Rheinheimera nanhaiensis sp. nov., isolated from marine sediments, and emended description of the genus Rheinheimera Brettar et al. 2002 emend. Merchant et al. 2007

Hui-Juan Li,1,2† Xi-Ying Zhang,1† Yan-Jiao Zhang,1 Ming-Yang Zhou,1 Zhao-Ming Gao,1 Xiu-Lan Chen,1 Hong-Yue Dang3 and Yu-Zhong Zhang1

1The State Key Laboratory of Microbial Technology, Marine Biotechnology Research Center, Shandong University, Jinan 250100, PR China
2College of Chemical and Environmental Engineering, Shandong University of Science and Technology, Qingdao 266510, PR China
3Centre for Bioengineering and Biotechnology, China University of Petroleum (East China), Qingdao 266555, PR China

A Gram-negative, facultatively aerobic, oxidase- and catalase-positive, rod-shaped bacterium, designated strain E407-8T, was isolated from a sediment sample from the South China Sea. Phylogenetic analysis of the 16S rRNA gene sequence revealed that strain E407-8T was affiliated with the genus Rheinheimera, sharing the highest sequence similarity with Rheinheimera pacifica KMM 1406T (97.5 %) and Rheinheimera aquimaris SW-353T (97.4 %) and showing less than 97 % sequence similarity to the type strains of other recognized Rheinheimera species. Levels of DNA–DNA relatedness of strain E407-8T to R. pacifica DSM 17616T and R. aquimaris JCM 14331T were 25.2 % (25.3 % in the duplicate measurement) and 9.4 % (6.5 %), respectively. The bacterium could grow at 10–48 °C (optimum 37 °C) and in the presence of 0–8 % (w/v) NaCl (optimum 0.5–2.5 %). The major cellular fatty acids of strain E407-8T were summed feature 3 (C16 : 1v7c and/or iso-C15 : 0 2-OH), C17 : 1ω8c, C16 : 0 and C18 : 1ω7c. The predominant respiratory quinone was ubiquinone Q-8. The DNA G+C content was 51.0 mol%. Based on the results of our polyphasic taxonomic study, strain E407-8T represents a novel species in the genus Rheinheimera, for which the name Rheinheimera nanhaiensis sp. nov. is proposed. The type strain is E407-8T (= CCTCC AB 209089T = KACC 140303T). An emended description of the genus Rheinheimera Brettar et al. 2002 emend. Merchant et al. 2007 is also proposed.

The genus Rheinheimera, belonging to the family Chromatiaceae in the class Gammaproteobacteria, was originally proposed by Brettar et al. (2002) for a small group of closely related Gram-negative, flagellated, blue-coloured bacterial strains isolated from seawater samples from different depths of the central Baltic Sea. At the time of writing, the genus Rheinheimera contains eight recognized species, Rheinheimera baltica (the type species; Brettar et al., 2002), R. pacifica (Romanenko et al., 2003), R. perlucida (Brettar et al., 2006), R. aquimaris (Yoon et al., 2007), R. chironomi (Halpern et al., 2007), R. texanensis (Merchant et al., 2007), R. soli (Ryu et al., 2008) and R. tangshanensis (Zhang et al., 2008). Most Rheinheimera species have originated from aquatic (marine or freshwater) environments, with the exceptions of R. soli from a playground soil sample and R. tangshanensis from rice-plant root samples, indicating that members of this genus may be widespread in various environments (Ryu et al., 2008; Zhang et al., 2008).

In our recent study on the diversity of protease-producing marine bacteria (Zhou et al., 2009), a protease-producing Rheinheimera-like strain, designated E407-8T, was isolated from a deep-sea sediment sample from the South China Sea. In this paper, based on a polyphasic taxonomic study, strain E407-8T is proposed to represent a novel Rheinheimera species.

