Echinicola rosea sp. nov., a marine bacterium isolated from surface seawater

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A novel Gram-stain-negative, rod-shaped, gliding, halotolerant, aerobic, light-pink-pigmented bacterium, strain JL3085T, was isolated from surface water of the South China Sea (16° 49′ 4″ N 112° 20′ 24″ E; temperature: 28.3°C, salinity: 34.5%). The major respiratory quinone was menaquinone 7 (MK-7). The polar lipids of strain JL3085T comprised phosphatidylethanolamine, four unidentified phospholipids and three unidentified lipids. The major fatty acids were iso-C₁₅:0 summed feature 3 (comprising iso-C₁₅:0 2-OH and/or C₁₆:1ω7c), iso-C₁₇:1ω9c, C₁₇:1ω6c, iso-C₁₇:1ω3-OH, iso-C₁₇:1ω6c, anteiso-C₁₅:0 and C₁₆:1ω5c. The DNA G+C content of strain JL3085T was 43.8 mol%. 16S rRNA gene sequence analysis indicated that strain JL3085T was affiliated with the genus Echinicola, a member of the phylum Bacteroidetes, and was related most closely to Echinicola vietnamensis KMM 6221T (96.8% similarity). DNA–DNA relatedness between strain JL3085T and E. vietnamensis KMM 6221T was 27.5%. Based on the evidence presented here, strain JL3085T is regarded as representing a novel species of the genus Echinicola, for which the name Echinicola rosea sp. nov. is proposed. The type strain is JL3085T (=NBRC 111782T=CGMCC 1.15407T).

The genus Echinicola was first proposed by Nedashkovskaya et al. (2006), and includes Echinicola pacifica, Echinicola vietnamensis, Echinicola jeungdonensis and ‘Echinicola shivajiensis’. Species of the genus Echinicola share the following characteristics: aerobic, heterotrophic, Gram-negative, gliding and non-diffusible carotenoid-pigmented bacteria with iso-C₁₅:0, C₁₆:1ω5c, iso-C₁₇:1ω9c, C₁₇:1ω6c, iso-C₁₇:1ω3-OH and summed feature 3 (comprising iso-C₁₅:0 2-OH and/or C₁₆:1ω7c) as the major fatty acids, menaquinone 7 (MK-7) as the major respiratory quinone, and DNA G+C content ranging from 44.0 to 46.9 mol% (Nedashkovskaya et al., 2006, 2007; Kim et al., 2011; Srinivas et al., 2012). Our objective in this study was to evaluate the phylogenetic, phenotypic and chemotaxonomic characteristics of a new strain, JL3085T, isolated from surface seawater of the South China Sea. The results suggest that this strain is affiliated to the genus Echinicola and represents a novel species.

We collected samples of surface seawater from Yongxing Island in the South China Sea (16° 49′ 4″ N 112° 20′ 24″ E) in May 2013. The samples were suspended in 20.0% (v/v) glycerol and stored at −80.0°C. Isolation of strains was performed using the standard dilution plating technique.

A 100 μl suspension was spread on marine agar 2216 (MA; Difco) medium and cultured at 30.0°C for 1 week, then a light-pink colony was picked and purified. Subsequently, the bacterial strain JL3085T was obtained and cultivated in an electrothermal incubator for further study.

Here, we describe the morphological, phylogenetic and biochemical characteristics of strain JL3085T according to the relevant minimal standards (Logan et al., 2009; Tindall et al., 2010). E. vietnamensis DSM 17526T, E. pacifica KCTC 12368T, E. jeungdonensis KCTC 23122T and ‘E. shivajiensis’ JCM 17847 were used as reference strains in parallel tests.

The cell morphology of strain JL3085T was observed by transmission electron microscopy (H600; Hitachi). Gliding motility was determined as described by Bowman (2000), and an aerobic/anaerobic respiration test was performed using the semi-solid agar puncture method (Dong & Cai, 2001). Tests for the Gram reaction and the presence of poly-β-hydroxybutyrate granules were conducted according to the methods described by Gerhardt et al. (1994) and Ostle & Holt (1982), respectively.

