Rubrivirga marina gen. nov., sp. nov., a member of the family Rhodothermaceae isolated from deep seawater

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Two aerobic, Gram-stain-negative, pale-red-pigmented and rod-shaped bacterial strains, designated SAORIC-26 and SAORIC-28T, were isolated from seawater (3000 m depth) from the Pacific Ocean. Phylogenetic analysis based on their 16S rRNA gene sequences revealed that the novel isolates could be affiliated with the family Rhodothermaceae of the class Cytophagia. Strains SAORIC-26 and SAORIC-28T shared 99.7% pairwise sequence similarity with each other and showed less than 92.6% similarity with other cultivated members of the class Cytophagia. The strains were found to be non-motile, oxidase-positive, catalase-negative and able to hydrolyse gelatin and aesculin. The DNA G+C contents were determined to be 64.8–65.8 mol% and MK-7 was the predominant menaquinone. Summed feature 9 (iso-C17:1ω9c and/or C16:1ω10c) and feature 3 (C16:1ω6c and/or C16:1ω7c) and iso-C15:0 were found to be the major cellular fatty acids. On the basis of this taxonomic study using a polyphasic approach, it was concluded that strains SAORIC-26 and SAORIC-28T represent a novel species of a new genus in the family Rhodothermaceae, for which the name Rubrivirga marina gen. nov., sp. nov. is proposed. The type strain of the type species of is SAORIC-28T (=KCTC 23867T=NBRC 108816T). An additional strain of the species is SAORIC-26.

The recently validated family Rhodothermaceae (Ludwig et al., 2011) belonging to the class Cytophagia in the phylum Bacteroidetes comprises four genera, Rhodothermus (Alfredsson et al., 1988), Salinibacter (Antón et al., 2002) Salisaeta (Vaisman & Oren, 2009) and Rubricoccus (Park et al., 2011). Three genera Rhodothermus, Salinibacter and Salisaeta have been isolated from extreme environments and exhibit thermophilic or halophilic characteristics. At the time of writing the genus Rubricoccus contains only one species with a validly published name, which was, in contrast to members of the other three genera, isolated from a euphotic zone of the Pacific Ocean and showed mesophilic and slightly halophilic characteristics. In this study we isolated two novel bacteria from deep seawater and investigated their biochemical and physiological characteristics to determine their taxonomic status.

Two aerobic bacteria were isolated from a deep seawater sample obtained from the western North Pacific Ocean (32°00′ N, 138°13′ E; depth, 3000 m) during the research cruise (KT-10-12) of RV ‘Tansei Maru’ [Atmospheric and Ocean Research Institute, The University of Tokyo, and Japan Agency for Marine-Earth Science and Technology (JAMSTEC)] on 3 July 2010. The seawater (200 μl) sample was inoculated on 1/5 strength marine agar (Difco) (agar 15 g, peptone 1 g, yeast extract 0.2 g, MgCl2 1.76 g, Na2SO4 0.65 g, CaCl2 0.36 g, KCl 0.1 g, NaHCO3 0.32 g, Ferric citrate 0.2 g, KBr 0.016 g, SrCl2 6 mg, H3BO3 4 mg, Na3HPO4 1.6 mg, Na2SiO3 0.8 mg, NaF 0.48 mg, NH4NO3 0.32 mg in 11 of 80% aged seawater) and incubated at 10°C for 30 days. Isolated strains were maintained on 1/2 strength marine agar 2216 (MA: Difco) supplemented with 1% NaCl at 30°C. The temperature (5, 10, 15, 20, 25, 30, 37 and 45°C) and pH (5–10) ranges for growth were determined by incubating the isolate on 1/2 MA supplemented with 1% NaCl at 30°C. The temperature (5, 10, 15, 20, 25, 30, 37 and 45°C) and pH (5–10) ranges for growth were determined by incubating the isolate on 1/2 MA supplemented with 1% NaCl. The NaCl concentration for growth was determined on 1/2 MA (agar 15 g, peptone 2.5 g, yeast extract 0.5 g, MgCl2 4.4 g, Na2SO4 1.62 g, CaCl2 0.9 g, KCl 0.27 g, NaHCO3 0.8 g, ferric citrate 0.5 g, KBr 0.04 g, SrCl2 0.015 g, H3BO3 0.01 g, Na2HPO4 4 mg, Na2SiO3 2 mg, NaF 1.2 mg, NH4NO3 0.8 mg l−1) containing 0–15% (w/
v) NaCl. Gram-staining was performed as described by Murray et al. (1994). Cell morphology and motility were observed by light microscopy (E600; Nikon) and transmission electron microscopy (TEM). Gliding motility was determined as described by Perry (1973). Growth under anaerobic conditions was determined after incubation for 4 weeks in an AnaeroPack (Mitsubishi Gas Chemical) on 1/2 MA supplemented with 1 % NaCl. Catalase activity was determined by bubble formation in a 3 % H₂O₂ solution. Oxidase activity was determined using cytochrome oxidase test paper (Nissui Pharmaceutical). API 20E, API 20NE, API 50CH and API ZYM strips (bioMérieux) were used to determine the physiological and biochemical characteristics. All suspension media for the API test strips were supplemented with 2 % (w/v) NaCl solution (final concentration). The results of API 20E, API 20 NE and API 50 CH tests were recorded after 5 days and those of API ZYM were recorded after 2 days at 30 °C. Determination of the respiratory quinone was carried out as described previously (Xie & Yokota, 2003). Bacterial strains grown on 1/2 MA supplemented with 1 % NaCl for 10 days at 30 °C was used for the analysis of fatty acid methyl esters. Fatty acid methyl esters were extracted and prepared according to standard protocols provided by the MIDI/Hewlett Packard Microbial Identification system (Sasser, 1990) using MIDI version 6.0 with TSBA6 database. Polar lipids were extracted according to the procedures described by Minnikin et al. (1984). They were identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin et al., 1984; Komagata & Suzuki, 1987). Phospholipids were detected with the Zinzadze reagent of Dittmer & Lester (1964). Whole-lipid profiles were detected by spraying with molybdophosphoric acid (5 g molybdophosphoric acid hydrate in 100 ml ethanol) followed by heating at 150 °C (Worliczek et al., 2007). DNA was prepared according to the method of Marmur (1961) from cells grown on 1/2 MA supplemented with 1 % NaCl and the DNA base composition was determined by using the HPLC method of Mesbah et al. (1989). A fragment of approximately 1450 bp from the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers 27F and 1492R (Lane, 1991: Weisburg et al., 1991). To ascertain the phylogenetic position of the novel isolate, the 16S rRNA gene sequences of strains SAORIC-26 and SAORIC-28T were compared with sequences obtained from GenBank (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). Multiple alignments of sequences were performed using CLUSTAL_X (version 1.83) (Thompson et al., 1997) and gaps and ambiguous bases were omitted for sequence comparison. Aligned sequences were analysed using MEGA version 4 (Tamura et al., 2007). Evolutionary distances were calculated using distance options according to the Kimura two-parameter model (Kimura, 1983). Clustering with the neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-likelihood (ML) (Felsenstein, 1981) methods was performed using ML version 2.4.4 (Guindon & Gascuel, 2003). Bootstrap values (Felsenstein, 1985) were calculated from 1000 replications. Pair-wise sequence similarities were calculated using the EzTaxon-e server (Kim et al., 2012).