A marine sediment sample was collected from station E407 (18° 29.810' N 112° 00.17' E) at a water depth of 1800 m using a core sampler, during the South China Sea Open...
Cruise of R/V Shiyan 3 in August 2007. Strain E407-8^T was isolated from the sediment sample using the dilution plating technique on a screening medium containing 0.2 % yeast extract (Oxoid), 0.3 % casein, 0.5 % gelatin, 1.5 % agar and artificial seawater (pH 7.0) at 15 °C (Zhou et al., 2009). Strain E407-8^T was able to form clear hydrolytic zones on this medium. The artificial seawater was prepared with synthetic sea salts (Marine Life Reef Salt, QingDao, China) and contained the following approximate ion concentrations (g l−1): Na^+ 9.1; K^+ 0.35; Mg^{2+} 1.25; Ca^{2+} 0.43; Sr^{2+} 0.0095; Cl^−, 17.1; SO_4^{2-}, 2.4; Br^−, 0.06; F^−, 0.001; HCO_3^−, 0.145. The isolated strain E407-8^T was routinely cultivated on a solid medium containing 0.5 % tryptone (Oxoid), 0.1 % yeast extract (Oxoid), 1.5 % agar and artificial seawater (hereafter referred to as marine agar) or in a liquid medium containing 0.5 % tryptone, 0.1 % yeast extract and artificial seawater (hereafter referred to as marine broth) at 30 °C and stored at −80 °C in marine broth supplemented with 20 % glycerol.

Cell morphology was observed by transmission electron microscopy (JEM-100CX II) using cells grown in marine broth at 15 or 30 °C for 24 h. The cells were stained with 1 % phosphotungstic acid. Gram-staining was performed using Hucker’s staining method (Murray et al., 1994). Growth at 10, 15, 20, 25, 30, 35, 37, 40, 42, 45, 48 and 50 °C was measured in marine broth. Growth at pH 5.0–11.0 (at intervals of 0.5 pH units) was determined in a medium containing 0.5 % tryptone, 0.1 % yeast extract and distilled water at 30 °C. The pH of the medium was adjusted with 1 M NaOH or HCl. The NaCl concentration range (0–9 %, w/v, in increments of 0.5 %) for growth was tested at 30 °C in the same medium used for pH tests with different NaCl concentrations. Anaerobic growth was checked in an anaerobic chamber (Forma 1029; Thermo Electron) in marine broth 2216 (Difco) at 28 °C for 15 days.

Catalase activity was assessed by bubble formation in 3 % (v/v) H_2O_2 solution. Oxidase activity was determined with commercial oxidase test strips (Merck). DNase activity was detected on DNase test agar (Oxoid) prepared using artificial seawater. Hydrolysis of starch, casein and Tweenes 40 and 80 was determined on marine agar at 30 °C according to standard methods (Smibert & Krieg, 1994). The ability to utilize various carbohydrates including D-glucose, N-acetyl-D-glucosamine, sucrose, maltose, citrate, xylose, mannose, trehalose, cellubiose, galactose, raffinose, L-arabinose and lactose as sole carbon and energy sources for growth was determined in a basal medium containing 0.054 % NH_4Cl, 3 % NaCl, 0.3 % MgCl_2, 6H_2O, 0.2 % K_2SO_4, 0.02 % K_2HPO_4, 0.001 % CaCl_2, 0.0006 % FeCl_3, 6H_2O, 0.0005 % Na_2MoO_4.7H_2O, 0.0004 % CuCl_2, 2H_2O, 0.6 % Tris and distilled water supplemented with 1 % carbohydrate as described by Shieh et al. (2004). Enzyme activities and other physiological and biochemical traits were determined by using API ZYM, API 20E and API 20NE strips (bioMérieux) according to the manufacturer’s instructions. Cells for inoculation of API ZYM, API 20E and API 20NE strips were suspended in artificial seawater; the API ZYM strip was read after 24 h and the API 20E and API 20NE strips were read after 72 h incubation at 30 °C. Antibiotic susceptibility tests were performed using the disc-diffusion method on marine agar and growth inhibition zones were observed after 3 days of incubation at 30 °C. R. aquimaris JCM 14331^T obtained from the Japan Collection of Microorganisms and R. pacifica DSM 17616^T obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) were used as reference strains in all the above tests.

