Oceanobacillus luteolus sp. nov., isolated from soil

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Two Gram-stain-positive, rod-shaped and endospore-forming bacteria, designated WM-1T and WM-4, were isolated from a paddy soil and a forest soil, respectively, in South China. Comparative 16S rRNA gene sequence analyses showed that both strains were members of the genus Oceanobacillus and most closely related to Oceanobacillus chironomi LMG 23627T with pairwise sequence similarity of 96.0 %. The isolates contained menaquinone-7 (MK-7) as the respiratory quinone and anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{15:0} as the major fatty acids (>10 %). Polar lipids consisted of a predominance of diphosphatidylglycerol and moderate to minor amounts of phosphatidylglycerol and phosphatidylinositol. The cell-wall peptidoglycan contained meso-diaminopimelic acid. The DNA G+C content was 38.6–39.2 mol%. The 16S rRNA gene sequence of strain WM-1T displayed 99.7 % similarity to that of strain WM-4, and DNA–DNA hybridization between the two strains showed a relatedness value of 91 %. Based on the results of this polyphasic study, strains WM-1T and WM-4 represent a novel species in the genus Oceanobacillus, for which the name Oceanobacillus luteolus sp. nov. is proposed. The type strain is WM-1T (=KCTC 33119T=CGMCC 1.12406T).

The genus Oceanobacillus, belonging to the family Bacillaceae, was first described by Lu et al. (2001) when the type species Oceanobacillus iheyensis was isolated from a deep-sea mud sample collected at a depth of 1050 m on the Iheyu Ridge of the Nansei Islands. The genus Oceanobacillus comprises aerobic, Gram-stain-positive, motile and rod-shaped bacteria that are characterized chemotaxonomically by the presence of menaquinone-7 (MK-7) as the major isorenoid quinone and anteiso-C_{15:0} as the predominant cellular fatty acid (Lee et al., 2006). At the time of writing, the genus Oceanobacillus comprises 18 species and subspecies as follows: Oceanobacillus iheyensis isolated from a deep-sea sediment (Lu et al., 2001), Oceanobacillus oncorhynchi subsp. oncorhynchi from freshwater fish (Yumoto et al., 2005), Oceanobacillus oncorhynchi subsp. incalldanensis from an algal mat (Romano et al., 2006), Oceanobacillus picturatus from a mural painting (Lee et al., 2006), Oceanobacillus chironomi from a chironomid egg mass (Raats & Halpern, 2007), Oceanobacillus profundus from deep-sea sediment (Kim et al., 2007), Oceanobacillus caeni from a wastewater treatment system (Nam et al., 2008), Oceanobacillus kapialis from fermented shrimp paste (Namwong et al., 2009), Oceanobacillus sojae from soy sauce production equipment (Tomimaga et al., 2009), Oceanobacillus localis from a marine solar saltern (Lee et al., 2010), Oceanobacillus neutriophilus from activated sludge (Yang et al., 2010), 'Oceanobacillus manasiensis' from a salt lake (Wang et al., 2010), Oceanobacillus kimchii from a traditional Korean fermented food (Whon et al., 2010), Oceanobacillus indicinducens from a fermented Polygonum indigo (Hirotta et al., 2013a), Oceanobacillus polygoni from indigo fermentation fluid (Hirotta et al., 2013b), Oceanobacillus chungangensis from a sand dune (Lee et al., 2013), Oceanobacillus pacificus from a deep-sea sediment (Yu et al., 2014) and Oceanobacillus limi from a salt lake (Amoozegar et al., 2014). In this study, two bacterial strains, WM-1T and WM-4, were isolated from a paddy field soil and a forest soil, respectively, in South China, and classified as representatives of a novel species of the genus Oceanobacillus.

The soil samples for isolation of strains WM-1T and WM-4 were obtained from a paddy field in Xuwen County and a forest soil in Sihui County, respectively, in Guangdong Province, South China. The paddy soil was saline with pH 8.3, which had an organic matter of about 3 % and carbon and nitrogen concentrations of 35 g kg\(^{-1}\) and 3.2 g kg\(^{-1}\), respectively. The organic matter in the forest soil was about 9.6 %, the moisture was 70 % and the carbon and nitrogen concentrations were 36 g kg\(^{-1}\) and 3.7 g kg\(^{-1}\), respectively. The soil temperature of the sampling sites was about 25 °C. Microbial enrichment was carried out using...
the medium and method described by Yang et al. (2013) at 30 °C. The enriched population was serially diluted (from 10^{-1} to 10^{-5}) and 100 μl of each dilution was spread onto LB agar (10.0 g peptone, 5 g yeast extract, 10 g NaCl and 15 g agar) and incubated at 30 °C until single colonies were present. The novel strains were preserved at -80 °C in LB medium supplemented with 15 % (v/v) glycerol. For phenotypic characterization of the novel isolates, LB was used as the basal medium (Raats & Halpern, 2007) and standard phenotypic tests were performed according to the recommended minimal standards for describing new taxa of aerobic, endospore-forming bacteria (Logan et al., 2009).

