Pseudogracilibacillus marinus sp. nov., isolated from a biofilm formed in coastal seawater

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A Gram-staining-positive, aerobic, motile, rod-shaped (0.4–0.5 × 2.0–4.0 μm), endospore-forming bacterium, designated strain NIOT.bflm.S4T, was isolated from biofilm formed on high-density polyethylene test coupons in coastal seawater. The strain required seawater for growth. It grew with 1.0–8.0 % (w/v) NaCl, at 4–45 °C and at pH 6.5–9.0, with optimum growth with 4.0–5.0 % (w/v) NaCl, at 30 °C and at pH 7.0–8.0. Phylogenetic analyses based on 16S rRNA and partial dnaK gene sequences showed that strain NIOT.bflm.S4T formed a phylogenetic lineage with Pseudogracilibacillus auburnensis P-207T, the only known species of the genus Pseudogracilibacillus and shared sequence identities of 96.9 and 83 %, respectively, with this strain. The identities of 16S rRNA and partial dnaK gene sequences with members of other related genera such as Gracilibacillus, Paralibacillus, Ornithinibacillus, Oceanobacillus, Virgibacillus and Lentibacillus were <95 and <78 %, respectively. The DNA G+C content of strain NIOT.bflm.S4T was 39.1 mol%. MK-7 was found as the sole isoprenoid quinone. The major polar lipids of strain NIOT.bflm.S4T were diphosphatidylglycerol, phosphatidylethanolamine and an unknown lipid. The diagnostic diamino acid of the cell-wall peptidoglycan was meso-diaminopimelic acid. Major cellular fatty acids were anteiso-C15:0 (27.9 %), anteiso-C17:0 (18.6 %), C12:0 (8.7 %) and iso-C15:0 (6.6 %). On the basis of phenotypic, phylogenetic and chemotaxonomic results, we propose that the isolate represents a novel species of the genus Pseudogracilibacillus, for which the name Pseudogracilibacillus marinus sp. nov. is proposed. The type strain is NIOT.bflm.S4T (=KACC 18456T=MTCC 12376T=1BRC 5831T).

The genus Pseudogracilibacillus is a member of the family Bacillaceae and was first proposed by Glaeser et al. (2014). Bacillaceae, the largest family of the phylum Firmicutes, contains Gram-stain-positive bacteria with low DNA G+C contents that are widely distributed in natural environments and are extremely robust due to their ability to form resistant endospores (Mandic-Mulec et al., 2015). Presently the genus Pseudogracilibacillus contains a single type species, Pseudogracilibacillus auburnensis, isolated from a rhizosphere soil sample in Auburn, USA. The genus includes rod-shaped, catalase-negative bacteria, which form smooth, glossy, butyrous, creamy to pinkish white colonies on nutrient-rich media. Chemotaxonomically, the type species of the genus contains MK-7 as the predominant menaquinone, and mainly iso- and anteiso-branched fatty acids. In this study, we determined the taxonomic position of a presumably novel strain of the genus Pseudogracilibacillus, NIOT.bflm.S4T, using a polyphasic approach. The strain was recovered during the course of an investigation of biofilm formed on high-density polyethylene test coupons.

The coupons were fastened to acrylic frames and immersed for 1 month (March, 2013) in the offshore waters of the Bay

Abbreviations: NSW, natural seawater; SSS, synthetic sea salts.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Pseudogracilibacillus marinus NIOT.bflm.S4T is KJ575074. The accession numbers for the partial dnaK gene sequences of P. marinus NIOT.bflm.S4T and P. auburnensis P-207T are KR153877 and KR153878, respectively.

Three supplementary figures and four supplementary tables are available with the online Supplementary Material.
of Bengal (latitude 14° 56′ 51.88″ N and longitude 80° 05′ 02.37″ E) at a depth of 2 m near the cages of the fin fish culture site in Kothachathiram village, Nellore district, Andhra Pradesh, India. The pH, temperature and salinity of the surrounding environment were pH 8.1, 28.2 °C and 32%, respectively. Strain NIOT.bflm.S4T was initially isolated and purified from marine agar 2216 (MA; HiMedia) plates incubated at 30 °C for 1 week, using a standard dilution-plating method. Sub-cultivation, and phenotypic, physiological, chemotaxonomic and molecular systematic studies were performed using cells grown on MA or in marine broth 2216 (MB; HiMedia) at 30 °C, unless otherwise stated. The purified strain was maintained on MA at 4 °C and in MB supplemented with 25% (v/v) glycerol at −80 °C. The preservation media contained a final concentration of NaCl of 2.5% (w/v). The type strain Pseudogracilibacillus auburnensis P-207T was provided by Dr John McInroy of Auburn University, USA, as a gift and was used as a reference strain for phenotypic comparisons and fatty acid analysis.