Genomic DNA of strain E407-8^T was extracted using a bacterial genomic DNA isolation kit (BioTek). The 16S rRNA gene was amplified by PCR from genomic DNA with primers 27F and 1492R (Lane, 1991). PCR products were purified using the E.Z.N.A. gel extraction kit (Omega BioTek). Purified PCR products were ligated into pMD 18-T vector (TaKaRa) and sequenced at Biosune Inc. (Shanghai, China) using an automated DNA sequencer (Applied Biosystems model 3730). The obtained 16S rRNA gene sequence of strain E407-8^T was compared with those deposited in GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and further aligned manually with sequences of related taxa retrieved from GenBank using the MEGA software version 4.0 (Tamura et al., 2007). Phylogenetic trees were constructed by the same software, using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods and using bootstrap analyses (1000 replications) (Felsenstein, 1985). Evolutionary distances for the neighbour-joining method were computed using the Jukes & Cantor (1969) model. DNA–DNA hybridization experiments between strain E407-8^T and the two closely related type strains R. aquimaris JCM 14331^T and R. pacifica DSM 17616^T were conducted at the DSMZ by using the methods of De Ley et al. (1970) as modified by Huß et al. (1983).

The genomic DNA G+C content was determined using HPLC (Mesbah et al., 1989). Respiratory lipoquinone analyses were carried out by the Identification Service of the DSMZ and Dr B. J. Tindall. Cellular fatty acid analyses, from cells grown in marine broth at 30 °C for 24 h, were performed according to the instructions of the Sherlock Microbial Identification System (MIDI) at the Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing, PR China.

Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain E407-8^T was affiliated with the genus Rheinheimera. The strain shared the highest 16S rRNA gene sequence similarity with R. pacifica KMM 1406^T (97.5 %) and R. aquimaris SW-353^T (97.4 %) and 94.7–96.7 % similarity with type strains of other recognized Rheinheimera species. The neighbour-joining phylogenetic tree showed that strain E407-8^T clustered with members of the genus Rheinheimera and formed an intragenus branch with R. pacifica KMM 1406^T with 80 % bootstrap support (Fig. 1). The maximum-parsimony phylogenetic tree showed a similar topology (Supplementary Fig. S1, Rheinheimera nanhaiensis sp. nov., from the South China Sea

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or less than 97% 16S rRNA gene sequence similarity are generally considered to belong to separate species. (Wayne et al., 1987; Stackebrandt & Goebel, 1994; Stackebrandt et al., 2002), the above genotypic data indicated that strain E407-8T represents a novel species in the genus Rheinheimera.

The DNA G+C content of strain E407-8T was 51.0 mol%, which was close to the highest G+C content reported for Rheinheimera species (50.5 mol%; Yoon et al., 2007). The major fatty acids (>5%) of strain E407-8T were summed feature 3 (C_{16:1\omega7c} and/or iso-C_{15:0} 2-OH; 21.1%), C_{17:1\omega8c} (14.5%), C_{16:0} (11.9%), C_{18:1\omega7c} (10.0%) and C_{17:0} (8.6%). The fatty acid profile of strain E407-8T was generally similar to those of other known Rheinheimera species, which are characterized by unsaturated fatty acids including C_{16:1\omega7c}, C_{17:1\omega8c} and C_{18:1\omega7c} and the straight-chain fatty acid C_{16:0} as major fatty acid components (Table 1). The dominant respiratory quinone of strain E407-8T was ubiquinone Q-8 (98%); minor amounts of Q-7 (2%) was also detected.

Cells of strain E407-8T were found to be able to produce prosthecae (Fig. 2), a cellular substructure formed by cytoplasmic extrusion from the cell wall and considered to help bacteria to improve nutrient uptake efficiency by

Table 1. Fatty acid compositions of strain E407-8T and type strains of other Rheinheimera species