The full and optimum growth ranges of temperature and pH for strain JL3085T were examined by assessing changes in the OD₆₀₀ values during the incubation period in marine broth 2216 (MB; Difco). The temperature range for growth was determined by incubation at 4.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0 and 50.0°C [at 2.0% (w/v) NaCl and pH 7.6]. The range of pH for growth was tested at pH 4.0,
Table 1. Differential phenotypic characteristics between strain JL3085\textsuperscript{T} and its close relatives within the genus *Echinicola*

<table>
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<tr>
<td>Cell shape\footnote{a}</td>
<td>Irregular rods</td>
<td>Rods\footnote{b}</td>
<td>Rods\footnote{c}</td>
<td>Rods\footnote{d}</td>
<td>Rods\footnote{d}</td>
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<td>Nitrate reduction</td>
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<td>–</td>
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<tr>
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<td>+</td>
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<td>Utilization of:</td>
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<tr>
<td>Dextrin, $\alpha$-d-galacturonic acid</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>–</td>
</tr>
<tr>
<td>Methyl $\beta$-d-glucoside</td>
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<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>DL-Lactic acid</td>
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<td>+</td>
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<td>Thymidine, $\alpha$-glucose 6-phosphate</td>
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<td>+</td>
<td>–</td>
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<tr>
<td>Uridine</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Phenylethylamine</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>DNA G+C content (mol%)*</td>
<td>43.8</td>
<td>45.9\footnote{a}</td>
<td>46.9\footnote{b}</td>
<td>44--45\footnote{d}</td>
<td>46.2\footnote{d}</td>
</tr>
</tbody>
</table>

\footnotetext{a}\textsuperscript{a}Data taken from: a, Nedashkovskaya \textit{et al.} (2007); b, Kim \textit{et al.} (2011); c, Nedashkovskaya \textit{et al.} (2006); d, Srinivas \textit{et al.} (2012).

5.0, 6.0, 7.0, 7.6, 8.0, 9.0, 10.0 and 11.0 [at 2.0% (w/w) NaCl and 30.0°C]. The full and optimal range of salinity for growth was assessed in marine rich organic medium (Yurkov \textit{et al.}, 1999) with 0, 0.5 and 1.0--15.0% (w/v) NaCl (increments of 1.0%) (at 30.0°C, pH 7.6).

Oxidase activity was measured using the method described by Smibert & Krieg (1994). Susceptibility to antibiotics was evaluated using the Kirby–Bauer disc diffusion method (Bauer \textit{et al.}, 1966). The following antibiotics were tested (µg per disc): oxacillin (1), lincomycin (2), clindamycin (2), ciprofloxacin (5), enrofloxacin (5), chloramphenicol (5), rifampicin (5), novobiocin (5), streptomycin (10), norfloxacin (10), gentamicin (10), ampicillin (10), carbenicillin (10), benzylpenicillin (10), oleandomycin (15), erythromycin (15), furazolidone (15), co-trimoxazole (25), cefazolin (30), kanamycin (30), vancomycin (30), tetracycline (30), neomycin (30), doxycycline (30), cefalexin (30), cephradine (30), minocycline (30), cefoperazone (75), piperacillin (100) and polymyxin B (300). Catalase activity and hydrolysis of casein, chitin, starch, DNA, agar, cellulose (filter paper) and Tween 20 were examined according to the methods described by Dong & Cai (2001). Acid production from various carbohydrates was carried out according to the method described by Cappuccino & Sherman (2002). The presence of flexirubin-type pigments was investigated using the method described by Fautz & Reichenbach (1980). To detect bacteriochlorophyll-α and carotenoid-type pigments, the absorption spectrum of acetone/methanol (7:2) extracts from the cells was determined via a Flexstation3 system using full wavelength scanning from 350 to 900 nm at increments of 4 nm. Other physiological and biochemical features of strain JL3085\textsuperscript{T} were investigated using API 20E, API 20NE and API ZYM galleries (BioMérieux) and Biolog GN2 microplates according to the manufacturers’ instructions.