The almost complete 16S rRNA gene sequence for strain SAORIC-26 and SAORIC-28T were determined and BLAST search results with the GenBank database showed that these strains belong to the family Rhodothermaceae. Phylogenetic analyses of 16S rRNA gene sequences showed that these strains formed a cluster with Rubricoccus marinus SG-29T with bootstrap confidence values of 100 % (NJ and ML) (Fig. 1). In particular, strains SAORIC-26 and SAORIC-28T showed 99.7 % sequence similarity with each other and displayed 92.6 % similarity with Rubricoccus marinus SG-29T, 86.4 % and 86.5 % sequence similarity, respectively with Salinibacter iranicus CB7T and 86.2 % and 86.3 % sequence similarity, respectively with Salinibacter luteus DGO T. Therefore, on the basis of these phylogenetic data, strains SAORIC-26 and SAORIC-28T should be placed into a novel species of a new genus within the family Rhodothermaceae.

Cells of strain SAORIC-28T grown on 1/2 MA supplemented with 1 % NaCl at 30 °C for 10 days were rod-shaped, pale-red pigmented, approximately 0.7 ± 0.5 μm wide and 3.0 ± 2.3 μm long. Spores were not observed microscopically (Fig. 2). Growth occurred only under aerobic conditions between 10 °C and 37 °C. Optimal temperature and pH ranges were 25–30 °C and pH 6–8, respectively. The strain required NaCl for growth and grew in 1–5 % NaCl (Table 1). Strains SAORIC-26 and SAORIC-28T were catalase-negative but oxidase, lipase (C14), α-galactosidase, β-glucosidase N-acetyl-β-glucosaminidase, Trypsin, z-chymotrypsin and acid phosphatase-positive. The polar lipid composition of strains SAORIC-26 and SAORIC-28T contained phosphatidylethanolamine, phosphatidylglycerol, diposphatidylglycerol, an unknown phospholipid and two unknown lipids (Table 1, Fig S1, available in IJSEM Online). The total polar lipids pattern of
strains SAORIC-26 and SAORIC-28<sup>T</sup> were similar to that of *Rubricoccus marinus* SG-29<sup>T</sup> but the presence of an unknown phospholipid distinguishes strains SAORIC-26 and SAORIC-28<sup>T</sup> from *Rubricoccus marinus* SG-29<sup>T</sup> (Fig. S1).