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{10:0}</td>
<td>0.2</td>
<td>0.1</td>
<td>--</td>
<td>--</td>
<td>0.1</td>
<td>--</td>
<td>3.7</td>
<td>1.2</td>
<td>2.2</td>
</tr>
<tr>
<td>C_{11:0} 3-OH</td>
<td>2.1</td>
<td>0.4</td>
<td>--</td>
<td>--</td>
<td>1.5</td>
<td>1.7</td>
<td>4.6</td>
<td>5.5</td>
<td>3.3</td>
</tr>
<tr>
<td>C_{12:0}</td>
<td>1.3</td>
<td>2.8</td>
<td>--</td>
<td>--</td>
<td>2.1</td>
<td>3.1</td>
<td>0.5</td>
<td>1.1</td>
<td>--</td>
</tr>
<tr>
<td>C_{12:0} 3-OH</td>
<td>3.9</td>
<td>4.7</td>
<td>--</td>
<td>--</td>
<td>3.6</td>
<td>5.6</td>
<td>9.5</td>
<td>8.5</td>
<td>7.2</td>
</tr>
<tr>
<td>C_{13:0} 3-OH</td>
<td>2.7a</td>
<td>1.0a</td>
<td>--</td>
<td>--</td>
<td>2.1a</td>
<td>3.0a</td>
<td>1.3a</td>
<td>1.9a</td>
<td>--</td>
</tr>
<tr>
<td>C_{14:0}</td>
<td>0.7</td>
<td>1.4</td>
<td>--</td>
<td>--</td>
<td>0.9</td>
<td>2.1</td>
<td>1.4</td>
<td>0.9</td>
<td>--</td>
</tr>
<tr>
<td>C_{14:0} 3-OH</td>
<td>2.0b</td>
<td>3.8b</td>
<td>--</td>
<td>--</td>
<td>2.1b</td>
<td>3.4b</td>
<td>0.3b</td>
<td>0.3b</td>
<td>--</td>
</tr>
<tr>
<td>C_{15:0}</td>
<td>2.8</td>
<td>1.0</td>
<td>2.4</td>
<td>--</td>
<td>3.2</td>
<td>2.7</td>
<td>6.0</td>
<td>6.9</td>
<td>2.1</td>
</tr>
<tr>
<td>C_{15:1\omega8c}</td>
<td>4.5</td>
<td>1.3</td>
<td>3.3</td>
<td>--</td>
<td>3.8</td>
<td>1.4</td>
<td>2.9</td>
<td>2.8</td>
<td>3.0</td>
</tr>
<tr>
<td>C_{16:0}</td>
<td>11.9</td>
<td>19.9</td>
<td>19.1</td>
<td>--</td>
<td>17.7</td>
<td>25.1</td>
<td>10.8</td>
<td>14.8</td>
<td>7.8</td>
</tr>
<tr>
<td>iso-C_{16:0}</td>
<td>3.7</td>
<td>1.5</td>
<td>3.7</td>
<td>--</td>
<td>2.1</td>
<td>0.7</td>
<td>0.8</td>
<td>1.5</td>
<td>--</td>
</tr>
<tr>
<td>C_{16:1\omega7c}</td>
<td>21.4</td>
<td>28.5</td>
<td>--</td>
<td>--</td>
<td>23.4</td>
<td>13.8</td>
<td>24.0</td>
<td>25.8</td>
<td>33.7</td>
</tr>
<tr>
<td>C_{16:1\omega9c}</td>
<td>0.6</td>
<td>2.1</td>
<td>25.5</td>
<td>--</td>
<td>2.1</td>
<td>2.3</td>
<td>0.7</td>
<td>--</td>
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</tr>
<tr>
<td>C_{17:0}</td>
<td>8.6</td>
<td>4.1</td>
<td>8.1</td>
<td>--</td>
<td>8.1</td>
<td>9.3</td>
<td>4.2</td>
<td>5.8</td>
<td>--</td>
</tr>
<tr>
<td>C_{17:1\omega8c}</td>
<td>14.5</td>
<td>5.6</td>
<td>11.7</td>
<td>--</td>
<td>11.6</td>
<td>9.7</td>
<td>12.5</td>
<td>7.6</td>
<td>15.2</td>
</tr>
<tr>
<td>C_{18:0}</td>
<td>0.6</td>
<td>1.5</td>
<td>--</td>
<td>--</td>
<td>0.8</td>
<td>1.5</td>
<td>0.4</td>
<td>0.3</td>
<td>--</td>
</tr>
<tr>
<td>iso-C_{18:0}</td>
<td>1.2</td>
<td>0.6</td>
<td>1.3</td>
<td>--</td>
<td>0.6</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>C_{18:1\omega7c}</td>
<td>10.0</td>
<td>15.9</td>
<td>15.7</td>
<td>--</td>
<td>8.1</td>
<td>11.0</td>
<td>7.1</td>
<td>6.7</td>
<td>13.4</td>
</tr>
</tbody>
</table>

