NA: HIMEDIA); (ii) at 5, 10, 16, 25, 30, 37, 42, 46, 52, 57°C in Nutrient Broth (NB); (iii) at pH 3, 4, 5, 6, 7, 8, 9, 10, 11 in NB using 0.1 N HCl and 0.1 N NaOH and (iv) in the presence of 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 15 and 18% (w/v) NaCl in NB initially devoid of NaCl. Endospore formation was determined by malachite green staining [13]. Gram staining was done according to Gram [14]. Motility was observed by hanging drop method using 18 hr BSH-3^T culture [15]. Different biochemical tests namely Indole test, MR-VP test, Simmons citrate, Starch hydrolysis, H₂S production, Catalase, Oxidase, Urease, Nitrate reduction test, Gelatin hydrolysis test were performed [16]. Effects of Tween 20, 40, 60 and 80 (1% w/v; Sigma) on BSH-3^T were determined [17].

16S rRNA sequencing

Genomic DNA of the BSH-3^T was isolated as described by Sambrook et al. [18]. Gene specific for 16S rRNA coding regions were amplified by PCR [19] using universal primers, forward (5'-AGTTTGATCCTGGCTCAG-3') and reverse (5'-GGCT/TACCTGTACGACTT-3'). The numbering of positions in the 16S rRNA gene fragments was based on the *E.coli* numbering system [20]. Amplified 16S rRNA gene products were purified and sequenced with an ABI PRISM big dye terminator cycle sequencing Ready Reaction kit on an ABI PRISM model 310 genetic analyzer. 16S rRNA gene sequence from the strain BSH-3^T was subjected to BLAST sequence for similarity search to identify the nearest taxa. The 16S rRNA gene sequence of the nearest taxa, belongs to family *Flavobacteriaceae*, were downloaded [21]. Multiple alignment of the sequence was aligned using the CLUSTAL_W in MEGA5 [22]. Phylogenetic trees were constructed using the neighbor-joining [23] as well as maximum parsimony algorithms and maximum likelihood algorithms [24]. Bootstrap analysis was performed to assess the confidence limits of the branching.

G+C content

Genomic DNA was isolated by using the method of Marmur [25] and the mol% G + C content was determined from melting point (T_m) curves [26] using Lambda 35; Perkin Elmer spectrophotometer equipped with Templab 2.0 software package.

Chemotaxonomic studies

For cellular fatty acids determination, strain BSH-3^T was grown on Nutrient agar plates at 37°C for 2 days. Cellular fatty acid methyl ester (FAME's) was isolated from cells by methylation, saponification and extraction following the standard protocol of Sherlock microbial identification system (MIDI). Separated cellular FAME's was identified by using Gas chromatography (Agilent 6890 series GC system) [27]. Polar lipids were extracted and analyzed from 36 hrs culture freeze dried cells. They were analyzed by two dimensional (2D) TLC followed by spraying with proper detection reagents [28]. Respiratory quinines were established and determined by HPLC (Agilent 1200 series) by using solvents acetonitrile: isopropanol (65:35), C18 column at 240nm wavelengths and the flow rate was maintained at 1mL min⁻¹ [29].

DNA- DNA hybridization was carried out to assess the DNA relatedness level between strains BSH-3^T and JCM 7460^T in triplicates using the DIG high prime DNA labeling kit (Roche Applied science) according to the manufacturer's instruction [30]. The consequential hybridization signals were captured and analyzed with Bio-Rad Quantity one software (version 4.62). The signals formed by hybridization of the probe to the homologous target DNA were calculated to be 100% and signal intensities by the self-hybridization of serial DNA dilutions were used for the calculation of the levels of DNA relatedness between strain BSH-3^T and JCM 7460^T. The hybridization experiments were always incorrigible by cross-hybridization.

