Gracilimonas rosea sp. nov., isolated from tropical seawater, and emended description of the genus Gracilimonas

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A Gram-staining-negative, non-motile, spore-forming, rod-shaped, marine bacterial strain, CL-KR², was isolated from tropical seawater near Kosrae, an island in the Federated States of Micronesia. Analysis of the 16S rRNA gene sequence of strain CL-KR² revealed a clear affiliation with the genus Gracilimonas. Based on phylogenetic analysis, strain CL-KR² showed the closest phylogenetic relationship to Gracilimonas tropica CL-CB462T, with 16S rRNA gene sequence similarity of 96.6%. DNA–DNA relatedness between strain CL-KR² and G. tropica CL-CB462T was 6.7% (reciprocal 9.5%). Strain CL-KR² grew in the presence of 1–20% sea salts and the optimal salt concentration was 3.5–5%. The temperature and pH optima for growth were 35°C and pH 7.5. The major cellular fatty acids (≥10.0%) of strain CL-KR² were iso-C₁₅ : 0, summed feature 3 (iso-C₁₅ : 02-OH and/or C₁₆ : 1ω7c) and iso-C₁₇ : ₁ω9c and the only isoprenoid quinone was MK-7. The polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, an unidentified phospholipid, two unidentified glycolipids and two unidentified lipids. The genomic DNA G+C content of strain CL-KR² was 43.2 mol%. The combined phenotypic, chemotaxonomic and phylogenetic data showed that strain CL-KR² could be distinguished from the only member of the genus Gracilimonas with a validly published name. Thus, strain CL-KR² should be assigned to a novel species in the genus Gracilimonas, for which the name Gracilimonas rosea sp. nov. is proposed. The type strain is CL-KR² (=KCCM 90206T=JCM 18898T).

The phylum Bacteroidetes occupies a large fraction of the domain Bacteria, but it is yet to be clarified taxonomically (Wang et al., 2012). The genus Gracilimonas was previously designated a genus incertae sedis within the order Sphingobacteriales (Choi et al., 2009), but its taxonomic position was later determined to be in the family Chitinophagaceae (http://www.bacterio.net). At the time of writing, the family Chitinophagaceae contains 16 genera with validly published names. To date, only one species, Gracilimonas tropica, isolated from a Synechococcus culture from seawater of the tropical Pacific Ocean, has been assigned to the genus. In this study, we describe a novel marine bacterial strain, CL-KR², isolated from tropical seawater near Kosrae, an island in the Federated States of Micronesia. Based on a polyphasic approach, we propose that the bacterium represents a novel species in the genus Gracilimonas.

Strain CL-KR² was isolated from tropical seawater sampled at a depth of 75 m. The seawater sample was diluted tenfold with 0.2 μm-filtered seawater from the same depth and then 50 μl diluted seawater was spread onto marine agar (MA; Difco) and incubated aerobically at 35°C for 3 days. Single, distinct colonies were subcultured onto MA every 3–4 days to ensure purity. Amongst the strains isolated from the seawater sample, a strain designated CL-KR² showed 3.4% 16S rRNA gene sequence divergence from G. tropica CL-CB462T (Choi et al., 2009). After salinity and temperature optima were
determined, strain CL-KR2T was routinely cultured onto MA and in marine broth (MB; Difco) at 35 °C. The strain was stored in MB supplemented with 30 % (v/v) glycerol at −80 °C.