*Reported as: a, summed feature 1 (C_{11:0} 3-OH and/or iso-C_{15:1\omega7c}); b, summed feature 2 (C_{14:0} 3-OH and/or iso-C_{16:0} 1\omega7c); c, summed feature 3 (C_{16:1\omega7c} and/or iso-C_{15:0} 2-OH). Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System.
increasing the cellular surface-to-volume ratio (Hedlund et al., 1997; Van Trappen et al., 2004a; Young, 2006). To the best of our knowledge, this is the first observation of prosthica formation in the genus Rheinheimera; members of the class Gammaproteobacteria able to produce prostheca have been reported from the genera Alteromonas (Van Trappen et al., 2004a; Chiu et al., 2007; Vandecandelaere et al., 2008), Glaciecola (Van Trappen et al., 2004b; Chen et al., 2009), Pseudidiomarina (Jean et al., 2006) and Simiduia (Shieh et al., 2008).

Other morphological, physiological and biochemical characteristics of strain E407-8T are given in the species description and in Table 2. Cells of strain E407-8T were Gram-negative, oxidase- and catalase-positive, flagellated rods (Fig. 2). Consistent with other known Rheinheimera species, the strain could grow across broad ranges of temperature (10–48 °C) and NaCl concentration (0–8 %) and could assimilate N-acetylgucosamine and hydrolyse gelatin, starch and aesculin. The above phenotypic features supported the assignment of the strain to the genus Rheinheimera. There was also a range of phenotypic characteristics that could be used to differentiate strain E407-8T from recognized Rheinheimera species, such as flagellum position and number, prostheta formation, reduction of nitrate, oxygen requirement, ranges of salt, temperature and pH for growth, enzyme activities, carbohydrate assimilation and DNA G+C content (Table 2).

Taken together, the results of phylogenetic analysis of 16S rRNA gene sequences, DNA–DNA hybridization experiments and analysis of fatty acid profiles and phenotypic characteristics indicate that strain E407-8T should be assigned to the genus Rheinheimera and represents a novel species of the genus, for which the name Rheinheimera nanhaiensis sp. nov. is proposed.

Some properties of strain E407-8T are not in agreement with or were not included in the original and emended descriptions of the genus Rheinheimera (Brettar et al., 2002; Merchant et al., 2007), such as facultatively aerobic growth, the ability to produce prostheta, growth at 48 °C or in 8 % NaCl and the presence of summed feature 3 among the major fatty acids. Similar discrepancies can also be observed for some other Rheinheimera species with validly published names, indicating the necessity to emend further the description of the genus, as follows.

**Emended description of the genus Rheinheimera Brettar et al. 2002 emend. Merchant et al. 2007**

The description is as given by Brettar et al. (2002) and Merchant et al. (2007) with the following amendments. Cells of some species can produce prostheta. Growth is aerobic or facultatively aerobic, chemoheterotrophic and occurs at 4–48 °C. Dominant fatty acids are C16:1ω7c or summed feature 3 (C16:1ω7c and/or iso-C15:0 2-OH), C16:0, C17:1ω6c and C18:1ω7c (Table 1). Strains do not necessarily need NaCl for growth and cannot tolerate >8 % NaCl.

**Description of Rheinheimera nanhaiensis sp. nov.**

*Rheinheimera nanhaiensis* (nan.hai.en’sis. N.L. fem. adj. nanhaiensis pertaining to NanHai, the Chinese name for the South China Sea, where the type strain was isolated).