Results and Discussion

Cells of the strain BSH-3^T were found to be Gram-negative, rod shaped, 0.3-0.4×1.4-1.6 μm in width and length (Figure 1) and non-motile. The diameter of *M. odoratus* is relatively very high (11-12 μm) compared to remaining species. All the cells of *Myroides* sps are having rod shaped morphology. Growth of strain BSH-3^T was found to occur in the pH range 5.0-9.0, with optimum growth at pH 7.5. The temperature range for growth was determined to be 10-40°C, with an optimum temperature being 37°C. No growth occurred at 50°C. The identified strain was found to be slightly halophilic depending on the extent of its salt tolerance. The optimum growth occurred between salinity of 2-4% (NaCl, w/v) and upto 6% NaCl (w/v) is tolerated.

Colonies were found to be circular, flat, slimy and yellowish color. Strain BSH-3^T was determined to be positive for catalase, oxidase, urease, voges- proskauer, gelatin hydrolysis, citrate and negative for

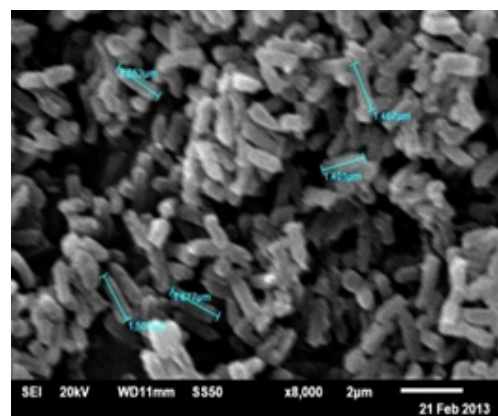


Figure 1: Scanning electron micrograph Image of Strain BSH-3^T grown on Nutrient agar medium for 24 hr at 37°C.

methyl red, casein hydrolysis, arginine dihydrolase, starch hydrolysis, esculin hydrolysis. Almost all strains of *Myroides* sps exhibits negative for hydrolysis of casein except *M. profundus*. The type strain BSH-3^T is sensitive to Vancomycin (30 µg), Ciproflaxacin (5 µg), Ofloxacin (5 µg), Sparfloxacin (5 µg), Gatifloxacin (5 µg), Cefradaine (30 µg), Erythromycin (10 µg), Linomycin (10µg), Cloxacillin (15 µg), but resistant to Aztreonam (30 µg), Azithromycin (15 µg), Dobyxy cycline hydrochloride (30 µg), Cefuroxime (30 µg), Penicillin-G (2 Units), Tetracycline (10 µg) and Co-trimoxazole (25 µg). Except *M. odoratus*, remaining five species showed positive for Leucinearyl amidase. The fatty acid profile of strain BSH-3^T found to be dominated by branched and hydroxy fatty acids, notably iso-C_{15:0} (48.6 %), aneteiso-C_{15:0} (5.8%), iso-C_{13:0} (9.7%) and iso-C_{15:0} 3-OH (7.5%). The iso-C_{15:0} value of BSH-3^T is high (48.6) compared to other five *Myroides* species i.e., *M. odoratus* (43.7), *M. odoratimimus* (44.7), *M. profundus* (27.6), *M. pelagicus* (29.3), *M. marinus* (24.2). The polar lipids of the strain BSH-3^T were one unidentified lipid (UL), five identified aminolipids (AL1-AL5) and one identified phosphatidyl ethanolamine (PE) and three phospholipids (PL1,PL2,PL3) identified by two dimensional thin layer chromatography (Figure 3). DNA-DNA hybridization of strain BSH-3^T with *M. odoratimimus* JCM 7460^T showed a relatedness of only 48.2 %, which is lower than the proposed threshold value of 70% [31]. The G+C content of strain BSH-3^T was determined to be 33.8 mol% (Tm), which is in the accordance with the levels (33.8 to 36.7 mol%) for the genus *Myroides* [5]. The strain BSH-3^T shows maximum enzyme activity was 85.7 IU/gds by 24 hr culture at 37°C under solid state fermentation.

Phylogenetic analysis based on 16S rRNA gene sequence (1449 base pairs) of strain BSH-3^T (GenBank/ EMBL / DDBJ accession number HF571338) and other closely related to *M. odoratimimus* with pairwise sequence similarity of 98.4%. Phylogenetic tree constructed with neighbor-joining, maximum-likelihood and maximum- parsimony Figure 2.