Genomic DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen), following the manufacturer’s protocol. The 16S rRNA gene amplification was carried out using the primers 27F (5′-AGGAGGTGWTCCARCC-3′) and 1525R (5′-AAGGAGGTGWTCARCC-3′) following standard PCR conditions with annealing at 55 °C for 1 min. As protein-encoding genes evolve much faster than rRNA genes and provide higher resolution than the use of the 16S rRNA gene (Yamamoto & Harayama, 1998), the partial dnaK gene sequence was determined as described by Thies et al. (1999). The PCR parameters were 94 °C for 60 s, 52 °C (42 °C during the first 8 cycles) for 60 s, and 72 °C for 1.2 min for 42 cycles. A reagent blank containing all components of the reaction mixture except template DNA was included in every PCR procedure. The sequencing reaction of the purified PCR product was performed using a BigDye terminator v3.1 cycle sequencing kit, according to the manufacturer’s instructions (Applied Biosystems), and the sequences were determined by using an ABI-3500 Genetic Analyzer (Applied Biosystems).

The almost-complete 16S rRNA gene sequence of strain NIOT.bflm.S4T (1521 nt) was analyzed for pairwise similarity with type strains of species with validly published names using EzTaxon-e (Kim et al. 2012). Strain NIOT. bflm.S4T showed the highest 16S rRNA gene sequence similarity of 96.9% to Pseudogracilibacillus auburnensis P-207T. The similarity with members of other related genera such as Gracilicibacillus, Paralobbacillus, Ornithinibacillus, Oceanobacillus, Virgibacillus and Lentibacillus was ≤95%. Partial dnaK gene sequences of NIOT.bflm.S4T (624 nt) and Pseudogracilibacillus auburnensis P-207T (587 nt) were checked for similarity against each other and searched against sequences in the GenBank and EMBL databases using tblastn and blastx. Strain NIOT.bflm.S4T and the most closely related reference strain (Pseudogracilibacillus auburnensis P-207T) showed an identity of 83% based on partial dnaK gene sequence, while the translated amino acid sequence identity between the two strains was 95%.

Supporting the low 16S rRNA gene sequence similarity values, the analysis of the additional phylogenetic marker dnaK showed that strain NIOT.bflm.S4T represents a distinct species of the genus Pseudogracilibacillus. The NIOT. bflm.S4T partial dnaK gene nucleotide and translated amino acid based identities with members of other related genera such as Gracilicibacillus, Ornithinibacillus, Oceanobacillus, Virgibacillus, Thalassobacillus, Bacillus and Lentibacillus were only 78–75% and 88–83%, respectively.

The 16S rRNA gene and dnaK amino acid sequences of the closest relatives were downloaded, and multiple sequence alignment was carried out using Clustal Omega (McWilliam et al., 2013). Phylogenetic trees were reconstructed on the basis of the neighbour-joining and maximum-parsimony methods, using the MEGAS program (Tamura et al., 2013) with 1000 bootstrap replicates to obtain a strict consensus tree. The phylogenetic analysis revealed that strain NIOT. bflm.S4T was a member of the family Bacillaceae. It formed an evolutionarily distinct lineage with Pseudogracilibacillus auburnensis P-207T, the only known species of the genus Pseudogracilibacillus (Figs 1 and 2). The node grouping strain NIOT.bflm.S4T and Pseudogracilibacillus auburnensis P-207T was well separated from species of other related genera, supported with very high bootstrap-resampling values. This supports the affiliation of strain NIOT.bflm.S4T to the genus Pseudogracilibacillus.

The genomic DNA was isolated following the method of Marmur (1961), and the DNA G+C content of strain NIOT.bflm.S4T was determined with a real-time PCR thermocycler and SYBR Green I (Bio-Rad) using the fluorometric method (Gonzalez & Saiz-Jimenez, 2002). The DNA G+C content of strain NIOT.bflm.S4T was 39.1 mol%, which differs by 5.1 mol% from the reference species Pseudogracilibacillus auburnensis P-207T (Table 1). Data from the literature suggest that when conventional methods are used for determining the DNA G+C content, the members of the same genus do not differ by more than 10 mol% (Schleifer, 2009) and variation within-species is at most 3 mol% (Mesbah et al., 2011). This supports the affiliation of strain NIOT.bflm.S4T to the genus Pseudogracilibacillus as a representative of a novel species.

