**Alkalimarinus sediminis** gen. nov., sp. nov., isolated from marine sediment

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Strain FA028ᵀ, a beige-pigmented, facultatively anaerobic, heterotrophic, catalase-negative and oxidase-positive, Gram-stain-negative bacterium, was isolated from marine sediment of the coast of Weihai, China. Cells of strain FA028ᵀ were rod-shaped, 1–3 μm in length and 0.5 μm in width. The strain was able to grow at 13–37 °C, at pH 7.0–9.5 and in the presence of 1.0–4.0 % (w/v) NaCl. Optimal growth was observed at 28 °C, with 3.0 % NaCl and at pH 7.5–8.0. Nitrate was not reduced. The G + C content of the DNA was 43.4 mol%. The isoprenoid quinone was Q-9 and the main cellular fatty acids (>10 %) were C₁₆ : ₀, C₁₆ : ₁ω9c and iso-C₁₅ : ₀ 2-OH/C₁₆ : ₁ω7c. The major polar lipids in strain FA028ᵀ were phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol; phospholipid was present in moderate to minor amounts in the polar lipid profile. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain FA028ᵀ was affiliated with the phylum Proteobacteria. 16S rRNA gene sequence comparisons showed that this isolate is unique, sharing <93 % similarity with species of the families Alteromonadaceae and Oceanospirillaceae. On the basis of the phenotypic and phylogenetic data, strain FA028ᵀ should be classified as representing a novel species of a new genus within the family Alteromonadaceae, for which the name Alkalimarinus sediminis gen. nov., sp. nov. is proposed. The type strain of Alkalimarinus sediminis is FA028ᵀ (=CICC 10906ᵀ=KCTC 42258ᵀ).

At the time of writing, the family Alteromonadaceae (Ivanova & Mikhailov, 2001) of the class Gammaproteobacteria, phylum Proteobacteria, includes 16 genera (LPSN, http://www.bacterio.net/-classifphyla.html). The type genus of the family is Alteromonas (Baumann et al., 1972) with Alteromonas macleodii as the type species. Bacteria of the family Alteromonadaceae are Gram-stain-negative, and most are motile by polar flagella. The vast majority of these genera have been isolated from the sea. In this study, a novel isolate from the sea, strain FA028ᵀ, was characterized by phenotypic and phylogenetic analyses and is proposed to be a member of a new genus.

During a study into the discovery of bacteria producing novel antibiotics from the marine environment, a novel facultatively anaerobic, beige-pigmented, Gram-stain-negative bacterium, designated FA028ᵀ, was isolated at 28 °C in MB medium, which consisted of the following ingredients in 1000 ml artificial seawater: 1 g yeast extract, 5 g peptone and 0.1 g ferric citrate. The artificial seawater was prepared with artificial sea salt [Sigma; 3.0 % (w/v)] and distilled water. The pH was adjusted to 7.5 and the medium was then autoclaved. This medium was used for all studies. Agar at 1.8 % was added for solid media (MA medium). The sample was collected from a marine environment (122° 03’ 44” E 37° 32’ 02” N) of the Weihai coast, Shandong Province, China. For isolation of bacterial strains, 1 g wet sediment was blended with 99 ml sterilized seawater and shaken vigorously with glass beads. The suspension was diluted in steps to 10⁻⁶ with sterilized seawater and 0.1 ml from each dilution was spread onto solid medium. The plates were incubated at 28 °C for 5–7 days. Strain FA028ᵀ was isolated and stored at (80 °C with 1 % (w/v) saline and 15 % (w/v) glycerol. Halicea salicicola DSM 19537ᵀ and Marinobacter hydrocarbonoclasticus DSM 8798ᵀ, obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), and Motiliproteus sediminis HS6ᵀ from our laboratory were used as reference strains and were cultured under the same conditions as strain FA028ᵀ, unless otherwise specified.

The GenBank accession number for the 16S rRNA gene sequence of Alkalimarinus sediminis FA028ᵀ is KP178167.

