**Epilithonimonas psychrotolerans** sp. nov., isolated from alpine permafrost

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A bacterial strain, designated TSBY 57<sup>T</sup>, was isolated during a study on the phylogenetic diversity of culturable bacteria from alpine permafrost in Tianshan Mountains, China, and was classified by means of a polyphasic taxonomic approach. The novel strain was found to belong to the genus *Epilithonimonas* and was distinguished from recognized species of this genus. Strain TSBY 57<sup>T</sup> grew aerobically, at 0–30 °C, with 0–1.5 % (w/v) NaCl and at pH 6–8. Cells were Gram-stain-negative, non-motile, non-spore-forming rods. Compared with the reference strains, the novel strain was psychrotolerant. The predominant fatty acids were summed feature 3 (consisting of C<sub>16 : 1</sub>v<sub>7c</sub> and/or C<sub>16 : 1</sub>v<sub>6c</sub>), anteiso-C<sub>15 : 0</sub> and iso-C<sub>15 : 0</sub>. The sole respiratory quinone was MK-6. Phosphatidylethanolamine was predominant in the polar lipid profile of strain TSBY 57<sup>T</sup>. These chemotaxonomic traits were in good agreement with the characteristics of the genus *Epilithonimonas*. On the basis of 16S rRNA gene sequence similarity, strain TSBY 57<sup>T</sup> was a member of the genus *Epilithonimonas* and was closely related to *Epilithonimonas tenax* DSM 16811<sup>T</sup> (99.0 %), *Epilithonimonas ginsengisoli* DCY78<sup>T</sup> (98.6 %) and *Epilithonimonas lactis* H1<sup>T</sup> (98.5 %). However, DNA–DNA reassociation values between strain TSBY 57<sup>T</sup> and *E. tenax* DSM 16811<sup>T</sup>, *E. ginsengisoli* DCY78<sup>T</sup> and *E. lactis* H1<sup>T</sup> were 39.5 ± 2.6, 37.7 ± 1.0 and 37.3 ± 1.1 %, respectively. The G + C content of the DNA was 34.4 ± 0.2 mol%. Based on data from this polyphasic taxonomic study, strain TSBY 57<sup>T</sup> represents a novel species of the genus *Epilithonimonas*, for which the name *Epilithonimonas psychrotolerans* sp. nov. is proposed. The type strain is TSBY 57<sup>T</sup> (=NRRL B-51307<sup>T</sup> = CCTCC AB 207182<sup>T</sup>).

The genus *Epilithonimonas* was defined by O’Sullivan et al. (2006) on the basis of rRNA cistron similarity studies and phenotypic characteristics. The first described species of the genus *Epilithonimonas* was isolated from the river epilithon (River Taff, Cardiff, UK), named *Epilithonimonas tenax* (type species). A second recognized species of the genus was then described, *Epilithonimonas lactis* (Shakéd et al., 2010), which was isolated from untreated cow’s milk. Recently, two novel species have been established: *Epilithonimonas xixisoli* (Feng et al., 2014), isolated from wetland, and *Epilithonimonas ginsengisoli* (Hoang et al., 2015), isolated from ginseng rhizosphere soil. Members of the genus *Epilithonimonas* are distributed in different freshwater and soil ecosystems.

During a previous study (Bai et al., 2006), strain TSBY 57<sup>T</sup> was isolated from a sample of alpine permafrost that was collected at the mouth of an ice-free cirque in Tianshan Mountains, China (43° 07’ 10.2” N 86° 49’ 28.2” E; altitude 3833 m). For phenotypic tests and fatty acid analysis, the reference strains *E. tenax* DSM 16811<sup>T</sup>, *E. ginsengisoli* KCTC 32174<sup>T</sup> and *E. lactis* H1<sup>T</sup> were grown under the same conditions as strain TSBY 57<sup>T</sup>. Cellular morphology was examined by transmission electron microscopy (JEM 1230; JEOL) using cells grown for 3 days at 20 °C on peptone-yeast extract-glucose (PYG) medium and negatively stained with 1 % phosphotungstic acid (pH 7.0). Motility was observed in semi-solid medium. The Gram reaction was carried out as described by Gerhardt et al. (1994). Flexirubin-type pigments were detected with 20 % (w/v) KOH according to the method of Fautz & Reichenbach (1980).