Growth was tested on tryptic soy agar (TSA; HiMedia), nutrient agar (NA; HiMedia) and Luria-Bertani agar (HiMedia), supplemented with 2% NaCl and with synthetic sea salts (SSS; HiMedia), and on MA at 30 °C. Macromolecular properties were determined using the classical characterization of colony appearance on MA. The motility of the cells was analysed by the hanging drop method using phase-contrast microscopy (Eclipse E600; Nikon) and the semi-solid agar method. Cell morphology and the presence of flagella were investigated using scanning electron microscopy (JEOL-6600; Nikon) and transmission electron microscopy (JEM-1010; JEOL) with cells grown on MA. Growth was assessed at 4 °C and 10–50 °C (at 5 °C intervals) and in buffered MB adjusted to various pH values (pH 5.0–10.0, in increments of 0.5 pH units). Growth at various NaCl

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concentrations (0.0–10.0 %, w/v) was investigated using MB prepared as per the commercial formula except for NaCl. Growth under anaerobic conditions was investigated as described previously (Verma et al., 2011). Gram staining and spore formation were determined according to the methods of Gerhardt et al. (1994), wherein for sporulation a week-old culture grown on MA or MA supplemented with MnSO₄ (0.3 g l⁻¹) was used.

Catalase and oxidase activities were determined using 3 % (v/v) aqueous hydrogen peroxide and Kovacs’ reagent (HiMedia), respectively. Production of enzymes such as urease, lipase, caseinase, amylase and gelatinase was determined according to Gerhardt et al. (1994) with media prepared in SSS. Oxidation of 31 different carbon substrates was determined using a Biolog EcoPlate, wherein for NIOT.bflm.S₄[^T] reactions, artificial seawater salts broth (HiMedia) was used.

**Fig. 1.** Phylogenetic tree based on nearly complete 16S rRNA gene sequences, showing the relationship of strain NIOT.bflm.S₄[^T] with *Pseudogracilibacillus auburnensis* P-207[^T] and representatives of some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % from neighbour-joining and maximum-parsimony methods, respectively, are shown at branch points. *Salinicoccus roseus* DSM 5351[^T] was used as an outgroup. Bar, 0.01 nucleotide substitutions per site.
Fig. 2. Phylogenetic tree based on partial dnaK amino acid sequences showing the phylogenetic position of strain NIOT.bflm.S4T with Pseudogracilibacillus auburnensis P-207T and representatives of some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) ≥50 % from neighbour-joining and maximum-parsimony methods, respectively, are shown at branch points. The amino acid sequence of Pseudomonas savastanoi 1448A (Q48E62) was used as an outgroup. Bar, 0.05 substitutions per amino acid position.

as the inoculation fluid. Combinations of 35 tests for utilization of carbohydrates were determined using a HiMedia KB009 HiCarbo kit (KB009A/KB009B/KB009C). Additional enzyme activities and biochemical features such as ONPG, lysine utilization, ornithine utilization, phenylalanine deamination, nitrate reduction, H2S production, citrate utilization, Voges–Proskauer’s reaction, methyl red, aesculin hydrolysis, indole, arginine, maltone utilization, and acid production from arabinobiose, xylose, adonitol, rhamnose, cellobiose, melibiose, sucrose, raffinose, trehalose, glucose, lactose, and mannitol were determined using a Hi25 Enterobacteriaceae Identification kit (KB003; HiMedia) and a HiBacillus Identification kit (KB013; HiMedia). Susceptibility against 20 antimicrobial compounds was determined by the conventional disc-diffusion method, using commercially available (HiMedia) antibiotic-impregnated discs, Icosa G-I Plus (IC002). Plates were incubated at 30 °C under aerobic conditions, and susceptibility was scored after 24 and 48 h on the basis of the distance from the edge of the clear zone to the disc. The strain was considered susceptible when the diameter of the inhibition zone was ≥25 mm, intermediate at 11–24 mm and resistant at <10 mm.

