Salibacter halophilus gen. nov., sp. nov., isolated from a saltern

De-Chen Lu,¹ Jun Xia,¹ Christopher A. Dunlap,² Alejandro P. Rooney² and Zong-Jun Du¹*¹

Abstract

A Gram-stain-negative and facultatively anaerobic bacterium, JZ3C3⁴, was isolated from a saltern in Feicheng, China (36° 8’ 24.45” E 116° 49’ 22.46” N). Cells of strain JZ3C3⁴ were 0.3–0.4 μm wide and 1.5–2.0 μm long, catalase-positive and oxidase-negative. Colonies on modified marine agar 2216 were orange, circular, convex, translucent and approximately 1 mm in diameter after incubation for 96 h at 33°C. Growth occurred at 20–50°C (optimally at 33°C), at pH 6.5–8.5 (optimally at 7.0–8.0) and in the presence of 2–18 % (w/v) NaCl (optimally in 6 % NaCl). Phylogenetic analysis of the 16S rRNA gene indicated that strain JZ3C3⁴ was a member of the family Cryomorphaceae within the order Flavobacteriales and the most closely related species was Owenweeksia hongkongensis DSM 17368T (89.2 % 16S rRNA gene sequence similarity). The major respiratory quinone of strain JZ3C3⁴ was menaquinone MK-7, and the dominant fatty acids were iso-C₁₅:₀ and iso-C₁₅:₁ G. The major polar lipids were two unidentified lipids and phosphatidylethanolamine, and the genomic DNA G+C content was 39.6 mol%. Polyphasic taxonomy clearly places the new strain as a novel species within a new genus of the family Cryomorphaceae, for which the name Salibacter halophilus gen. nov., sp. nov. The type strain of Salibacter halophilus is JZ3C3⁴ (=KCTC 52047T=MCCC 1K02288T).

At the time of writing, the family Cryomorphaceae in the order Flavobacteriales of the phylum Bacteroidetes contains ten genera; nine of them are of marine origin – Phaeocystidibacter [1], Salinitrepsis [2], Wandonia [3], Lishizhenia [4], Brumimicrobium, Cryomorpha, Crocinomix [5], Luteibaculum [6] and Owenweeksia [7] – and one of freshwater origin – Fluviicola [8]. Members of the family Cryomorphaceae are Gram-stain-negative, strictly aerobic or facultatively anaerobic and chemo-organotrophic [1]. Most require sea salts for growth [1]. Recently, two genera that are phylogenetically classified within the family Cryomorphaceae have been described and named as Phaeocystidibacter [1] and Salinitrepsis [2]. In this study, an orange-pigmented bacterium, JZ3C3⁴, was isolated during the study of the diversity of halophilic bacteria in saltern environments. The phenotypic, physiological and chemotaxonomic characteristics of the new isolate, along with the results of phylogenetic analysis, support the establishment of a novel species of a new genus within the family Cryomorphaceae.

During a study of the diversity of halophilic bacteria in saltern environments, a novel strain, designated JZ3C3⁴, was isolated at 37°C on marine agar 2216 (MA; Becton Dickinson), was collected from a saltern in Feicheng, China (36° 8’ 24.45” E 116° 49’ 22.46” N), and was treated using the standard dilution plating technique. The suspension was serially diluted to 1×10⁻⁶ with sterilized seawater. Colonies of strain JZ3C3⁴ were orange, smooth, convex, glistening and translucent with an entire margin. Strain JZ3C3⁴ was routinely grown on modified MA at 37°C, consisting of distilled water with the following additions (all units g l⁻¹): sea salt Sigma-Aldrich, 40; NaCl, 30; yeast extract, 1; peptone, 5; ferric citrate, 0.1; and agar, 18. Modified MA was used as the medium for all experiments except where otherwise specified. Modified marine broth 2216 (modified MB) is of the same composition as the modified MA (excluding agar). For long-term preservation, cells were stored at −80°C in sterile 1 % (w/v) saline medium containing 15 % (v/v) glycerol. Phaeocystidibacter luteus MCCC 1F01079T and Owenweeksia hongkongensis JCM 12287T were obtained from the Marine Culture Collection of China (MCCC) and Japan Collection of Microorganisms (JCM), respectively. They were studied in parallel with strain JZ3C3⁴ as reference strains for physiological and chemotaxonomic comparisons except analysis of polar lipids.

