Croceifilum oryzae gen. nov., sp. nov., isolated from rice paddy soil

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A mesophilic, aerobic, Gram-stain-positive, filamentous bacterial strain, designated ZY11a\(^3\)T, was isolated from rice paddy soil in Japan. This strain grew on a solid medium with formation of substrate mycelium; endospores were produced singly along the mycelium. Formation of aerial mycelium was not observed on any of the media tested. This strain produced a characteristic saffron yellow soluble pigment. Cloned 16S rRNA gene sequences of strain ZY11a\(^3\)T yielded three different copies (similarity between the three sequences: 99.8–99.9 %). One of these sequences had one base deletion. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain ZY11a\(^3\)T belongs to an independent phylogenetic lineage of the family Thermoaclinomycetaceae. The cell wall of strain ZY11a\(^3\)T contained meso-diaminopimelic acid, alanine and glutamic acid, but no characteristic sugars. It contained menaquinone 7 as the sole menaquinone. The major cellular fatty acids were iso-C\(_{15}\) : 0 and anteiso-C\(_{15}\) : 0. The major polar lipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl-N-methylethanolamine and unidentified aminophospholipids. The DNA G + C content was 42.5 mol%. From phylogenetic analysis based on 16S rRNA gene sequences and phenotypic characteristics, this strain is considered to represent a novel species in a new genus, for which the name Croceifilum oryzae gen. nov., sp. nov. is proposed. The type strain of Croceifilum oryzae is ZY11a\(^3\)T (= JCM 30426\(^T\) = CCUG 66446\(^T\) = DSM 46876\(^T\)).

The family Thermoaclinomycetaceae, which includes the genera Thermoaclinomyces, Laceyella, Seinonella, Thermoflavimicrobium, Planifilum and Mechercharimyces, of the phylum Firmicutes was originally proposed by Matsuo et al. (2006), and the description has been amended by Yassin et al. (2009), von Jan et al. (2011) and Li et al. (2012). Recently, numerous novel genera including Desmospora (Yassin et al., 2009), Shimazuela (Park et al., 2007), Kroopenstedtia (von Jan et al., 2011), Lihuaxuella (Yu et al., 2012), Marininema (Li et al., 2012), Melghirimyces (Addou et al., 2013), Hazenella (Buss et al., 2013), Polycladonmyces (Tsubouchi et al., 2013), Geothermomicrobium (Zhou et al., 2014) and Salinitrix (Zarparvar et al., 2014) were added to this family. Members of this family form substrate mycelia and may form aerial mycelia (aerial mycelia are not observed in the genera Planifilum, Lihuaxuella, Marininema, Hazenella and Geothermomicrobium). They are mesophilic or thermophilic.

During the isolation of various bacteria producing pigment from environmental samples, we isolated strain ZY11a\(^3\)T, which formed substrate mycelium and produced saffron yellow soluble pigment, from Japanese rice paddy soil by cultivating on IPM plates (per litre: 0.5 g yeast extract, 0.5 g proteose peptone (Difco no. 3), 0.5 g tryptone, 0.5 g sucrose, 0.3 g sodium pyruvate, 0.3 g K\(_2\)HPO\(_4\), 0.3 g MgSO\(_4\) \(\cdot\) 7H\(_2\)O, 0.3 g CaCl\(_2\) \(\cdot\) 2H\(_2\)O and 15 g Gellan gum, pH 6.6) aerobically at 25 °C. In this study, the taxonomic position of this strain was investigated.

Genomic DNA was extracted from strain ZY11a\(^3\)T using an UltraClean Microbial DNA Isolation kit (Mo Bio). PCR amplification of the 16S rRNA gene was performed by using PrimeSTAR HS DNA polymerase (Takara Bio) with primers Eubac27F and 1492R (DeLong, 1992). The PCR product was cloned into the Smal site of vector pUC19 and sequenced with an automatic DNA sequencer (Applied Biosystems 3130 Genetic Analyzer; Life Technologies). Similarity-based searches were performed using the EzTaxon-e server (http://etzaxon-e.ezbiocloud.net/; Kim et al., 2012). The 16S rRNA gene sequences of strain ZY11a\(^3\)T (1427 or 1428 bp) were aligned with those of the type strains of related genera using the Clustal X software package (Thompson et al., 1997). Evolutionary
distances were calculated using the Kimura two-parameter method (Kimura, 1980), and were then used by the MEGA6 program (Tamura et al., 2013) to reconstruct a phylogenetic tree through the neighbour-joining method (Saitou & Nei, 1987) with bootstrap values (Felsenstein, 1985) based on 1000 replications. Another phylogenetic tree was reconstructed with the maximum-likelihood method (Tamura & Nei, 1993) in the MEGA6 program, with bootstrap values based on 1000 replications.