Therefore, on the basis of phenotypic and phylogenetic differences with the most closely related type strain (Table 1), BSH-3^T is considered as a representative of novel species in the genus *Myroides*, for which the name *Myroides gitamensis* sp. nov.is proposed

Description of *Myroides gitamensis* sp. Nov.

Myroides gitamensis (gi.tam.en'sis. L. masc. adj. GITAM is the university place in Visakhapatnam, where the type strain was isolated).

The strain BSH-3^T were determined to be Gram-negative, rod shaped, 0.3-0.4×1.4-1.6 µm (Figure 1) and non-motile. Colonies were circular, flat, slimy and yellowish color (Table 2). The strain was observed to grow between 10 to 40°C and from pH 5–9, with

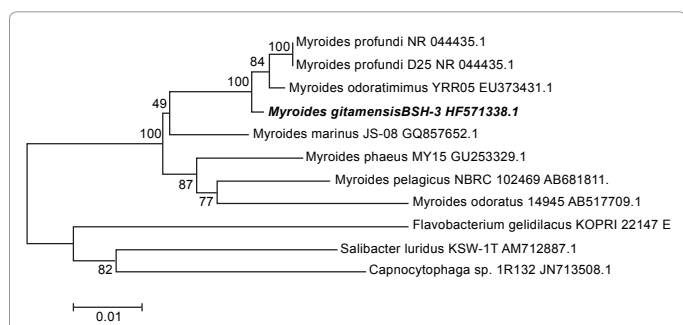


Figure 2: Neighbor-joining tree showing the phylogenetic relationship of strain BSH-3^T and phylogenetically related species of the genus *Myroides* based on 16S rRNA gene sequences. Bar, 1 nucleotide substitution per 100 nucleotides.

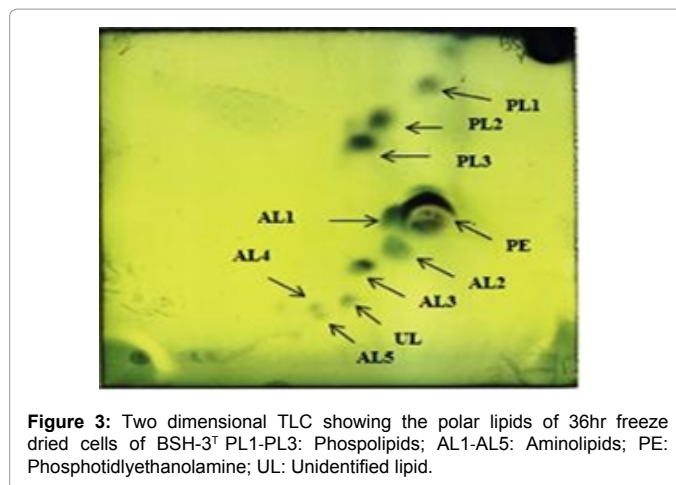


Figure 3: Two dimensional TLC showing the polar lipids of 36hr freeze dried cells of BSH-3^T PL1-PL3: Phospholipids; AL1-AL5: Aminolipids; PE: Phosphatidylethanolamine; UL: Unidentified lipid.

Fatty acid	1	2	3	4	5	6
i-C _{15:0} 3OH	5.8	4.7	5.8	4.6	6.1	5.8
i-C _{17:1} w9C	7.5	2.4	15.1	20.1	13.1	13.8
C _{16:0} 3OH	5.9	6.9	4	3.6	2.8	1.5
i-C _{17:0} 3OH	10.4	18.1	12.5	10.5	10.1	21.2
Summed Feature 3	11.7	16.8	9.5	6.7	1.9	1.3

Strains: (1) BSH-3^T, (2) *M. marinus* JS-08^T, (3) *M. odoratimimus* JCM7460^T, (4) *M. profundus* DSM19823^T, (5) *M. pelagicus* KCTC12661^T, (6) *M. odoratus* JCM7458^T Data were obtained in this study are taken from Cho et al. [5] and are percentages of total fatty acids, 'tr' indicates trace amount less than 1.0% of the total. Prefix esi, OH and Cre presents iso-branched, hydroxyl fatty acids and cis isomer respectively

Table 1: Cellular fatty acid contents of strain BSH-3^T (*Myroides gitamensis*) and closest related strains of genus *Myroides*.