For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony by the boiling method (Englen & Kelley, 2000). Crude extracts served as DNA template for PCRs, which included primers 27F and 1492R (Lane, 1991) and Taq DNA polymerase (Promega). The PCR product was purified by using an AccuPrep PCR purification kit (Bioneer). Direct sequencing of the purified 16S rRNA gene PCR product was performed using sequencing primers 27F, 518F, 800R and 1492R (Lane, 1991; Anzai et al., 1997) with an Applied Biosystems sequencer (ABI 3730XL) at Macrogen (Seoul, Korea). The nearly complete 16S rRNA gene sequence (1458 bp) of strain CL-KR2T was obtained and compared against the GenBank and EzTaxon-e databases using BLASTN (Altschul et al., 1997; Kim et al., 2012). Sequence similarity was calculated using the EzTaxon-e server. The 16S rRNA gene sequences of the type strain of G. tropica and members of phylogenetically related genera were obtained from GenBank and the Ribosomal Database Project II database (Cole et al., 2007). The 16S rRNA gene sequences were aligned manually using the jPHYDIT program (Jeon et al., 2011). To assess the robustness of the tree topology, bootstrap analyses were performed based on 1000 replications for the neighbour-joining, maximum-parsimony and maximum-likelihood methods.

G. tropica CL-CB462T (Choi et al., 2009) was used as a reference strain in all morphological, physiological and biochemical analyses. Morphological and physiological characteristics described hereafter were based on cultures grown for 5 days in MB at 35 °C unless otherwise specified. Morphological and physiological tests were performed as follows. Cell morphology was examined by epifluorescence microscopy and transmission electron microscopy (EX2; JEOL). Cells for microscope observation were grown for 1 day at 35 °C in MB. Anaerobic growth was tested on MA using the GasPak anaerobic system (BBL) at 35 °C for 3 weeks. Anaerobic acid production from D-glucose was also tested on ZO medium (Lemos et al., 1985) supplemented with 1 % D-glucose. The temperature range for growth was determined by assessing changes in OD600 over time in MB at 5–45 °C (in increments of 5 °C) for 7 days. The pH range for growth was also determined by assessing changes in the OD600 in MB adjusted to pH 5.0–10.0 (in increments of 0.5 pH units), incubated at 35 °C for 7 days. The following buffers were used for pH experiments: citrate/phosphate (pH 5.0 and 5.5), MOPS (Sigma) (pH 6.0–8.0), borate/boric acid (pH 8.5 and 9.0), carbonate/bicarbonate (pH 9.5 and 10.0), borax/NaOH (pH 9.5 and 10.0) and Tris/HCl (pH 8.5–10.0), each at a final concentration of 50 mM (Scheidle et al., 2011; Jang et al., 2013). The pH of a subsample of autoclave-sterilized medium was measured before inoculation of cells to check for changes in pH. There were no significant changes in the pH of the medium after autoclave sterilization. Salt tolerance tests were carried out on the basis of changes in the OD600 in synthetic ZoBell broth (per litre distilled water: 5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate) supplemented with 0–25 % sea salts (Sigma) [in increments of 0.5 % (w/v) from 0 to 10 % and at 12, 15, 18, 20, 23 and 25 %] at 35 °C over 7 days. Gram-staining was performed using the method of Smibert & Krieg (1994). Cell motility was observed by the hanging drop method (Suzuki et al., 2001). Catalase activity was determined by bubble formation after adding 3 % (v/v) H2O2 and oxidase activity was determined using 1 % (w/v) tetramethyl p-phenylenediamine (Cappuccino & Sherman, 2002). Endospores were detected by malachite green staining (Smibert & Krieg, 1994). The presence of flexirubin-type pigments and poly-β-hydroxybutyrate granules was examined by methods described by Choi et al. (2009). Genomic DNA–DNA relatedness was determined by using dot-blot hybridization (Kim et al., 2007, 2008). Prehybridization, hybridization and detection were performed using a DIG labelling and detection kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. The experiment was repeated on different days, as done by Goris et al. (2007).