Two supplementary figures and two supplementary tables are available with the online Supplementary Material.
Routine growth was performed at 28 °C on MA medium. On MA medium, optimum growth could be obtained after 3–5 days. Colony morphology examination of strain FA028T was performed on cultures from MA medium after 4 days of incubation at 28 °C. Cell size, morphology, motility and the presence of flagella were observed using transmission electron microscopy (JEM-1200EX; JEOL). Supplementary light microscopy (E600; Nikon) examination was also performed. Motility was assessed via the hanging-drop method. Gram staining was carried out as described by Gerhardt et al. (1994). The antibiotic sensitivity profile of the isolate was determined with the Kirby–Bauer disc diffusion method on MA plates (as strain FA028T showed poor growth on Muller-Hinton agar) using various antibiotics. In this method, bacterial lawns were first made from a bacterial suspension of 0.5 McFarland units in 0.9 % NaCl. Two or three antibiotic discs were then applied to each plate. The result was determined from the measurements of the inhibition zones produced after 1–2 days of incubation according to Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards guidelines. For determination of the effects of different growth temperatures, the bacteria were inoculated on MA media and incubated for about 7 days until growth could be indicated by visible colonies at various temperatures (4, 8, 13, 17, 24, 28, 30, 37 and 42 °C). To test for effects of pH on growth, the standard medium was separately modified by addition of buffers MES (pH 5.5 and 6.0), PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), Tricine (pH 8.5) and CAPSO (pH 9.0 and 9.5) (Sangon) at concentrations of 20 mM. The pH of the medium was adjusted by addition of 1 M HCl or NaOH before autoclaving. Tolerance to NaCl was tested using MA-NaCl media (1.8 % agar, 0.5 % peptone, 0.1 % yeast extract, 0.32 % MgSO4, 0.12 % CaCl2, 0.07 % KCl, 0.02 % NaHCO3), with the NaCl concentrations ranging from 0 to 9.0 % (w/v) at intervals of 0.5 %. The standard solid medium was used to determine hydrolysis of agar. In addition, hydrolysis of starch, lipid, cellulose and alginate was determined by supplementing the standard solid medium with 0.2 % (w/v) soluble starch, 1 % (v/v) Tween 80, 0.5 % (w/v) carboxymethylcellulose and 0.5 % (w/v) sodium alginate, respectively (Barrow & Cowan, 1993).

Physiological and biochemical characteristics were tested with API 20E and API ZYM kits (bioMérieux) according to the manufacturer’s instructions. Utilization of different compounds as sole carbon and energy sources was determined using the Biolog system (bioMérieux). Tests for acid production from carbohydrates were performed by using the API 50CHB fermentation kit (bioMérieux) according to the manufacturer’s instructions. The API 50CHB strips were read every 24 h after incubation at 28 °C. All the tests were performed in duplicate, and appropriate positive and negative controls were included. Reduction of nitrate and oxidation-fermentation tests were performed as described by Dong & Cai (2001). Growth under anaerobic conditions was determined after cultivation in an anaerobic chamber on the solid medium mentioned above with or without 0.1 % (w/v) KNO3 for at least 2 weeks at 28 °C. Oxidase activity was tested by using the bioMérieux oxidase reagent kit according to the manufacturer’s instructions, and catalase activity was evaluated based on the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. Further information on the morphological, physiological and biochemical characteristics of strain FA028T is given in the species description.

Respiratory isoprenoid quinones were isolated and separated according to Tindall et al. (2007), and analysed by HPLC (Kroppenstedt, 1982). Cultures for fatty acid analysis were incubated on MA plates at 28 °C for 3 days, and analysis was performed as described by Sasser (1990). The extract was separated using the Sherlock Microbial Identification System (MIS) (MIDI). Peaks were automatically integrated and fatty acid names were identified and percentages calculated by the MIS Standard Software (Microbial ID). Polar lipids were separated by two-dimensional silica gel TLC. Total lipid material was detected using molybdatephosphoric acid and specific functional groups were detected using spray reagents specific for each one. Full details are given by Tindall et al. (2007). Cellular polar lipid analysis was carried out by the Identification Service of the DSMZ.

The genomic DNA of strain FA028T was extracted and purified using a bacteria genomic DNA Mini kit (Takara). The DNA G+C content of the study strain was determined according to the method of Gonzalez & Saiz-Jimenez (2002). The 16S rRNA gene was amplified by PCR with universal primers 27f and 1492r according to the method described by Liu et al. (2014). The purified PCR product was ligated to the vector pGM-T (Tiangen) and cloned according to the manufacturer’s instructions. Sequencing was performed by Shanghai Sunny Biotechnology. The nearly complete 16S rRNA gene sequence of strain FA028T was submitted to GenBank to search for similar sequences using the BLAST program and EzBioCloud algorithm. 16S rRNA gene sequences of several closely related species were first aligned using the SINA online server (Pruesse et al., 2012) and the alignments were then adjusted manually. The ARB software package (Ludwig et al., 2004) and the ‘All-Species Living Tree’ project (Yarza et al., 2008) were used to find close phylogenetic relatives. The phylogenetic tree was generated in ARB using RAxML with the GTR+GAMMA model (Stamatakis, 2006).