Characteristics	1	2	3	4	5	6
Habitat	Slaughter house soil	Seawater	Wound swab	Deep Sea sediment	Seawater	Feaces
Cell diameter	1.4-1.8	0.6-1	3.5-4	1.6-2.4	0.5-1	12-12
Pigmentation	Yellow	Yellow to orange	Pale yellow	Pale yellow	Yellow to orange	Yellow
Rods	Short rods	Rods	Rods	Spindle-shaped rods	Short rods	Rods
Growth temperature (°C)	10-37	10-37	10-37	10-42	10-37	20-37
pH range	5-9	5-9	6-9	5-9	5-9	6-9
NaCl range (% w/v)	0.5-6	0-5	0-6	0-8	0-9	0-5
Urease	+	+	+	-	-	+
Nitrate reductase	-	-	+	-	+	+
Hydolysis of:						
Casein	-	-	-	+	-	-
Tween 20	+	-	+	+	-	-
Tween 80	+	-	-	+	-	-
Activity of:						
Trypsin	-	+	-	-	-	-
Leucinearyl amidase	+	+	+	+	+	-
Valinearyl amidase	-	+	-	-	-	-
Esterase (C4)	-	+	+	-	-	+
DNA G+C content (mol%)	33.8	34.2	31.7	33	33.6	34.7

Strains: (1) BSH-3^T, (2) *M. marinus* JS-08^T, (3) *M. odoratimimus* JCM7460^T, (4) *M. profundus* DSM19823^T, (5) *M. pelagicus* KCTC12661^T, (6) *M. odoratus* JCM7458^T [2-4]

Table 2: Features that distinguish *Myroides gitamensis* sp. nov. from the related *Myroides* species.



Figure 4: Screening of L-asparaginase producing bacteria using modified czapekdox's containing L-asparagine and phenol red indicator agar.

optimum growth at pH 7.5. Optimum growth was observed to occur between salinity 0.5 to 6.0 % (NaCl, w/v). Strain BSH-3^T was determined to be positive for catalase, oxidase, urease, voges- proskauer, gelatin hydrolysis, citrate and negative for methyl red, casein hydrolysis, arginine dihydrolase, starch hydrolysis, esculin hydrolysis. The acid production form is negative for adinotol, sorbitol, dextrose, fructose, inositol, lactose, maltose, mannitol, raffinose and rhamnose. Hydrolysis of tween 40, 60, 80 compounds shows positive. The major fatty acids are iso-C_{15:0}, iso-C_{17:0} 3-OH. The polar lipids of the strain BSH-3^T were three identified phospholipids, one unidentified lipids, five identified aminolipids and one identified phosphatidyl ethanolamine by two dimensional thin layer chromatography and summed feature 3 contains iso-C_{15:0} 2OH and/or C_{16:1} ω7c. The major quinone system is menaquinone-7 (MK-7). The DNA G + C content of this type strain is 33.8 mol%.

From the present investigation, we conclude that the type strain, BSH-3^T (=MTCC 11601^T = BCC 64301^T = MCC 2182^T), isolated from slaughter house soil sample in Visakhapatnam, India was found to be novel. The strain was deposited with the GenBank/ EMBL / DDBJ with accession number for the 16S rRNA gene sequence of strain BSH-3^T is HF571338.

Acknowledgement

The authors are thankful to GITAM university management for providing research facilities for carry out this work. The authors also acknowledge the help of MTCC (Chandigarh), MCC (Pune) and BCC (Thailand) for deposition of this strain as a type strain and providing accession number.

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