Hydrolysis of gelatin, starch and Tweens 40, 60 and 80 was assessed according to Høvik Hansen & Sorheim (1991). Decomposition of casein, hypoxanthine and xanthine was determined according to the protocols described by Smibert & Krieg (1994). Carbon utilization was tested according to Bruns et al. (2001). Cultures of strain CL-KR2T and the reference strain grown on MA for 5 days at 35 °C were used, and cell suspensions were prepared using the same basal broth medium. Carbon utilization was recorded as negative when growth was equal to, or less than, that in the negative control with no carbon source. Growth was measured by monitoring changes in the OD600 (Ultraspec 2000; Pharmacia Biotech) after 7 days of incubation at 35 °C. In addition, other biochemical activities of strain CL-KR2T were determined by using API 20NE and API ZYM kits (bioMérieux) according to the manufacturer’s instructions except that cell suspensions were prepared using artificial seawater (ASW) (per litre distilled water: 24 g NaCl, 5.1 g MgCl2, 4 g Na2SO4, 1.1 g CaCl2, 0.7 g KCl, 0.2 g NaHCO3, 0.1 g KBr, 0.027 g H3BO3, 0.024 g SrCl2, 0.003 g NaF; Lyman & Fleming, 1940). For API ZYM and API 20NE tests, strain CL-KR2T and the reference strain were cultivated in MB at 35 °C for 5 days. Cells were harvested by centrifugation and
resuspended in ASW at a cell density corresponding to tubes no. 5 and no. 1 (for API ZYM and API 20NE tests, respectively) of the MacFarland scale of standard opacities.

Cultures of strain CL-KR2\textsuperscript{T} and the reference strain \textit{G. tropica} CL-CB462\textsuperscript{T} grown in MB at 35 °C for 5 days were used for all chemotaxonomic analyses (isoprenoid quinones, fatty acids and total polar lipids). Cells were collected by centrifugation and washed with 0.2 μm-filtered autoclaved PBS and the collected cells were lyophilized for 24 h for further analyses (Hwang \textit{et al.}, 2006). Isoprenoid quinones were extracted according to the method of Minnikin \textit{et al.} (1984) and were analysed by HPLC (Collins, 1985). Polar lipids were extracted by the method described by Minnikin \textit{et al.} (1984), separated by two-dimensional TLC and identified by spraying with detection reagents (Komagata & Suzuki, 1987). TLC plates were scanned immediately after the end of the reaction with detection reagents. Fatty acid methyl esters in whole cells of strain CL-KR2\textsuperscript{T} and \textit{G. tropica} CL-CB462\textsuperscript{T} were analysed by GC according to the instructions of the Microbial Identification System at the Korean Culture Center of Microorganisms (KCCM), Seoul, Korea. For DNA G+C content analysis, DNA was extracted by the method of Marmur (1961) and analysed by HPLC (HP 100; Hewlett Packard) as described by Mesbah \textit{et al.} (1989). Lambda phage DNA was analysed together for calibration. Experiments were performed in duplicate in all morphological, physiological and chemotaxonomic tests except fatty acids analysis.

Cells of strain CL-KR2\textsuperscript{T} grown for 1 day at 35 °C in MB were rod-shaped, 0.2–0.4 μm wide and 1.2–4.2 μm long. Strain CL-KR2\textsuperscript{T} grown for 9 days on MA at 35 °C formed circular, convex, entire, pink and opaque colonies, 2.6–3.4 mm in diameter. Cells were Gram-staining-negative, non-motile and spore-forming. Strain CL-KR2\textsuperscript{T} grew well under aerobic conditions, and could also grow slowly under anaerobic conditions. Anaerobic acid production occurred from D-glucose. The optimal temperature for growth was 35 °C. No growth occurred at 10 or 45 °C over 7 days of incubation. The pH range for growth was pH 6.5–9.0 and the optimum was pH 7.5. Strain CL-KR2\textsuperscript{T} was not able to grow in ZoBell broth in the absence of sea salts; salt tolerance ranged from 1 to 20 %. The results of oxidase and catalase tests were positive. Gelatin, casein, starch and Tween 60 were hydrolysed, but Tweens 40 and 80, hypoxanthine and xanthine were not. Flexirubin pigments were not produced and poly-β-hydroxybutyrate granules were not observed inside the cells. Other morphological and physiological characteristics of strain CL-KR2\textsuperscript{T} are given in the species description and a comparison of selected morphological, physiological and biochemical characteristics of strain CL-KR2\textsuperscript{T} and \textit{G. tropica} CL-CB462\textsuperscript{T} is given in Table 1.