Growth of strain ZYf1a\textsuperscript{T} on IPM plates, R2A agar (Reasoner & Geldreich, 1985), yeast extract-malt extract agar (ISP medium 2) (Shirling & Gottlieb, 1966), tyroside agar (ISP medium 7) (Shirling & Gottlieb, 1966), Luria–Bertani (LB) agar or CYC agar (Lacey & Cross, 1989) was tested. Cell morphology was observed under a phase-contrast light microscope (BH-2; Olympus) using cells grown on R2A agar, on a slide culture with R2A agar and in R2A liquid medium at 30 °C for 1, 4 and 7 days. Scanning electron microscopy (JSM-6320F; JEOL) was used to observe endospore-forming cells of strain ZYf1a\textsuperscript{T} grown on R2A agar at 30 °C for 5 days. The Gram reaction of cells was determined via the traditional staining method and non-staining (KOH) method (Buck, 1982). The temperature range for growth was tested on R2A agar at 8, 20, 25, 30, 37, 40, 45 and 55 °C. The pH range for growth was determined in liquid R2A medium that was adjusted to pH 3.0–11.0 (in increments of 1 pH unit) using 100 mM citrate buffer (pH 3.0, 4.0), 50 mM MES (pH 5.0, 6.0), 50 mM HEPES (pH 7.0, 8.0), 50 mM CHES (pH 9.0, 10.0) and 50 mM CAPS (pH 11.0). NaCl tolerance was studied using R2A agar supplemented with 1–5 % (w/v) NaCl. Catalase activity was tested with hydrogen peroxide solution at a concentration of 3 % (w/w) in H\textsubscript{2}O (Sigma). Oxidase activity was tested with oxidase reagent (bioMérieux). Degradation of substrates was assayed using R2A agar with 1 % (w/v) casein, 1 % (w/v) soluble starch, 0.5 % (w/v) gelatin, 0.5 % (w/v) L-tyrosine, 0.5 % (w/v) hypoxanthine, 0.5 % (w/v) xanthine, 1 % (v/v) Tween 20, 1 % (v/v) Tween 40 or 1 % (v/v) Tween 80. Degradation of cellulose was tested using liquid R2A medium, with cellulose filter paper as the substrate. Urea hydrolysis was tested with urea agar (Christensen, 1946). Nitrate reduction was determined as described by Claus & Berkeley (1986) except that liquid R2A medium with 0.1 % (w/v) KNO\textsubscript{3} was used. Resistance to novobiocin was determined with the API 20 E and API 20 NE systems (bioMérieux). Acid production from carbon sources was tested with the API 50 CH system (bioMérieux). Utilization of respiratory quinones and cellu-}

The cell-wall peptidoglycan were performed by using cells grown aerobically in R2A liquid medium for 1 day at 30 °C. Analysis of cellular fatty acids was performed by using cells grown aerobically in R2A liquid medium for 2 days at 35 °C. Profiles of respiratory quinones and cellular fatty acids were determined by TechnoSuruga Laboratory. Extraction of respiratory quinones was performed according to the method of Nishijima et al. (1997) prior to analysis by HPLC. Cellular fatty acids were identified using the Sherlock Microbial Identification System (version 6) (MIDI) with the TSBA6 library. Polar lipids were extracted from cells, and examined by two-dimensional TLC (Minnikin et al., 1984). Polar lipids were detected by staining with molybdatophosphoric acid (Merck Millipore) for total lipids, molybdenum blue (Sigma) for phospholipids, 2-naphthol sulphuric acid for glycolipids or ninhydrin for aminolipids. Compositions of amino acids and sugars in the cell-wall peptidoglycan were determined according to the method of Kawamoto et al. (1981) using cellulose TLC plates (Merck Millipore). The DNA G+C content was determined by the HPLC method of Tamaoka & Komagata (1984).

Strain ZYf1a\textsuperscript{T} grew well on IPM plates and R2A agar, weakly on ISP medium 2, LB agar and CYC agar, but not on ISP medium 7. R2A agar was used for routine cultivation of this strain. Growth of strain ZYf1a\textsuperscript{T} was observed at 20–37 °C (with an optimum at 30 °C) and over a pH range of 6.0–9.0 (with an optimum pH of 7.0). Strain ZYf1a\textsuperscript{T} formed circular, flat colonies with a saffron yellow centre that were 3–5 mm in diameter on IPM plates and R2A agar at 30 °C after 6 days, saffron yellow colonies with radial wrinkles (1–3 mm in diameter) on ISP medium 2 after 9 days, and irregular, raised, saffron yellow colonies (1–1.5 mm in diameter) on LB agar after 9 days. Cells were aerobic, Gram-stain-positive, non-motile and filamentous. Strain ZYf1a\textsuperscript{T} grew on solid medium with the formation of substrate mycelium. Endospores (0.4–0.5 × 1.2–1.4 μm) were produced singly on short, unbranched sporophores along the mycelium (Figs S1 and S2, available in the online Supplementary Material). However, formation of aerial mycelium was not observed on any of the media tested. Production of saffron yellow soluble pigment was observed in the liquid culture of this strain.