Respiratory quinones were extracted using methanol/hexane (Tindall, 1990a, b), followed by phase separation into hexane, and analysis by HPLC. The G+C content was calculated from the ratio of deoxyguanosine to thymidine according to the method of Mesbah \textit{et al.} (1989). DNA–DNA hybridization was carried out as described by Ley \textit{et al.} (1970) following the modifications described by Huss \textit{et al.} (1983). Analyses of respiratory quinones, G+C content and DNA–DNA hybridization were carried out by the Identification
purity and concentration were assessed using the tech following the manufacturer JCM 17847 and/or iso-C

Genomic DNA was extracted and purified from cells cultured in 2216E medium for 2 days at 30.0°C, as described by Kates et al. Cells of strain JL3085 were Gram-stain-negative, flexible, aerobic, chemo-organotrophic rods, approximately 0.5–0.8 µm wide and 1.6–3.8 µm long (Fig. S1, available in the online Supplementary Material). Poly-β-hydroxybutyrate granules were observed. Growth occurred at 4.0–50.0°C, at pH 4.0–11.0 and in the presence of 0–15.0% (w/v) NaCl. Cells grew optimally at 35.0°C, with 1.0–2.0% (w/v) NaCl and at pH 8.0. Details of other phenotypic characteristics of strain JL3085 are given in the species description and Table 1.

The phenotypic and chemotaxonomic characteristics of strain JL3085 are compared with the type strains of closely related species within the genus Echinicola in Table 1. Strain JL3085 shared common characteristics with other type strains of the genus Echinicola, including the ability to produce carotenoid pigments and being oxidase- and catalase-positive. However, it differed from the reference strains in several characteristics as listed in Table 1. Thus, strain JL3085 could be distinguished clearly from these recognized species of the genus Echinicola.

The major fatty acids of strain JL3085 were iso-C₁₅:₀ (24.3%), summed feature 3 (comprising iso-C₁₅:₀ 2-OH and/or C₁₆:₁ ω7c; 14.2%), iso-C₁₇:₀ 3-OH (7.9%), iso-C₁₇:₁ ω9c (7.8%), iso-C₁₇:₁ ω6c (6.8%), anteiso-C₁₅:₀ (5.1%) and C₁₆:₁ ω5c (4.8%) (Table 2). Comparison of the cellular fatty acid profiles of strain JL3085 and related species indicated that they contained a similar composition of major fatty acids, but the amount of C₁₅:₀ was significantly higher and summed feature 9 was not detected in strain JL3085. Analysis of polar lipids using two-dimensional TLC revealed that strain JL3085, E. vietnamensis DSM 17526, E. pacifica KCTC 12368, E. jeungdonensis KCTC 23122 and

Genomic DNA was extracted and purified from cells cultivated in 2216E medium for 2 days at 30°C using a Tguide Bacteria Genomic DNA Kit (OSR-M502; TIANGEN Biotech) following the manufacturer’s instructions. The DNA purity and concentration were assessed using the A₂₆₀/A₂₆₀ and A₂₃₀/A₂₆₀ ratios (Johnson, 1994). The 16S rRNA gene was amplified by PCR with the universal primers 27F (5'-AGAGTTTGATCCTGCTCAG-3') and 1492R (5'-GGA TACCTGGTTACGACTT-3') (Embley et al., 1991). The 16S rRNA gene sequence of strain JL3085 was then submitted to the GenBank database (NCBI) and identified using the BLAST search tool and the IDENTIFY program of the EzBio-Cloud database (Kim et al., 2012). The 16S rRNA gene sequences of representatives of the genus Echinicola and related genera of the family Cyclobacteriaceae were downloaded from GenBank for phylogenetic analysis. Multiple alignments were carried out using CLUSTAL X and phylogenetic trees were reconstructed using MEGA 5.0 with the neighbour-joining, maximum-likelihood and maximum-parsimony methods (Tamura et al., 2011). Bootstrap percentages were based on 1000 replications. The aligned sequences were analysed by the neighbour-joining distance method using the pairwise gap deletion option and relative distances were calculated by using the Kimura two-parameter model. For the maximum-likelihood analysis, the complete gap deletion option was chosen and distances were computed with a correction for multiple substitution using Kimura’s two-parameter model. Phylogenetic relationships were analysed by maximum-likelihood using the Nearest-Neighbor-Interchange method. For the maximum-parsimony analysis, the complete gap deletion option and Mini-Mini Heuristic method was used.