The major cellular fatty acids (>10%) of strains SAORIC-26 and SAORIC-28<sup>T</sup> were summed feature 9 (iso-C<sub>17:1</sub>v<sub>9c</sub> and/or C<sub>16:0</sub>10-methyl; 15.7% and 16.2% respectively), summed feature 3 (C<sub>16:1</sub>v<sub>6c</sub> and/or C<sub>16:1</sub>v<sub>7c</sub>; 17.7% and 16.0% respectively), iso-C<sub>17:0</sub> (12.6% and 13.5% respectively) and

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**Table 1.** Differential characteristics of the genus *Rubrivirga* gen. nov. and related members of genera in the family *Rhodothermaceae*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigment</td>
<td>Pale red</td>
<td>Reddish</td>
<td>Red</td>
<td>Red</td>
<td>Reddish</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Rod</td>
<td>Coccus</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
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<tr>
<td>Cell length (μm)</td>
<td>0.7–5.0</td>
<td>0.3–0.5</td>
<td>15.0–30.0</td>
<td>0.4–2.6</td>
<td>2.0–2.5</td>
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<tr>
<td>Temperature for growth (°C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl (% w/v)</td>
<td>1–5</td>
<td>1–5</td>
<td>5–20</td>
<td>15–30</td>
<td>2–6</td>
</tr>
<tr>
<td>Catalase</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Oxidase</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>Lipase (C14)</td>
<td>+</td>
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</tr>
<tr>
<td>α-Galactosidase</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>β-Glucosidase</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>3-acetyl-β-glucosaminidase</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Polar lipids</td>
<td>DPG, PE, PG, PL, 2L</td>
<td>DPG, PE, PG, 2L</td>
<td>ND</td>
<td>DPG,PC 3GL, L</td>
<td>DPG, PE</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>64.8–65.8</td>
<td>68.9&lt;sup&gt;*&lt;/sup&gt;</td>
<td>62.9</td>
<td>66.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>64.4</td>
</tr>
</tbody>
</table>

<sup>*</sup>Data from (Park et al., 2011).
iso-C_{15:0} (10.4 % and 10.6 % respectively). The portions of iso-C_{15:0}, C_{16:1}ω9c and C_{17:1}ω8c distinguished strains SAORIC-26 and SAORIC-28\textsuperscript{T} from the closely related *Rubricoccus marinus* SG-29\textsuperscript{T} (Table 2).

Phenotypic differences among strains SAORIC-26, SAORIC-28\textsuperscript{T} and members of related genera are shown in Tables 1 and 2. These accumulated data showed that strains SAORIC-26 and SAORIC-28\textsuperscript{T} were sufficiently different from related genera and thus represented a novel species in a new genus of the family Rhodothermaceae, with the proposed name *Rubrivirga marina* gen. nov., sp. nov.

### Description of Rubrivirga gen. nov.

*Rubrivirga* (Ru.bri.vir’ga. L. adj. ruber -bra -brum red; L. fem. n. virga a rod; N.L. fem. n. *Rubrivirga* a red rod.)

Cells are pale-red-pigmented, rod-shaped, non-motile, Gram-stain-negative and obligately aerobic. The major respiratory quinone is MK-7. Predominant cellular fatty acids are iso-C\textsubscript{17:1}ω9c and/or C\textsubscript{16:0} 10-methyl (summed feature 9), C\textsubscript{16:1}ω6c and/or C\textsubscript{16:1}ω7c (summed feature 3), iso-C\textsubscript{17:0} and iso-C\textsubscript{15:0}. The type species is *Rubrivirga marina*.

### Description of Rubrivirga marina sp. nov.

*Rubrivirga marina*. (ma.ri’na. L. fem. adj. marina of the sea, marine.)

*Rubrivirga marina* exhibits the following properties in addition to those given in the genus description. Colonies are circular and 1–2 mm in diameter after 10 days incubation of 1/2 MA supplemented with 1 % NaCl. Cells are approximately 0.7 ± 0.5 μm wide and 3.0 ± 2.3 μm long (n=22). The temperature range for growth is 10–37 °C. The optimal temperature for growth is 25–30 °C. No growth occurs above 45 °C. The pH range for growth is 6.0–9.0. NaCl is required for growth and strains can tolerate up to 5 % (w/v). NaCl Catalase-negative but oxidase-positive. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, tryptase, x-chymotrypsin, acid phosphatase, x-galactosidase and N-acetyl-β-galactosaminidase activities, but negative for naphthol-AS-BI-phosphohydrolase, β-galactosidase, x-glucosidase, β-glucuronidase, x-mannosidase and x-fucosidase activities. Produces acetoin. Gelatin and aesculin are hydrolysed. Acid is produced from aesculin, maltose, 5-ketoglucanate and arabinitol. Assimilation of D-glucose, L-arabinose, mannose, mannitol, N-acetylglucosamine, maltose, gluconate, capric acid, adipic acid, malic acid and citrate were not detected according to API 20NE.

The type strain, SAROIC-28\textsuperscript{T} (=KCTC 23867\textsuperscript{T}=NBRC 108816\textsuperscript{T}), was isolated from deep seawater (depth; 3000 m) from the western North Pacific Ocean near Japan. The DNA G+C content of the type strain is 68.9 mol%. Strain SAORIC-26 (=KCTC 23866=NBRC 108815), is an additional strain of the species.

### Acknowledgements

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### References