To determine the phylogenetic position of strain ZYf1a\textsuperscript{T}, the 16S rRNA gene sequence was analysed. Cloned 16S rRNA gene sequences of this strain yielded three different copies (similarity between the three was 99.8–99.9 %). One of these had one base deletion. The 16S rRNA gene sequences of strain ZYf1a\textsuperscript{T} showed closest similarity to that of Geothermococcus terrae YIM 77562\textsuperscript{T} (92.8–92.9 %). Phylogenetic analyses based on 16S rRNA gene sequences, performed using the neighbour-joining and maximum-likelihood methods, showed that strain ZYf1a\textsuperscript{T} belonged to the family Thermotogaceae, and that it formed a lineage deeply branched from related genera (Figs 1 and S3). The phylogenetic analyses and the relatively high sequence divergence values (>7.1 %) indicated that this strain represents a novel species of a new genus.
The physiological and chemotaxonomic characteristics of strain ZYf1a3T were determined. Strain ZYf1a3T did not produce acid from any of the carbon sources in the API 50 CH system. It did not use any of the carbon sources in the test using ISP medium 9. Similar results have been reported for members of the family Thermoactinomycetaceae (Hatayama et al., 2005; Addou et al., 2013). These media might not be rich enough for growth of this strain. Melanoid pigment was produced by strain ZYf1a3T grown on R2A agar with 0.5 % (w/v) L-tyrosine or 1 % (w/v) casein. The physiological characteristics of strain ZYf1a3T are summarized in Table 1 and in the species description. Strain ZYf1a3T contained menaquinone 7 (MK-7) as the sole respiratory quinone. The major cellular fatty acids (10 % of the total) were iso-C15 : 0 (50.3 % of the total) and anteiso-C15 : 0 (31.8 %). The cellular fatty acids iso-C13 : 0 (6.9 %), iso-C14 : 0 (5.5 %), anteiso-C13 : 0 (2.3 %), C14 : 0 (1.8 %), C16 : 0 (0.4 %), iso-C17 : 0 (0.4 %), iso-C16 : 0 (0.4 %) and C15 : 0 (0.2 %) were also detected (Table S1). The polar lipids were phosphatidylethanolamine, diphasphatidylglycerol, phosphatidylglycerol, phosphatidyl-N-methylethanolamine, three unidentified aminophospholipids, six unidentified aminolipids and an unidentified polar lipid (Fig. S4). The cell-wall peptidoglycan contained meso-diaminopimelic acid, alanine and glutamic acid. No sugars were detected in the cell wall. The DNA G+C content was 42.5 mol%.

Differential phenotypic characteristics between strain ZYf1a3T and its closest phylogenetic neighbours (G. terrae JCM 18057T, Shimazuella kribbensis JCM 14729T and Seinonella peptonophila JCM 10113T) are shown in Table 1. In particular, strain ZYf1a3T was easily distinguished from its closest phylogenetic neighbours by production of a saffron yellow soluble pigment and catalase activity. In addition, strain ZYf1a3T could be distinguished from G. terrae JCM 18057T based on growth at 50 °C, resistance to novobiocin, degradation of starch, L-tyrosine and Tween 80, profile of cellular fatty acids (Table S1) and DNA G+C content. Shimazuella kribbensis JCM 14729T differed from strain ZYf1a3T in formation of aerial mycelium, resistance to novobiocin, melanin production, profile of cellular fatty acids, major menaquinone and DNA G+C content. Seinonella peptonophila JCM 10113T differed from strain ZYf1a3T in formation of aerial mycelium, melanin production, degradation of gelatin, Tween 20 and Tween 40, profile of cellular fatty acids and DNA G+C content.
Table 1. Differential phenotypic characteristics between strain ZY1a3T and its closest phylogenetic neighbours