Table 2. Cellular fatty acid composition (%) of strain JL3085 and related species within genus Echinicola

<table>
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<th>Fatty acid</th>
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<td>Straight-chain:</td>
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<tr>
<td>C₁₅:₀</td>
<td>3.5</td>
<td>–</td>
<td>1.0</td>
<td>TR</td>
<td>–</td>
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<tr>
<td>C₁₆:₀</td>
<td>TR</td>
<td>TR</td>
<td>1.7</td>
<td>3.7</td>
<td>TR</td>
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<tr>
<td>Branched saturated:</td>
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<tr>
<td>iso-C₁₅:₀</td>
<td>24.3</td>
<td>24.8</td>
<td>29.1</td>
<td>17.0</td>
<td>35.0</td>
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<tr>
<td>iso-C₁₆:₀</td>
<td>2.2</td>
<td>1.7</td>
<td>1.6</td>
<td>1.7</td>
<td>1.8</td>
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<tr>
<td>iso-C₁₇:₀</td>
<td>1.3</td>
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<td>1.0</td>
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<td>anteiso-C₁₅:₀</td>
<td>5.1</td>
<td>2.3</td>
<td>20.0</td>
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<td>1.7</td>
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<td>Unsaturated:</td>
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<tr>
<td>C₁₅:₁ ω6c</td>
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<td>TR</td>
<td>TR</td>
<td>2.1</td>
<td>1.2</td>
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<tr>
<td>C₁₆:₁ ω5c</td>
<td>4.8</td>
<td>12.6</td>
<td>TR</td>
<td>17.5</td>
<td>6.0</td>
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<tr>
<td>C₁₇:₁ ω6c</td>
<td>6.8</td>
<td>4.2</td>
<td>4.8</td>
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<td>3.3</td>
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<tr>
<td>C₁₇:₁ ω8c</td>
<td>1.1</td>
<td>TR</td>
<td>–</td>
<td>1.0</td>
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<tr>
<td>iso-C₁₇:₁ ω9c</td>
<td>7.8</td>
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<td>C₁₅:₀ 2-OH</td>
<td>1.1</td>
<td>TR</td>
<td>1.3</td>
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<td>TR</td>
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<tr>
<td>C₁₆:₀ 3-OH</td>
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<td>2.5</td>
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<td>1.8</td>
<td>1.3</td>
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<td>2.8</td>
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<td>7.9</td>
<td>8.7</td>
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<td>20.0</td>
<td>7.2</td>
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<td>8.0</td>
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<td>Summed feature 9</td>
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<td>–</td>
<td>6.0</td>
<td>3.7</td>
<td>9.4</td>
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</table>

The predominant fatty acids are shown in bold type; TR, trace amount (<1%); –, not detected. Summed feature 3: iso-C₁₅:₀ 2-OH and/or C₁₆:₁ ω7c; summed feature 4: anteiso-C₁₇:₁ B and/or iso-C₁₇:₁ I; summed feature 9: iso-C₁₇:₁ ω9c and/or C₁₆:₀ 10-methyl.

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Echinicola rosea sp. nov.
‘E. shivajiensis’ JCM 17847 contained phosphatidylethanolamine, unidentified phospholipids and unidentified lipids (Fig. S2). Additional types of unidentified lipids were detected in strain JL3085T and ‘E. shivajiensis’ JCM 17847, while E. jeungdonensis KCTC 23122T contained additional types of unidentified phospholipids. The major quinone of JL3085T was MK-7, which is also the major quinone of the genus Echinicola.