Genomic DNA was extracted using a DNA extraction kit (Aidlab, China). The bacterial universal primer set, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTGACGAC-3 ′) (Lane, 1991), was used for PCR amplification of the 16S rRNA gene. The PCR product was gel-purified using a gel extraction kit (D2500; Omega Bio-tek) and sequenced, and almost complete 16S rRNA gene sequences were determined for strains WM-1T and WM-4 (1464 bp). The calculation of pairwise 16S rRNA gene sequence similarities was performed using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). The CLUSTAL_X program was used for multiple alignments (Thompson et al., 1997). Phylogenetic trees with 1000 bootstrap replications were reconstructed using MEGA software version 5.0 (Tamura et al., 2011). Clustering was performed with the neighbour-joining, maximum-likelihood, minimum-evolution and maximum-parsimony methods.

Strains WM-1T and WM-4 displayed the highest 16S rRNA gene sequence similarities to Oceanobacillus chironomi LMG 23627T (96.0 % similarity), Oceanobacillus indicireducens A21T (95.2 %–95.4 %) and Oceanobacillus profundus CL-MP28T (95.1 %). In addition to the genus Oceanobacillus, these two isolates were also related to species of the genera Ornithinibacillus, Paraliobacillus and Virgibacillus, with the highest 16S rRNA gene sequence similarities of 94.4 %, 94.2 % and 94.0 % to Oceanobacillus bavariensis SL6-1T, Paraliobacillus quinghaiensis YIM-C158T and Virgibacillus xinjiangensis WSBC 24001T, respectively. Phylogenetic analysis indicated that the novel isolates formed a robust cluster with species of the genus Oceanobacillus and were relatively far from the genera Ornithinibacillus, Paraliobacillus and Virgibacillus (Fig. 1 and Figs. S1, S2 and S3, available in the online Supplementary Material), which supported the affiliation of the novel isolates to the genus Oceanobacillus.

DNA–DNA hybridization was carried out between strains WM-1T and WM-4 with photobiotin-labelled probes in microplate wells as described by Ezaki et al. (1989) in triplicate. The hybridization value is the mean of at least two hybridization experiments (reciprocal and non-reciprocal values). Random amplified polymorphic DNA (RAPD)-PCR types of the two isolates were performed using three different previously reported primers (González et al., 2012) at a concentration of 20 mM: Ser 6 (GTGAGCGTTC; OPS 19 (GAGTCAGCAG), and OPB 6 (TGCTCTGCCC). Reproducibility was examined by comparing the profiles of at least three RAPD-PCR repeats.

The two isolates displayed 99.7 % similarity over the length of the 16S rRNA gene sequences. DNA–DNA hybridization between the two strains showed a relatedness value of 91 %. This value was above 70 %, confirming that the novel strains were members of the same species (Wayne et al., 1987). Three distinct stable RAPD-PCR types of the two isolates were revealed in Fig. S4. For RAPD-PCR, the banding patterns of strains WM-1T and WM-4 were almost the same when using primers Ser 6 and OPB 6, but were different when using primer OPS 19, suggesting that the two isolates represent two different strains.