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<tr>
<td>Observation of aerial mycelium</td>
<td>–</td>
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<td>+</td>
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<td>Growth at 50 °C</td>
<td>–</td>
<td>+</td>
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<td>Growth on 25 µg novobiocin ml⁻³</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>Soluble pigment</td>
<td>Saffron yellow</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Catalase</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Melanin production</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Degradation of:</td>
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<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
<td>†</td>
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<tr>
<td>Gelatin</td>
<td>+</td>
<td>+</td>
<td>†</td>
<td>–</td>
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<tr>
<td>L-Tyrosine</td>
<td>–</td>
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<td>Tween 20</td>
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<td>+</td>
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<td>Tween 40</td>
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<td>Tween 80</td>
<td>–</td>
<td>+</td>
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<td>–</td>
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<tr>
<td>Major cellular fatty acid(s)</td>
<td>iso-C₁₅:₀, anteiso-C₁₅:₀</td>
<td>C₁₅:₀, anteiso-C₁₅:₀</td>
<td>iso-C₁₄:₀, anteiso-C₁₄:₀</td>
<td>iso-C₁₄:₀, anteiso-C₁₄:₀</td>
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<tr>
<td>(&gt;10 % peak area ratio)</td>
<td>C₁₅:₀, anteiso-C₁₅:₀</td>
<td>C₁₅:₀, anteiso-C₁₅:₀</td>
<td>C₁₄:₀, anteiso-C₁₄:₀</td>
<td>C₁₆:₁₀₁₁c</td>
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<tr>
<td>Major menaquinone</td>
<td>MK-7</td>
<td>MK-7</td>
<td>MK-9</td>
<td>MK-7</td>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>42.5</td>
<td>45.5</td>
<td>39.4</td>
<td>40</td>
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</table>

*Negative for degradation of starch, gelatin and Tween 20 as described by Zhou et al. (2014).
†Positive for degradation of starch and negative for degradation of gelatin as described by Park et al. (2007).
§Cells grown aerobically in R2A liquid medium for 2 days at 35 °C (ZY1a3T, Shimazuella kribbensis ICMP 14729T and Seinonella peptonophila ICMP 10113T) or 50 °C (G. terrae ICMP 18057T) were used for the analysis. Percentages of cellular fatty acids are shown in Table S1.

Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain ZY1a3T could be classified as representing a novel species in a new genus of the family Thermoactinomycesaceae. Differential phenotypic characteristics of this strain from related genera (Table 1) support this proposal. Therefore, we propose that strain ZY1a3T be classified as representing Croceifilum oryzae gen. nov., sp. nov.

Description of Croceifilum gen. nov.

Croceifilum (Cro.ce.i.fi’lum. L. adj. croceus saffron, yellow; L. neut. n. filum a thread; N.L. neut. n. Croceifilum a saffron yellow thread).

Cells are Gram-stain-positive, aerobic, mesophilic, non-motile and filamentous. Substrate mycelium is formed on solid medium. Endospores are produced singly on short, unbranched sporophores along the substrate mycelium. The cell-wall peptidoglycan contains meso-diaminopimelic acid, alanine and glutamic acid but no diagnostic sugars. The major menaquinone is MK-7. The major cellular fatty acids are iso-C₁₅:₀ and anteiso-C₁₅:₀. The major polar lipids are phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl-N-methylethanolamine and unidentifiable aminophospholipids. The type species is Croceifilum oryzae.

Description of Croceifilum oryzae sp. nov.

Croceifilum oryzae (o.ry’zae. L. gen. n. oryzae of rice, pertaining to the isolation of the type strain from rice paddy soil).

Displays the following properties in addition to those given in the genus description. Colonies are circular, flat with saffron yellow centre on IPM plates and R2A agar at 30 °C after 6 days, saffron yellow with radial wrinkles on ISP medium 2 after 9 days, and irregular, raised and saffron yellow on LB agar after 9 days. Growth occurs at pH 6.0–9.0 (with an optimum pH range of 7.0), at 20–37 °C (with an optimum at 30 °C) and in the presence of 0–1 % NaCl. Produces a saffron yellow soluble pigment. Positive for oxidase and melanin production. Negative for catalase and nitrate reduction. Casein, gelatin, Tween 20 and Tween 40 are degraded, but starch, L-tyrosine, hypoxanthine, xanthine, cellulose, urea and Tween 80 are not. Sensitive to novobiocin. In API 20E and 20 NE tests, hydrolysis of gelatin is positive, but nitrate reduction, indole production, H₂S production, acetoin production, urease, β-glucosidase, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and trypstatin deaminase, assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid, and fermentation/oxidation of D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, melibiose, amygdalin and L-arabinose are negative. Negative for all reactions in API 50 CH acid production tests.

The type strain, ZY1a3T (=JCM 30426T=CCUG 66446T=DSM 46876T), was isolated from a rice paddy soil sample collected in Zama (Kanagawa, Japan). The DNA G+C content of the type strain is 42.5 mol%.

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References