The isolate NIOT.bflm.S4T grew on all media tested prepared with SSS, with luxurious growth on MA and TSA. Requirement of SSS or natural seawater (NSW) for growth indicate its marine nature. Cells are aerobic, Gram-staining-positive slender rods, 0.4–0.5 µm in width and 2.0–4.0 µm in length and motile by single polar flagellum (Figs 3, S1 and S2, available in the online Supplementary Material). Cells are sensitive to (µg per disc), cefalotin (30), clindamycin (2), co-trimoxazole (25), erythromycin (15), ofloxacin (5), penicillin (10), ampicillin (10), oxacillin (1), linezolid (30), teicoplanin (10), metillicin (5), amoxycilav (30) and novobiocin (5), but resistant to gentamycin (10), clari- thrromycin (15) and tetracycline (30). Detailed results of physiological and biochemical characteristics are listed in Tables S1, S2 and S3, and the species description. Many properties of strain NIOT.bflm.S4T such as smooth, glossy, creamy to pinkish colonies on TSA, slender rods with terminally located spherical endospores, negative catalase reaction, utilization of lysine and ornithine and hydrolisis of urea and carbon compounds were consistent with the description of the genus Pseudogracilibacillus. However, the novel isolate clearly differed from the closely related type strain Pseudogracilibacillus auburnensis P-207T in having motility by means of a single polar flagellum, requiring seawater for growth, oxidase activity and the absence of nitrate reduction. Other characteristics differentiating strain NIOT.bflm.S4T and Pseudogracilibacillus auburnensis P-207T are presented in Table 1.

The cellular fatty acids were determined using the cell mass of strains NIOT.bflm.S4T and Pseudogracilibacillus auburnensis P-207T grown on NA, with and without SSS, respectively, at 30 °C for 48 h, wherein the standardization of physiological age of cultures was taken into account. Fatty acids were extracted, saponified and methylated using the standard MIDI protocol. Fatty acid methyl esters were identified with the Sherlock System version 6.1, RTSBA6.
Table 1. Differential characteristics of strain NIOT.bflm.S4\textsuperscript{T} and Pseudogracilibacillus auburnensis P-207\textsuperscript{T}

Data for both strains were from the present study except where indicated. +, Positive; –, negative; R, resistant; S, sensitive; I, intermediate.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NIOT.bflm.S4\textsuperscript{T}</th>
<th>P-207\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of isolation</td>
<td>Marine biofilm</td>
<td>Soil rhizosphere</td>
</tr>
<tr>
<td>Cell motility</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Oxidase activity</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Citrate utilization</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Growth at/with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 °C</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>45 °C</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>pH</td>
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<td>5.5–10.5</td>
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<td>Acid production from:</td>
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<tr>
<td>Glucose</td>
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<td>–</td>
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<tr>
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<tr>
<td>Sucrose</td>
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<tr>
<td>Xylose</td>
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<tr>
<td>Rhamnose</td>
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<td>–</td>
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<tr>
<td>Utilization of:</td>
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<tr>
<td>Maltose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Inulin</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Oxidation of:</td>
<td></td>
<td></td>
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<tr>
<td>Tween 80</td>
<td>–</td>
<td>+</td>
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<tr>
<td>α-Cyclodextrin</td>
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<td>–</td>
</tr>
<tr>
<td>Glycogen</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>β-Malic Acid</td>
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<td>–</td>
</tr>
<tr>
<td>Antimicrobial sensitivity (µg):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gentamicin (10)</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td>Tetracycline (30)</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td>Novobiocin (5)</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Phosphatidyglycerol content</td>
<td>Absent</td>
<td>Major</td>
</tr>
<tr>
<td>iso-C\textsubscript{16,0} fatty acid</td>
<td>3.3 %</td>
<td>11.2 %</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>39.1</td>
<td>34†</td>
</tr>
</tbody>
</table>

*Without sea salts, strain NIOT.bflm.S4\textsuperscript{T} did not show any growth.
†Data from Glaser et al. (2014).