Cell morphology was observed using a transmission electron microscope and a light microscope after 24 h of growth on LB agar at 30 °C. The endospore formation experiment was conducted using a staining solution kit (HB8300; Qingdao Hope-Bio Technology, China). The motility of cells was tested by observing the growth spread in semi-solid LB medium. The Gram reaction was determined by the conventional Gram-staining method and confirmed by a Gram staining kit (HB8278; Qingdao Hope-Bio Technology). The temperature range for growth was determined at 4, 15, 25, 30, 37, 40, 45, 50 and 55 °C. The pH range for growth was determined at pH 4.0–11.0 using the following buffer systems: pH 4.0–5.0, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0, 0.1 M KH2PO4/0.1 M NaOH; pH 9.0–10.0, 0.1 M NaHCO3/0.1 M Na2CO3; pH 11.0, 0.05 M Na2HPO4/0.1 M NaOH (Zhang et al., 2009). The tolerance for 0–10 % (w/v) NaCl was determined in a complex medium [containing (1%)]: 10 g tryptone and 5 g yeast extract; pH 8.0]. Oxidase activity was tested with oxidase test paper (bioMérieux), and catalase activity was determined based on bubble production in 3 % (v/v) H2O2 solution. Hydrolysis of Tween 20, 60 and 80, casein, gelatin and starch was tested as described by Dong & Cai (2001). The use of acetate, lactate, pyruvate, D-glucose, L-arabinose, sucrose, D-mannitol, D-fructose, glycerol and starch as sole carbon source was examined using a basal medium [containing (1%)]: 0.5 g KH2PO4, 0.1 g NaCl, 2H2O, 0.05 g yeast extract; pH 8.0] supplemented with 2.0 g/l (NH4)2SO4, 2.0 g/l NH4Cl, 2.0 g/l glycine, 1.5 g/l lalanine, 2.0 g/l l-serine, 2.0 g/l l-histidine, 2.0 g/l l-tyrosine or 2.0 g/l l-proline as sole nitrogen source. Other enzyme activities and biochemical characteristics were tested using the API 20E and API 50CH systems (bioMérieux) according to the manufacturer’s instructions. All of the tests were performed in duplicate.

Cells of strains WM-1T and WM-4 were Gram-stain-positive, aerobic, motile and rod-shaped (1.5–2.4 μm long and 0.4–0.7 μm wide) (Fig. S5). Ellipsoidal endospores were produced terminally or subterminally positioned within swollen sporangia (Fig. S6). Colonies were circular, convex and pale yellow with a diameter of 1.8–2.5 mm after incubation at 30 °C for 48 h on LB agar. Strains WM-1T and
WM-4 were positive for catalase but negative for oxidase activities. Strain WM-1\textsuperscript{T} could hydrolyse Tweens 60 and 80 but not Tween 20, casein or starch. In contrast to strain WM-1\textsuperscript{T}, strain WM-4 could hydrolyse Tweens 20, 60 and 80 and casein, but not starch. Acid was not produced from all tested substrates. The isolates could not utilize acetate, lactate, pyruvate, D-glucose, L-arabinose, sucrose, D-mannitol, D-fructose, glycerol or starch as a sole carbon source, or utilize (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, NH\textsubscript{4}Cl, glycine, L-alanine, L-serine, L-histidine, L-tyrosine or L-proline as a nitrogen source. The physiological characteristics of strains WM-1\textsuperscript{T} and WM-4 are summarized in the species description and a comparison of selective characteristics with type strains of closely related species are given in Tables 1 and S1.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strains WM-1\textsuperscript{T} and WM-4, and representatives of some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) $\geq 50\%$ are shown at the branching points. Bar, 0.02 substitutions per nucleotide position.
Table 1. Differential characteristics of strains WM-1<sup>T</sup> and WM-4, and the type strains of related members of the genus *Oceanobacillus*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>3</th>
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<th>5</th>
<th>6</th>
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<tr>
<td><strong>Temperature for growth (°C)</strong></td>
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<tr>
<td>Range</td>
<td>15–50</td>
<td>15–50</td>
<td>12–45</td>
<td>18–45</td>
<td>15–40</td>
<td>15–40</td>
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<tr>
<td>Optimum</td>
<td>30</td>
<td>30</td>
<td>37</td>
<td>37</td>
<td>30</td>
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<tr>
<td>Range</td>
<td>5.0–10.0</td>
<td>5.0–10.0</td>
<td>6.5–10.0</td>
<td>7.0–11.0</td>
<td>6.0–9.5</td>
<td>6.0–10.0</td>
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<tr>
<td>Optimum</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>9.5</td>
<td>7.0–8.0</td>
<td>8.0</td>
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<tr>
<td>Optimum NaCl for growth (%)</td>
<td>0.5–1</td>
<td>0.5–1</td>
<td>2–3</td>
<td>4</td>
<td>1.5–2.5</td>
<td>3</td>
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<td><strong>Growth with 10 % (w/v) NaCl</strong></td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Nitrate reduction</td>
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<td>+</td>
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<td>Citrate utilization</td>
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<td>+</td>
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<td>Oxidase</td>
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<td>β-Galactosidase</td>
<td>+</td>
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<td>Arginine dihydrolase</td>
<td>–</td>
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<td>Urease</td>
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<td><strong>Hydrolysis of</strong></td>
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<td>Tween 20</td>
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<td>+</td>
<td>–</td>
<td>+</td>
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<td>Tween 80</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Tween 60</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Casein</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Starch</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Gelatin</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>38.6</td>
<td>39.2</td>
<td>38.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35.8&lt;sup&gt;d&lt;/sup&gt;</td>
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*Data from: a, Raats & Halpern (2007); b, Hirota et al. (2013a); c, Kim et al. (2007); d, Lu et al. (2001).*