Strain FA028T was isolated after 5 days of culture at 28 °C. Colonies were circular with entire edge, beige-pigmented and opaque. Transmission electron microscopy revealed that the cells were rod-shaped with a single polar flagellum (Fig. S1, available in the online Supplementary Material). Strain FA028T contained Q-9 (100 %) as the sole ubiquinone. The DNA G+C content
of strain FA028T was 43.4 mol%. It was resistant to tetracycline, trimethoprim and lincomycin. Strain FA028T could be distinguished from related genera Marinobacter, Marinobacterium and Motiliproteus based on its physiological and biochemical characteristics. Strain FA028T was able to grow in the presence of 1.0–4.0 % (w/v) NaCl and was negative for catalase activity and positive for lipase activity. By contrast, members of Marinobacter can grow in the presence of 0.5–20 % (w/v) NaCl and are positive for catalase activity; members of Marinobacterium are positive for catalase activity and negative for lipase activity. Furthermore, Motiliproteus sediminis HS6T can hydrolyze gelatin and has bipolar flagella, in contrast to strain FA028T. Details of characteristics that differentiate strain FA028T from related genera are shown in Table 1 and Table S1.

The major polar lipids in strain FA028T were phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and diphostatidylglycerol (DPG). Phospholipid (PL) was present in moderate to minor amounts in the polar lipid profile (Fig. S2). The predominant cellular fatty acids of strain FA028T were C16:0 (26.8 %), C16:1ω9c (19.6 %) and iso-C15:0 2-OH/C16:1ω7c (25.8 %). However, Marinobacter hydrocarbonoclasticus DSM 8798T contained C18:1ω9c (30.1 %), Haliea salxigenes DSM 19537T contained C17:1ω8c (24.8 %) and Motiliproteus sediminis HS6T contained C18:1ω7c (12.3 %) as one of the predominant cellular fatty acids. Thus, the fatty acid profiles of strain FA028T and reference strains appeared to be significantly different. Comparison of the fatty acid profiles of strain FA028T and reference strains is provided in Table S2.

16S rRNA gene sequence analysis placed strain FA028T within the class Gammaproteobacteria. The sequence obtained was aligned with sequences deposited in the GenBank database through the BLAST software program (Altschul et al., 1997). The highest degree of 16S rRNA gene sequence similarity for strain FA028T was found to be with Marinobacter xestospongiae JCM 17469T (Lee et al., 2012) (92.77 %). Alignment of the 16S rRNA gene sequence with that of Marinobacter xestospongiae JCM 17469T using EzTaxon Server 2.0 (Chun et al., 2007) gave 92.68 % similarity. The phylogenetic tree obtained by using the neighbour-joining method (Fig. 1) revealed that the novel isolate formed a separate branch with the genera Marinobacter (Gauthier et al., 1992), Haliea (Urios et al., 2008), Microbulbifer (González et al., 1997), Marinimicrobium (Lim et al., 2006) and Saccharophagus (Ekborg et al., 2005).

We therefore propose to establish a new genus to accommodate this organism, namely Alkalimarinus gen. nov. Strain FA028T is the type strain of the type species, Alkalimarinus sediminis sp. nov. We also included 16S rRNA gene sequences of members of the family Oceanospirillaceae (Garrity et al., 2005) in the phylogenetic tree, and they formed a separate cluster distinct from the genus Alkalimarinus. Based on the phylogenetic tree and the physiological and biochemical characteristics, we suggest that strain FA028T should be classified in the family Alteromonadaceae.

**Description of Alkalimarinus gen. nov.**

Alkalimarinus (Al.ka.li.ma.rin.us. N.L. masc. n. alkali alkali; L. adj. marinus of the sea, marine; N.L. masc. n. Alkalimarinus marine bacterium living under alkaline conditions).