Phylogenetic analyses based on the 16S rRNA gene sequence showed that strain CL-KR2\textsuperscript{T} belonged to the genus \textit{Gracilimonas}. Strain CL-KR2\textsuperscript{T} was most closely related to \textit{G. tropica} CL-CB462\textsuperscript{T}, with 16S rRNA gene sequence similarity of 96.6 %. The 16S rRNA gene similarity between strain CL-KR2\textsuperscript{T} and other phylogenetically close strains was relatively low, with 92.0 % similarity to \textit{Balneola alkaliphila} CM41_14b\textsuperscript{T} (Urios \textit{et al.}, 2008) being the next highest. In all three of the phylogenetic trees,

\begin{table}[h]
\centering
\caption{Selected physiological and biochemical characteristics of strain CL-KR2\textsuperscript{T} and \textit{G. tropica} CL-CB462\textsuperscript{T}}
\begin{tabular}{|l|l|l|}
\hline
Characteristic & CL-KR2\textsuperscript{T} & \textit{G. tropica} CL-CB462\textsuperscript{T} \\
\hline
Cell size (μm) & 1.2–4.2 × 0.2–0.4 & 3.4–9.8 × 0.2–0.5 \\
Colony colour & Pink & Orange \\
Temperature for growth (°C) & 15–40 & 20–40 \\
Salt tolerance (%) & 1–20 & 1.5–20 \\
Optimum & 3.5–5 & 2.5–5 \\
Utilization as a sole carbon source & & \\
Mannitol & + & - \\
Pyruvic acid & + & - \\
Sucrose & + & - \\
1-Rhamnose & - & + \\
L-Proline & + & - \\
α-Chymotrypsin (API ZYM) & - & + \\
Unidentified glycolipid(s) & GL1, GL2 & GL1 \\
DNA G+C content (mol%) & 43.2 & 42.4 \\
\hline
\end{tabular}
\end{table}
strain CL-KR2T formed a robust clade with *G. tropica* CL-CB462T (Fig. 1). The level of DNA–DNA relatedness between *G. tropica* CL-CB462T and strain CL-KR2T was 6.7 ± 0.5% (reciprocal 9.5 ± 1.0%). This value is well below the currently accepted limit for DNA relatedness (70%) for the phylogenetic definition of a species (Stackebrandt & Goebel, 1994). Thus, it indicates that strain CL-KR2T represents a novel species in the genus *Gracilimonas*. The DNA G+C content of strain CL-KR2T was 43.2 mol%.

The only isoprenoid quinone of strain CL-KR2T was MK-7. The major cellular fatty acids (>10.0%) of strain CL-KR2T were iso-C15:0 (34.5%), summed feature 3 (iso-C15:0 2-OH and/or C16:1ω7c 19.0%) and iso-C17:1ω9c (10.0%) (Table 2). Overall, the fatty acid composition of strain CL-KR2T appeared to be generally similar to that of *G. tropica* CL-CB462T. The polar lipids detected from both strain CL-KR2T and *G. tropica* CL-CB462T were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, an unidentified phospholipid, an unidentified glycolipid (GL1) and unidentified lipids L1 and L2 (Fig. S1, available in IJSEM Online).