†These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TSBY 57<sup>T</sup> is DQ173014.

Three supplementary figures are available with the online Supplementary Material.
Growth was investigated on PYG and in trypticase soy broth (TSB; Oxoid) with 0.5–2 % NaCl (w/v) (at intervals of 0.5 % NaCl) and at 0, 4, 7, 15, 22, 25, 28, 30 and 37 °C for 14 days. Growth at pH 4.0–11 (at intervals of 0.5 pH units) was tested in TSB, with the pH adjusted prior to sterilization by the addition of HCl or NaOH and measured again after sterilization. Growth under anaerobic conditions was determined by incubation in an anaerobic chamber on trypticase soy agar (TSA) supplemented with 0.5 % (w/v) glucose or 0.1 % (w/v) potassium nitrate. Growth was evaluated on TSA, R2A agar, Luria–Bertani (LB) agar, nutrient agar and MacConkey agar (Oxoid). Catalase activity was determined by bubble production (LB) agar, nutrient agar and MacConkey agar (Oxoid). Growth at 30 °C was also tested in TSB, with the pH adjusted prior to sterilization. Growth under anaerobic conditions was determined by incubation in an anaerobic chamber on trypticase soy agar (TSA) supplemented with 0.5 % (w/v) glucose or 0.1 % (w/v) potassium nitrate.

Cells of strain TSBY 57T were Gram-stain-negative (Fig. S1, available in the online Supplementary Material), non-motile, non-spore-forming rods and produced flexirubin-type pigments. Growth of the novel strain occurred at 0–30 °C with an optimum temperature of 20 °C and pH 6–8 on PYG, TSA, R2A agar, LB agar and nutrient agar, but not on MacConkey agar. Compared with the reference strains, the novel strain was psychrotolerant. This trait matched with its isolation source, namely alpine permafrost. Colonies of strain TSBY 57T were slimy, yellow and circular with entire edges and became mucoid and unidentifiable as single entities after prolonged incubation. Colonies of the reference strains are orange. Catalase and oxidase were positive. Indole was not produced. Strain TSBY 57T assimilated L-arabinose in contrast to all reference strains. In addition, the novel strain clearly differed from its closest relative, E. tenax, according to results of acid production from carbohydrates (Table 1). Details of the morphological, physiological and biochemical characteristics of strain TSBY 57T are given in Table 1 and the species description.

The 16S rRNA gene sequence of strain TSBY 57T was amplified using the bacterial universal primers 8f and 1492r (Bai et al., 2006), which correspond to positions 8–27 and 1492–1511, respectively, in the 16S rRNA gene sequence of Escherichia coli. The almost-complete 16S rRNA gene sequence (1375 bp) was compiled using the SeqMan software (DNASTAR) and compared with those of related taxa available in GenBank. Evolutionary distances were calculated using the method of Jukes & Cantor (1969) and phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. Tree topologies were evaluated by bootstrap analysis of 1000 replications using MEGA version 5.0 (Tamura et al., 2011). In the neighbour-joining phylogenetic tree and the maximum-parsimony tree (Fig. S2), strain TSBY 57T formed a separate clade within the cluster containing E. tenax, E. lactis, E. ginsengisoli and E. xixisoli and clearly separated from the cluster comprising species of the genus