Cells are Gram-negative, straight or curved rods (1.0–2.0 μm long and 0.3–0.5 μm wide). Motile by a single polar flagellum. Able to form prostheta. Facultatively aerobic. Colonies on marine agar are slightly yellow, circular (1.5–2.0 mm in diameter) and convex with smooth surfaces after incubation for 48 h at 30 °C. Oxidase- and catalase-positive. Reduces nitrate to nitrite. Grows at 10–48 °C (optimum, 37 °C), but not at 50 °C. Grows in 0–8 % (w/v) NaCl (optimum, 0.5–2.5 %) and at pH 5.5–10.0 (optimum, pH 7.5–8.5). Hydrolyses starch, gelatin, casein, DNA and Tween 40 and 80. Able to utilize D-glucose, N-acetyl-D-glucosamine, sucrose, trehalose, cellobiose and maltose as sole carbon and energy sources. In API 20NE tests, positive for reduction of nitrate to nitrite, gelatinase, assimilation of D-glucose, N-acetylglucosamine and maltose and hydrolysis of aesculin and negative for indole production, arginine dihydrolase, urease, β-galactosidase,
Table 2. Differential characteristics of strain E407-8\textsuperscript{T} and type strains of other Rheinheimera species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
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<tbody>
<tr>
<td>Cell morphology*</td>
<td>SR/CR</td>
<td>R</td>
<td>R/C</td>
<td>SR/CR</td>
<td>R</td>
<td>R</td>
<td>R/C</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>Width</td>
<td>0.3–0.5</td>
<td>0.6–0.8</td>
<td>0.3–0.7</td>
<td>0.9–1.5</td>
<td>0.3–0.7</td>
<td>0.4–1.0</td>
<td>0.5–1.5</td>
<td>0.7–0.8</td>
</tr>
<tr>
<td></td>
<td>Length</td>
<td>1.0–2.0</td>
<td>1.8–2.0</td>
<td>0.3–5.0</td>
<td>2.0–3.5</td>
<td>1.0–2.4</td>
<td>1.3–2.5</td>
<td>0.9–2.5</td>
<td>1.2–2.5</td>
</tr>
<tr>
<td>Flagella†</td>
<td>S, P</td>
<td>M, P and L</td>
<td>S, P</td>
<td>S, P</td>
<td>S, P or L</td>
<td>S, P</td>
<td>S, P or M and L</td>
<td>S, P</td>
<td></td>
</tr>
<tr>
<td>Oxygen requirement‡</td>
<td>FA</td>
<td>A</td>
<td>A</td>
<td>FA</td>
<td>FA</td>
<td>A</td>
<td>A</td>
<td>FA</td>
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</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+§</td>
<td>+§</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>–§</td>
<td>+§</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td></td>
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<tr>
<td>NaCl concentration for growth (%, w/v)</td>
<td>Range</td>
<td>0–8</td>
<td>0–8</td>
<td>0–8</td>
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<tr>
<td></td>
<td>Optimum</td>
<td>0.5–2.5</td>
<td>0.5–2.5§</td>
<td>1–3</td>
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<td>0.5–1</td>
<td>1</td>
<td>1–3</td>
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<td>6.0–8.5</td>
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<td>N-Acetylglucosaminidase</td>
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<td>–§</td>
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<td>+</td>
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<td>+</td>
<td>+§</td>
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<td>Acid phosphatase</td>
<td>+</td>
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<td>+§</td>
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<td>Esterase (C4)</td>
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<td>l-Arabinose</td>
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<td>+§</td>
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</table>

* C, Cocci; CR, curved rods; R, rods; SR, straight rods.
† M, Multiple; s, single; l, lateral; p, polar.
‡ A, Aerobic; FA, facultatively aerobic.
§ Result from this study.

...content...
lipoquinoines are Q-8 (98 %) and Q-7 (2 %). The major fatty acids (>5%) are summed feature 3 (C₁₆:1ω7c and/or iso-C₁₅:0 2-OH), C₁₇:1ω8c, C₁₆:0, C₁₈:1ω7c and C₁₇:0. The DNA G+C content of the type strain is 51.0 mol%.

The type strain is E407-8T (= CCTCC AB 209089T = KACC 14030T), isolated from marine sediments of the South China Sea.

Acknowledgements

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References