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**Table 1. Differential characteristics between strain FA028T and related genera**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
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<tbody>
<tr>
<td><strong>Cell size (μm)</strong></td>
<td>0.4–0.6 × 1–1.3</td>
<td>0.3–1.3 × 1–2.3</td>
<td>0.6–0.8 × 1.3–2.2</td>
<td>0.3–0.7 × 1.1–2.7</td>
<td>0.5–0.9 × 0.9–2.0</td>
<td>0.5–0.8 × 1.5–6.0</td>
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<tr>
<td><strong>Flagella</strong></td>
<td>Single polar</td>
<td>Single polar</td>
<td>Single polar</td>
<td>Single polar or triple polar or none</td>
<td>Single polar or bipolar</td>
<td>Bipolar</td>
</tr>
<tr>
<td><strong>Growth at/with:</strong></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4 °C</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+∗</td>
<td>—</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0–4.0 %</td>
<td>0.5–20.0 %</td>
<td>ND</td>
<td>0.35–15 %</td>
<td>0.3–9.0 %</td>
<td>0.5–7.0 %</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>—</td>
<td>—</td>
<td>+∗</td>
<td>+</td>
<td>+</td>
<td>+∗</td>
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<tr>
<td>Oxidase reaction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Catalase reaction</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
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<td>Amylase</td>
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<td>Gelatinase</td>
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<td>—</td>
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<tr>
<td>Lipase</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>43.4</td>
<td>52.7–58.9</td>
<td>55.0–57.0</td>
<td>61.4–65.0</td>
<td>46.6–52.2</td>
<td>61.2</td>
</tr>
<tr>
<td>Quinone(s)</td>
<td>Q9</td>
<td>Q9/Q8†</td>
<td>ND</td>
<td>Q8</td>
<td>Q8†</td>
<td>Q8/Q7</td>
</tr>
</tbody>
</table>

*≥50 % positive/negative.  †Data are incomplete in the literature.
Cells are Gram-stain-negative, rod-shaped, facultatively anaerobic, heterotrophic and have a single polar flagellum; alkaline-resistant, catalase-negative and oxidase-positive. No growth without NaCl. The isoprenoid quinone is Q-9. The genus is affiliated to the family Alteromonadaceae in the class Gammaproteobacteria. The type species is Alkalimarinus sediminis.

**Description of Alkalimarinus sediminis sp. nov.**

Alkalimarinus sediminis (se.di.mi’nis. L. n. sedimen -inis sediment; L. gen. n. sediminis of sediment).

Displays the following properties in addition to those given in the genus description. Cells are approximately 1–3 μm in length and 0.4–0.6 μm in width. Colonies on MA are...
beige-pigmented, circular and about 2 mm in diameter after 3 days of growth at 28 °C. Able to grow at 13–37 °C, at pH 7.0–9.5 and in the presence of 1.0–4.0 % (w/v) NaCl. Optimal growth at 28 °C, with 3.0 % NaCl and at pH 7.5–8.0. Nitrate is not reduced. Acid is produced from fermentation of glucose under anaerobic conditions, but no gas. Tween 80 is hydrolysed, but starch, carboxymethylcellulose, sodium alginate and agar are not. Acid is produced from glycerol, D-ribose, D-glucose, dulcitol, aesculin and potassium 5-ketogluconate. Acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and naphthol-AS-BI-phosphohydrolase are produced, but trypsin, \( \alpha \)-galactosidase, \( \beta \)-galactosidase, \( \alpha \)-glucosidase, \( \beta \)-glucosidase, N-acetyl-\( \beta \)-glucosaminidase, \( \alpha \)-fucosidase, lipase (C14), cystine arylamidase, \( \alpha \)-chymotrypsin, \( \beta \)-glucuronidase and \( \alpha \)-mannosidase are not. Positive oxidations are observed with methyl \( \beta \)-D-glucoside, glucuronamide, acetocacetic acid, D-fructose 6-phosphate and D-glucuronic acid in Biolog GEN III microplates. The main fatty acids are C\(_{16:0}\), C\(_{16:1\,\Delta 9c}\) and iso-C\(_{15:0}\) 2-OH(C\(_{16:1}\))\(_{07c}\). The major polar lipids are phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and diphasatidylglycerol (DPG).

The type strain, FA028T (= CICC 10906\(^T\) = KCTC 42258\(^T\)), was isolated from marine sediment from the coast of Weihai, China. The DNA G+C content of the type strain is 43.4 mol%.

Acknowledgements

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References