The following phenotypic features were characteristic of strain CL-KR2T in comparison with its closest phylogenetic neighbour, *G. tropica* CL-CB462T. Growth occurred at 15 °C for strain CL-KR2T, but not for *G. tropica* CL-CB462T over 7 days of incubation in MB and on MA. Strain CL-KR2T could be distinguished from *G. tropica* CL-CB462T by the ability to utilize mannitol, pyruvic acid, sucrose and L-proline and the inability to utilize L-rhamnose as a sole carbon source. According to the results of API ZYM tests, strain CL-KR2T differed from *G. tropica* CL-CB462T. The polar lipids detected from both strain CL-KR2T and *G. tropica* CL-CB462T were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, an unidentified phospholipid, an unidentified glycolipid (GL1) and unidentified lipids L1 and L2 (Fig. S1, available in IJSEM Online).

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Table 2. Cellular fatty acid compositions of strain CL-KR2<sup>T</sup> and G. tropica CL-CB462<sup>T</sup>

Data were obtained in this study from cells grown in MB (Difco) at 35 °C for 5 days. The test conditions in this study were different from those used by Choi et al. (2009) (MA, 30 °C, 3 days). Values are percentages of total fatty acids. —, Not detected; TR, trace amount (<1%).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>CL-KR2&lt;sup&gt;T&lt;/sup&gt;</th>
<th>G. tropica CL-CB462&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
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<tr>
<td>C&lt;sub&gt;13.0&lt;/sub&gt;</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C&lt;sub&gt;14.0&lt;/sub&gt;</td>
<td>TR</td>
<td>TR</td>
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<tr>
<td>C&lt;sub&gt;15.0&lt;/sub&gt;</td>
<td>5.6</td>
<td>3.2</td>
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<tr>
<td>C&lt;sub&gt;16.0&lt;/sub&gt;</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C&lt;sub&gt;18.0&lt;/sub&gt;</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Unsaturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;14:1o5c&lt;/sub&gt;</td>
<td>—</td>
<td>TR</td>
</tr>
<tr>
<td>C&lt;sub&gt;15:1o6c&lt;/sub&gt;</td>
<td>4.7</td>
<td>4.1</td>
</tr>
<tr>
<td>C&lt;sub&gt;15:1o8c&lt;/sub&gt;</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:1o5c&lt;/sub&gt;</td>
<td>1.4</td>
<td>1.2</td>
</tr>
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<td>3.4</td>
<td>3.6</td>
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<td>1.9</td>
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<tr>
<td>C&lt;sub&gt;18:1o7c&lt;/sub&gt;</td>
<td>TR</td>
<td>—</td>
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<tr>
<td>Branched</td>
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<tr>
<td>iso-C&lt;sub&gt;12.0&lt;/sub&gt;</td>
<td>—</td>
<td>—</td>
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<tr>
<td>iso-C&lt;sub&gt;13.0&lt;/sub&gt;</td>
<td>3.1</td>
<td>2.4</td>
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<td>3.5</td>
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<td>44.6</td>
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<tr>
<td>iso-C&lt;sub&gt;15:1&lt;/sub&gt;F&lt;sup&gt;*&lt;/sup&gt;</td>
<td>TR</td>
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</tr>
<tr>
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<td>2.9</td>
<td>1.7</td>
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<tr>
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<td>1.4</td>
<td>—</td>
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<tr>
<td>iso-C&lt;sub&gt;17:0&lt;/sub&gt;</td>
<td>1.0</td>
<td>1.6</td>
</tr>
<tr>
<td>iso-C&lt;sub&gt;17:1o9c&lt;/sub&gt;</td>
<td>10.0</td>
<td>10.0</td>
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<tr>
<td>anteiso-C&lt;sub&gt;15:0&lt;/sub&gt;</td>
<td>2.1</td>
<td>5.5</td>
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<tr>
<td>anteiso-C&lt;sub&gt;17:1o9c&lt;/sub&gt;</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>Hydroxy</td>
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<tr>
<td>C&lt;sub&gt;15.0&lt;/sub&gt; 2-OH</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Summed features†</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>3</td>
<td>19.0</td>
<td>10.2</td>
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<tr>
<td>4</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>Unknowns‡</td>
<td></td>
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<tr>
<td>13.565</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>14.959</td>
<td>—</td>
<td>TR</td>
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</tbody>
</table>