Strains WM-1<sup>T</sup> and WM-4 contained MK-7 as the respiratory quinone. Polar lipids of the two strains consisted of major amounts of diphasphatidylglycerol and moderate to minor amounts of phosphatidylglycerol and phosphatidylinositol (Fig. S7). As shown in Table S2, the fatty acid profiles of the isolates contained anteiso-C<sub>15:0</sub>, anteiso-C<sub>17:0</sub> and iso-C<sub>15:0</sub> as the major fatty acids (>10%), which were similar to their phylogenetic neighbours *Oceanobacillus chironomi* DSM 18262<sup>T</sup>, *Oceanobacillus indicireducens* JCM 17251<sup>T</sup> and *Oceanobacillus profundus* DSM 18246<sup>T</sup> in the sense that anteiso-C<sub>15:0</sub> is the predominant fatty acid. As for the type species *Oceanobacillus iheyensis* DSM 14371<sup>T</sup>, the predominant fatty acid is variable in different reports, iso-C<sub>15:0</sub> in this study and by Kim et al. (2007), but anteiso-C<sub>15:0</sub> by Hirota et al. (2013a). The DNA G+C content of the two novel strains was 38.6–39.2 mol%, which is in accordance with values for the genus *Oceanobacillus* (35.8–40.1 mol%; Yang et al., 2010). Both strains contained meso-diaminopimelic acid (*meso*-DAP) as the diamino acid, and this is the same with other species of the genus *Oceanobacillus* (Hirota et al., 2013a). In conclusion, the above characteristics supported the affiliation of strains WM-1<sup>T</sup> and WM-4 to the genus *Oceanobacillus*.
and positive for the hydrolysis of Tween 60 and 80 but not starch. Both strains are negative for arginine dihydrolase, trisodium citrate utilization, urease and tryptophan deaminase, and cannot produce acid from erythritol, L-arabinose, D-ribose, D-xyllose, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, N-acetylglucosamine, amydalin, arbutin, salicin, cellobiose, asuscin ferric citrate, maltose, trehalose, raffinose, glycozen, starch or gentiobiose, but at least one of their reference strains are positive for these items. Therefore, on the basis of the data presented, strains WM-1T and WM-4 are considered to be representatives of a novel species of the genus Oceanobacillus, for which the name Oceanobacillus luteolus sp. nov. is proposed.

**Description of Oceanobacillus luteolus sp. nov.**

*Oceanobacillus luteolus* (lu.te.o.lus. L. masc. adj. luteolus yellowish, referring to the colour of the colonies on LB agar).

Cells are Gram-stain-positive, motile, aerobic, rod-shaped (1.5–2.4 μm long and 0.4–0.7 μm wide) and produce ellipsoidal endospores terminally or subterminally positioned within swollen sporangia. Colonies are circular, convex and pale yellow with a diameter of 1.8–2.5 mm after incubation at 30 °C for 48 h on LB agar. Cells can grow without NaCl and can tolerate up to 5% (w/v) NaCl in a complex medium. Growth occurs at pH 5.0–10.0 (optimum pH 8.0) and at 15–50 °C (optimum 30 °C). Catalase-positive but oxidase-negative. Tween 60 and 80 are hydrolysed but not starch or aesculin. Hydrolysis of Tween 20, casein and gelatin is variable. H2S and indole are not produced. Cells test positive for β-galactosidase activity, but negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, trisodium citrate, urease, tryptophan deaminase, Voges–Proskauer reaction and nitrate reduction. Acetate, lactate, pyruvate, D-glucose, L-arabinose, sucrose, D-mannitol, D-fructose, glyceroand starch are not utilized as a sole carbon source, and (NH4)2SO4, NH4Cl, glycine, L-alanine, L-serine, L-histidine, L-tyrosine or L-proline are not utilized as a nitrogen source. Acid is not produced from any carbohydrates in the API 50 CH system. The major cellular fatty acids are anteiso-C15:0 (14.3%) and iso-C15:0 (12.9%). The type strain, WM-1T (=KCTC 33119T=CGMCC 1.12406T), was isolated from a paddy field soil in Guangdong, South China. The DNA G+C content of the type strain is 38.6 mol%.

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