Morphological and physiological features of strain JZ3C3⁴ were examined after incubation at 33°C for 96 h on modified MA. Transmission electron microscopy was used to assess cell size and morphology (Jem-1200; Jeol). Gliding...
motility was examined according to the method described by Bowman [9]. The Gram-reaction was carried out as described by Smibert and Krieg [10]. Anaerobic growth was tested for 7 days at 33 °C on modified MA with or without 0.1 % (w/v) NaNO₃ in an anaerobic jar with an atmosphere of 60 % CO₂, 30 % N₂ and 10 % H₂. Susceptibility to antibiotics was determined using filter-paper discs containing various antibiotics as described by Du et al. [11] and according to the Clinical and Laboratory Standards Institute (2012). Growth was evaluated at various temperatures (4, 15, 20, 25, 28, 30, 33, 37, 42, 45, 50, 55 °C) on modified MA until growth was indicated by visible colonies. The effects of different salt concentrations on growth were detected by using a medium comprising 0.1 % (w/v) yeast extract, 0.5 % (w/v) peptone and 1.8–2.0 % (w/v) agar, artificial seawater (0.32 % MgSO₄, 0.12 % CaCl₂, 0.07 % KCl and 0.02 % NaHCO₃), all w/v and containing different concentrations of NaCl [0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 25 % (w/v)]. The effect of pH on growth was investigated in modified MB from pH 5.5 to 10.0 adding the following buffers during pH adjustment: 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, 9.5 and 10.0), at concentrations of 20 mM. OD₆₀₀ values of the culture were measured after 96 h of incubation at 33 °C. The pH of the medium was adjusted by adding 1 M HCl or NaOH before autoclaving. The requirement for Mg²⁺ and Ca²⁺ was examined according to Zhou et al. [1]. Hydrolysis of starch, lipids, cellulose, alginate, and weens 20, 40 and 80 was determined on modified MA as described previously [10].

Tests for other physiological or biochemical characteristics were performed using the API 20E, API ZYM and API 50CHB kits (bioMérieux) and the Biolog GEN III system, according to the manufacturer’s instructions (except for salinity, which was adjusted to 6 % with sea salts). Oxidation-fermentation and nitrate reduction were tested as described by Dong and Cai [12]. Oxidase activity was tested using an oxidase reagent kit (bioMérieux) according to the manufacturers instructions. Catalase activity was tested by measuring the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution.

The 16S rRNA gene was amplified by PCR using two universal primers as described by Liu et al. [13]. The PCR product was ligated into the pGM-T vector (Tiangen) for cloning, as described by Liu et al. [13]. Sequencing reactions were carried out using an ABI BigDye 3.1 Sequencing Kit (Applied Biosystems) and an automated DNA sequencer (model ABI 3730; Applied Biosystems). A nearly complete sequence (1448 bp) was submitted to GenBank. The EzTaxon server (eztaxone.ezbiocloud.net; [14]) was used to obtain sequences of reference type strains and to determine which taxa possessed 16S rRNA gene sequences that shared a high level of nucleotide similarity with strain JZ3C34ᵀ. Alignment of sequences was carried out using the alignment program CLUSTAL X (version 1.81) [15]. Phylogenetic analysis based on almost-complete 16S rRNA gene sequences of strain JZ3C34ᵀ and members of the family Cryomorphaceae was used to reconstruct the tree using the neighbour-joining method [16] implemented in the software package MEGA (version 6.0) [17]. The maximum-likelihood [18] and minimum-evolution [19] methods were also used to estimate and verify the taxonomic position of the novel isolate. Genomic DNA was extracted using a commercial genomic DNA extraction kit (TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver. 3.0). The DNA G+C content was determined using HPLC according to the methods described by Tamaoka and Komagata [20] and Mesbah et al. [21].