*Double bond positions indicated by capital letters are unknown.
†Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 1 comprised iso-C<sub>15:1</sub>I and/or C<sub>13:0</sub> 3-OH; summed feature 3 comprised iso-C<sub>15:0</sub> 2-OH and/or C<sub>16:1o7c</sub>; summed feature 4 comprised anteiso-C<sub>17:1</sub>B and/or iso-C<sub>17:1</sub>L.
‡Unknown fatty acids have no name listed in the peak library file of the MIDI system and therefore can be identified only by their equivalent chain-lengths.

CL-CB462<sup>T</sup> by the absence of z-chymotrypsin activity (Table 1). In the total polar lipid profile, strain CL-KR2<sup>T</sup> differed from G. tropica CL-CB462<sup>T</sup> by the presence of a second unidentified glycolipid (GL2) (Fig. S1).

In conclusion, based on the phenotypic, chemotaxonomic and phylogenetic data described above, we suggest that strain CL-KR2<sup>T</sup> represents a novel species of the genus Gracilimonas, for which the name Gracilimonas rosea sp. nov. is proposed.

**Description of Gracilimonas rosea sp. nov.**

_Gracilimonas rosea_ (ro’s.e.a. L. fem. adj. rosea rose-coloured, pink, referring to the colony colour).

After 9 days of incubation on MA at 35 °C, colonies are circular, convex, entire, opaque, pink and 2.6–3.4 mm in diameter. Growth occurs at 15–40 °C (optimum 35 °C) and at pH 6.5–9.0 (optimum pH 7.5). No growth occurs at 10 or 45 °C. No growth occurs in ZoBell broth without sea salts. Growth occurs in the presence of sea salts at 1–20% (w/v) (optimum 3.5–5%). Aerobic and facultatively anaerobic. Gram-staining-negative, non-motile, spore-forming and positive for catalase and oxidase activity. Gelatin, casein, starch and Tween 60 are hydrolysed, but Tetens 40 and 80, hypoxanthine and xanthine are not hydrolysed. Does not produce flexirubin pigments. Poly-β-hydroxybutyrate granules are absent. According to API ZYM tests, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase are positive, but tests for trypsin, z-chymotrypsin, x- and β-galactosidases, β-glucuronidase, x- and β-glucosidases, β-mannosidase and x-fucosidase are negative. In API 20NE tests, aesculin is hydrolysed, but nitrate reduction, indole production, fermentation of glucose and activities of arginine dihydrolase and urease are absent. Acid is produced from d-glucose in ZOF medium under anaerobic conditions. In sole carbon utilization tests, fructose, glucose, mannitol, mannose, raffinose, trehalose, glyc erol, L-arabinose, N-acetyl-D-glucosamine, pyruvic acid, succrose, DL-aspartate and L-proline are utilized, but acetate, ascorbate, citrate, cellobiose, salicin, inositol, L-rhamnose, maleic acid and succinate are not utilized. The only isoprenoid quinone is MK-7. The polar lipids detected are diphasphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, an unidentified phospholipid, two unidentified glycolipids and two unidentified lipids.

The type strain is CL-KR2<sup>T</sup> (KCCM 90206<sup>T</sup> = JCM 18898<sup>T</sup>), isolated from tropical seawater near Kosrae, an island in the Federated States of Micronesia. The genomic DNA G+C content of the type strain is 43.2 mol%.

**Emended description of the genus Gracilimonas**

Choi et al. 2009

The characteristics of the genus are as described by Choi et al. (2009), with the following amendment. The major polar lipids are diphasphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and an unidentified phospholipid. The only isoprenoid quinone is MK-7.
Acknowledgements

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Gracilimonas rosea sp. nov.