The major cellular fatty acids (>6.0% of the total fatty acids) of strain NIOT.bflm.S4\textsuperscript{T} were anteiso-C\textsubscript{15:0} (27.9%), anteiso-C\textsubscript{17:0} (18.6%), C\textsubscript{12:0} (8.7%) and iso-C\textsubscript{15:0} (6.6%). The fatty acid profile of strain NIOT.bflm.S4\textsuperscript{T} was similar to that of the reference strain grown under the same conditions. However, there were some differences in the proportions and types of some fatty acid components, especially in the monounsaturated fatty acids, that allowed the differentiation of strain NIOT.bflm.S4\textsuperscript{T} from Pseudogracilibacillus auburnensis P-207\textsuperscript{T} (Tables 1 and S4). The polar lipids profile was composed of the major lipids diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and an unknown lipid not detectable with any specific spray reagent (UL1) with minor amounts of two unidentified phospholipids (PL1, PL2) and a minor amount of another unknown lipid (UL2) (Fig. S3). In Pseudogracilibacillus auburnensis P-207\textsuperscript{T}, along with DPG and PE, phosphatidyglycerol was also reported as a major lipid, which was not detected in strain NIOT.bflm.S4\textsuperscript{T} (Table 1). Strain NIOT.bflm.S4\textsuperscript{T} contained MK-7 as the sole isoprenoid quinone. However, Pseudogracilibacillus auburnensis P-207\textsuperscript{T} was reported to contain MK-7 as the predominant quinone and MK-5 as a minor quinone. meso-Diaminopimelic acid was identified to be the diagnostic diamino acid of the cell-wall peptidoglycan, which is in accordance with Pseudogracilibacillus auburnensis P-207\textsuperscript{T}.

Fig. 3. Negatively stained cells of strain NIOT.bflm.S4\textsuperscript{T} grown on MA for 24 h at 30 °C and visualized by transmission electron microscopy.
Therefore, the results of physiological, biochemical and chemotaxonomic (fatty acid, peptidoglycan, quinone and polar lipid compositions) experiments, in combination with phylogenetic analysis (16S rRNA and dnaK gene sequences) demonstrated that strain NIOT.bflm.S4<sup>T</sup> represents a novel species of the genus *Pseudogracilibacillus*, for which the name *Pseudogracilibacillus marinus* sp. nov. is proposed.

**Description of Pseudogracilibacillus marinus** sp. nov.

*Pseudogracilibacillus marinus* (ma’ri’nuς L. masc. adj. marinus pertaining to the sea).

On MA and TSA, colonies grown aerobically at 30°C for 48 h are circular, slightly convex, smooth, glistening, creamy to pinkish in appearance, with translucent opacity, butyrous consistency and approximately 2.0–3.0 mm in diameter. Cells are Gram-staining-positive and slender rod-shaped (0.4–0.5×2.0–4.0 μm) and motile by means of a single polar flagellum with no chains or filaments observed. Terminally located endospores are present in slightly swollen sporangia. Requires SSS or NSW for growth, which occurs with 1.0–8.0% (w/v) NaCl, at 4–45°C and at pH 6.5–9.0, with optimum growth with 4.0–5.0% (w/v) NaCl, at 30°C and at pH 7.0–8.0. Tests for production of lipase, caseinase, amylase, gelatinase, reduction of nitrate and catalase activity are negative. Positive for oxidase activity, production of urease, and utilization of lysine, ornithine and citrate. Negative for the utilization of malonate, hydrolysis of ascelin, production of indole and H₂S, phenylalanine deaminase and methyld red, ONPG, and Voges–Proskauer reactions. Produces acid from xylose, rhamnose, raffinose, glucose and sucrose but not from arabinose, adonitol, cellobiose, melibiose, saccharose, trehalose and lactose. Utilization of carbohydrate is positive for dextrose, inulin, glycerol, salicin and sorbose.

On the basis of Biolog tests, gives a positive result for oxidation of Tween 40, α-cyclodextrin, glycovgen, α-DL-glycerol phosphate, α-ketobutyric acid, D-malic acid, L-serine, L-threonine and glycol-L-glutamic acid, but negative for the oxidation of Tween 80, pyruvic acid methyl ester, cellobiose, α-lactose, methyl β-D-glucoside, D-xylene, i-erythritol, D-mannitol, N-acetyl-D-glucosamine, D-glucosaminic acid, glucose 1-phosphate, D-galactonic acid γ-lactone, D-galacturonic acid, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, γ-hydroxybutyric acid, itaconic acid, L-arginine, L-asparagine, L-phenylalanine, phenylethylamine and putrescine. MK-7 is the sole isoprenoid quinone. The major fatty acids (>6%) are anteiso-C<sub>15:0</sub>, anteiso-C<sub>17:0</sub> C<sub>12:0</sub> and iso-C<sub>15:0</sub>. Other phenotypic characteristics are given in Tables 1, S2, S3 and S4.

The type strain, NIOT.bflm.S4<sup>T</sup> (=KACC 18456<sup>T</sup>=MTCC 12376 =TBRCC 5831<sup>T</sup>), was isolated from biofilm formed on high-density polyethylene test coupons immersed in the offshore waters of the Bay of Bengal. The DNA G+C content of the type strain is 39.1 mol%.

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**References**