To determine the major menaquinone, whole-cell fatty acids and polar lipid profiles, cells shaken (120 r.p.m.) in modified MB at 33 °C for 96 h were harvested and subjected to freeze-drying. The major menaquinone was detected according to the method described by Minnikin et al. [22]. Fatty acid methyl esters were extracted and prepared according to the

<table>
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<th>Characteristic</th>
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<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<tbody>
<tr>
<td>Colony colour</td>
<td>O</td>
<td>O</td>
<td>Y-O</td>
<td>Y-O</td>
<td>Y-O</td>
<td>O</td>
<td>Y-O</td>
<td>O</td>
<td>Y</td>
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<tr>
<td>Growth with 0%–15% (w/v) NaCl</td>
<td>−/+</td>
<td>−/+</td>
<td>−/+</td>
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<td>−/+</td>
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<td>Growth at 4 °C/50 °C</td>
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<td>Gliding motility</td>
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<td>+</td>
<td>v</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>Hydrolysis of:</td>
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<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>v</td>
<td>−</td>
<td>−</td>
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<td>−</td>
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<tr>
<td>Gelatin</td>
<td>+</td>
<td>+</td>
<td>v</td>
<td>+</td>
<td>v</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<td>Utilization of glucose</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
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standard protocol of the MIDI/Hewlett Packard Microbial Identification System [25]. Polar lipids were determined using two-dimensional TLC using the method of Minnikin et al. [22]. Four separate TLC plates (EMD Millipore, 1.16487.0001) were prepared for each sample and individually stained using phosphomolybdic acid solution (total lipids), molybdenum blue solution (phosphates), α-naphthyl sulfuric solution (carbohydrates) and ninhydrin (amines) all reagents were from Sigma-Aldrich.

Cells of strain JZ3C34T rods, 0.3–0.4μm in width and 1.5–2.0μm in length (Fig. S1, available in the online Supplementary Material). Like all members of the Cryomorphaceae, strain JZ3C34T Gram-stain-negative and hydrolyse starch. However, strain JZ3C34T be readily distinguished from other members of the family Cryomorphaceae by certain physiological features, such as tolerance to high levels of salinity (as much as 15%, w/v, NaCl) and temperature (as high as 50°C). Other characteristics that differentiate this strain from other representative members of the family Cryomorphaceae are shown in Table 1.

A number of other traits and characteristics of strain JZ3C34T, including antibiotic susceptibility, enzymatic activities and substrate oxidation, were also assessed. With respect to antibiotic susceptibility, strain JZ3C34T was sensitive to penicillin (10μg), streptomycin (10μg), cefotaxime (30μg), rifampicin (5μg), chloramphenicol (30μg), erythromycin (15μg), lincomycin (2μg) and acetylsalicylic acid (30μg). The novel isolate was positive for activities of alkaline phosphatase, leucine arylamidase, acid phosphatase, esterase (C4) and esterase lipase (C8), but negative for alkaline phosphatase, leucine arylamidase, adhesin, l-arabinose, sorbitol and d-arabinose.

Table 2. Characteristics that distinguish strain JZ3C34T from the type strains of the two genera in the family Cryomorphaceae to which it is most closely related

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
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<tbody>
<tr>
<td>Isolation source</td>
<td>Land</td>
<td>Marine</td>
<td>Marine</td>
</tr>
<tr>
<td>Salinity (% w/v NaCl)</td>
<td>2–18</td>
<td>1.0–7.5</td>
<td>0.25–7.5</td>
</tr>
<tr>
<td>Optimum</td>
<td>6</td>
<td>3–5</td>
<td>2–5</td>
</tr>
<tr>
<td>Temperature for growth (°C)</td>
<td>20–50</td>
<td>4–37</td>
<td>10–40</td>
</tr>
<tr>
<td>Oxidase reaction</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Hydrolysis of</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tween 20</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Tween 80</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Gentiobiose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Fructose</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>l-Galactonic acid lactone</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>d-Glucuronic acid</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>l-Rhamnose</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Bromosuccinic acid</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Fructose 6-phosphate</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Acid production from</td>
<td></td>
<td></td>
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<tr>
<td>Mannose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>d-Ribose</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>d-Fructose</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>l-Sorbose</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Aesculin</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Trehalose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>d-Tagatose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Major quinone</td>
<td>MK-7</td>
<td>MK-6</td>
<td>MK-6</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>39.6</td>
<td>39–40</td>
<td>44.6</td>
</tr>
</tbody>
</table>

shown in Table S1. The major polar lipids of strain JZ3C34T were two unidentified lipids and phosphatidylethanolamine. Diphasphatidylglycerol, glycolipid, two unidentified phospholipids and two unidentified lipids were present in moderate to minor amounts in the polar lipid profile (Fig. 1).