Phylogenetic analysis of 16S rRNA gene sequences revealed that strain JL3085T occupied a distinct lineage within the genus Echinicola of the family Cyclobacteriaceae (Fig. 1). Strain JL3085T was related most closely to the type strains of species of the genus Echinicola (96.8% similarity with E. vietnamensis KMM 6221T, 95.7% with ‘E. shivajiensis’ AK12, 95.6% with E. jeungdonensis HMD3054T and 93.3% with E. pacifica KMM 6172T), and formed a distinct cluster with them. Additionally, the DNA–DNA hybridization value between strain JL3085T and E. vietnamensis KMM 6221T was 28.4%, well below the cutoff point (70.0%) for the definition of bacterial species recommended by Wayne et al. (1987) and Tindall et al. (2010).

Based on the phenotypic and phylogenetic analyses above, strain JL3085T is considered to represent a novel species of the genus Echinicola, for which the name Echinicola rosea sp. nov. is proposed.

It should be noted that the DNA G+C content of strain JL3085T was 43.8 mol%, which is outside the range reported for members of the genus Echinicola (44.0–46.9 mol%). Thus, the DNA G+C content range of the genus Echinicola should be updated to 43.8–46.9 mol%. In addition, the main cellular fatty acids of the genus Echinicola are iso-C15:0, C17:1ω6c, iso-C17:0 3-OH and summed feature 3 (comprising iso-C15:0 2-OH and/or C16:1ω7c). The major polar lipids are unidentified phospholipids, unidentified lipids and phosphatidylethanolamine.

**Description of Echinicola rosea sp. nov.**

**Echinicola rosea** (ro’s.e.a. L. fem. adj. rosea pink).

Cells are flexible and rod-shaped, gliding, aerobic, halotolerant, Gram-stain-negative, approximately 0.5–0.8 μm wide and 1.6–3.8 μm long. On MA, colonies are convex, glossy, uniformly circular and light pink when grown for 3 days at 35.0 °C. Oxidase and catalase are positive. Chitin and aesculin are hydrolysed, but casein, starch, gelatin, DNA, urea, agar, cellulose (filter paper), and Tweens 20, 40 and 80 are not. Cells are susceptible to clindamycin, ciprofloxacin, enrofloxacin, chloramphenicol, rifampicin, novobiocin, carbenicillin, oleandomycin, erythromycin, furazolidone, co-trimoxazole, doxycycline, cefalexin, cephradine, minocycline, lincomycin, cefoperazone and
piperacillin, but resistant to oxacillin, streptomycin, nor-
foxacin, gentamicin, ampicillin, benzylpenicillin, cefazolin,
kanamycin, vancomycin, tetracycline, neomycin and poly-
myxin B. Carotenoid pigments are formed, while bacterio-
chlorophyll-α and flexirubin-type pigments are not. Arginase dihydrodase, β-galactosidase and acetoac conflict are
detected. In the API ZYM system, cells are positive for
alkaline phosphatase, acid phosphatase, esterase (C4),
leucine arylamidase, valine arylamidase, cystine arylami-
dase, naphthol-AS-BI-phosphohydrolase, α-galactosidase,
α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase,
α-fucosidase and α-mannosidase, but negative for lipase
(C14), trypsin, α-chymotrypsin and β-gluconidase. In
sole-carbon-source utilization tests on Biolog GN2 micro-
plates, dextrin, N-acetyl-d-glucosamine, i-erythritol,
L-fucose, gentiobiose, lactulose, methyl β-D-glucoside, tre-
halose, turanose, xylitol, pyruvic acid methyl ester,
d-sorbitose, turanose, xylitol, pyruvic acid methyl ester,
N-acetylglucosamine, adonitol or dulcitol. The dominant fatty acids are iso-C15:0, summed feature 3, iso-C17:0 3- O H , iso-C17:ω9c, C17:ω6c, anteiso-C15:0 and
C16:ω5c. The polar lipids are phosphatidylethanolamine,
similar to those described for E. rosea sp. nov., iso-
fractions of a new isolates. The isoprenoid quinone is MK-7.
The type strain, JL3085T (=NBRC 111782T=CGLMCC
1.15407T), was isolated from the surface seawater of Yongxi-
ing Island in the South China Sea. The DNA G+C content
of the type strain is 43.8 mol%.

Acknowledgements
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03-01-02-03 and GASI-03-01-02-05.

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lated from the surfaces of Antarctic algae, and reclassification of Cytophaga
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