The DNA G+C content of strain JZ3C34T was 39.6 mol%, which is within the range of values for members of the family Cryomorphaceae (35–45 mol%). The results of the phylogenetic analysis of the 16S rRNA gene sequences showed that strain JZ3C34T formed a distinct lineage within the family Cryomorphaceae and was most closely related to Owenweeksia hongkongensis [7] (Fig. 2), which was also confirmed by the maximum-likelihood and minimal-
evolution algorithms (Figs S2 and S3). However, strain JZ3C34\textsuperscript{T} was considerably divergent from the type strain of \textit{O. hongkongensis} and shared only 89.2\% sequence similarity, which suggests that the new isolate represents a member of a new genus in the family \textit{Cryomorphaceae}.

In light of these results and the aforementioned characteristics of the phenotypic, physiological and biochemical analyses, we propose that strain JZ3C34\textsuperscript{T} is recognized as the type strain of a new taxon within the family \textit{Cryomorphaceae}, for which the name \textit{Salibacter halophilus} gen. nov., sp. nov. is proposed.

**DESCRIPTION OF \textit{SALIBACTER} GEN. NOV.**

\textit{Salibacter} (Sa.li.bac’ter. L. masc. n. salis, salt; N.L. masc. n. bacter a rod; N.L. masc. n. \textit{Salibacter} a salted rod).

Cells are Gram-stain-negative, halophilic, facultatively anaerobic, oxidase-negative, catalase-positive, non-flagellated rods that do not form endospores. The main respiratory quinone is MK-7. The major polar lipids include phosphatidylethanolamine and two unidentified lipids. The major cellular fatty acids are iso-C\textsubscript{15} : 0 and iso-C\textsubscript{15} : 1\textsubscript{G}. The type species is \textit{Salibacter halophilus}.

**DESCRIPTION OF \textit{SALIBACTER HALOPHILUS} SP. NOV.**

\textit{Salibacter halophilus} [ha.lo’phi.lus. Gr. n. hals, halos salt; N.L. adj. philus -a -um (from Gr. adj. philos -e -on) friend, loving; N.L. masc. adj. halophilus salt-loving].

Has the following properties in addition to those given in the genus description. Cells are non-motile rods, approximately 0.3–0.4 µm in width and 1.5–2.0 µm in length. Colonies on modified MA are orange, circular, convex, translucent and approximately 1 mm in diameter after incubation for 96 h at 33°C. Growth occurs at 20–50°C (optimum 33°C), at pH 6.5–8.5 (optimum pH 7.0–8.0) and in the presence of 2.0–
18.0 % (w/v) NaCl (optimum 6.0 %). Growth occurs on modified MA and requires Na⁺, Mg²⁺ and Ca²⁺. Gelatin, and Tweens 20, 40 and 80 are hydrolysed, but starch, agar and alginate are not hydrolysed. Nitrate is not reduced to nitrite. Cells are negative for H₂S production, indole production, the Voges–Proskauer reaction, ONPG test and Simmons’ citrate utilization. Diphosphatidylglycerol, glycolipid, two unidentified phospholipids and two unidentified lipids are present in moderate to minor amounts in the polar lipid profile.

The type strain is JZ3C34ᵀ (=KCTC 52047ᵀ=MCCC 1K02288ᵀ), isolated from a saltern in Feicheng, China. The DNA G+C content of the type strain is 39.6 mol%.

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Conflicts of interest
This work was supported by the National Natural Science Foundation of China (Project nos. 31370057 and 31290231 to Z-J.D.) and 2013 Shandong Provincial Second Group Projects on Resource Platforms for Marine Economic and Innovative Development Regions: Marine Microorganisms Preservation Platform (Project no. 2150299 to Z-J.D.).

Ethical statement
The authors declare that they have no conflicts of interest.

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