![Image](https://example.com/image.png)
Chryseobacterium. Therefore, the tree topologies supported that strain TSBY 57T not only belonged to the genus Epilithonimonas but also represented a novel species. The EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012) was used to calculate sequence similarity. The EMBL-EBI web services tool FASTA and DNAman 8.0 were used to calculate sequence similarity between strain TSBY 57T and E. ginsengisoli DCY78T. Strain TSBY 57T showed high 16S rRNA gene sequence similarity to E. tenax DSM 16811T (99.0 %), E. ginsengisoli DCY78T (98.6 %) and E. lactis H1T (98.5 %), but lower similarity to Chryseobacterium xinjiangense TSBY 67T (Zhao et al., 2011; 95.9 %). Sequence similarities of 95.6–93.5 % were found with the type strains of other recognized species of the genus Chryseobacterium.

The genomic DNA of strain TSBY 57T was extracted and degraded enzymically into nucleosides and the DNA G+C content was determined as described by Tamaoka & Komagata (1984) with the following modifications. Nucleosides were separated by HPLC using a Kromasil C18 column (5 mm, 4.66 × 150 mm; Shimadzu) at room temperature. The solvent was 0.6 M NH₄H₂PO₄ (pH 5.6) with 2.5 % acetonitrile. The DNA G+C content of strain TSBY 57T was 34.4 ± 0.2 mol%, a value that falls within the range reported for members of the genus Epilithonimonas (33.3–38.0 %).

DNA–DNA hybridization was carried out by using the optical renaturation method (De Ley et al., 1970; Huss et al., 1983; Jahnke, 1992). The most closely related strains, E. tenax DSM 16811T, E. ginsengisoli KCTC 32174T and E. lactis H1T, were used as reference strains. Hybridization was conducted with three replications for each sample and the mean values from these were used as final results. DNA relatedness values between strain TSBY 57T and its closest relatives E. tenax DSM 16811T, E. ginsengisoli KCTC 32174T and E. lactis H1T were 39.5 ± 2.6, 37.7 ± 1.0 and 37.3 ± 1.1 %, respectively. According to current theory, these results demonstrate that strain TSBY 57T represents a novel species of the genus Epilithonimonas (Wayne et al., 1987; Stackebrandt & Goebel, 1994; Keswani & Whitman, 2001).

The fatty acid methyl esters of strain TSBY 57T and E. tenax DSM 16811T, E. ginsengisoli KCTC 32174T and E. lactis H1T were obtained from cells grown on TSA at 20 °C for 2 days by saponification, methylation and extraction as described by Kämper & Kroppenstedt (1996) and were separated using GC (6890; Agilent). Peaks were automatically integrated and fatty acid identities and percentages were determined using the Sherlock Microbial Identification system (MIDI, version 6.0B). Respiratory quinones were extracted from 100 mg of lyophilized cells with a mixture of chloroform/methanol (2 : 1, v/v). After filtration, evaporation under reduced pressure at low temperature (36 °C) and dissolution in aceton, the menaquinone solution was identified by one-dimensional TLC and analysed by HPLC (Waters) according to the methods described by Collins et al. (1977) and Nakagawa & Yamasato (1993). Polar lipids were extracted, separated by two-dimensional TLC and identified as described by Minnikin et al. (1979) at Guangdong Institute of Microbiology. The major fatty acids of strain TSBY 57T were summed feature 3 (consisting of C₁₆ : 0 3-OH and/or C₁₆ : 1ω₇c, 22.2 %), anteiso-C₁₅ : 0 (11.7 %) and iso-C₁₇ : 0 3-OH (8.3 %). Overall, this composition was consistent with those of the closest phylogenetic neighbours grown under the same conditions (Table 2). However, strain TSBY 57T differed by having higher proportions of iso-C₁₆ : 0 3-OH (9.9 %) and C₁₇ : 0 2-OH (6.7 %) and lower proportions of iso-C₁₅ : 0 (10.5 %) and C₁₆ : 0 3-OH (2.6 %).

The predominant respiratory quinone of strain 57T was MK-6. Phosphatidylethanolamine and one unidentified aminolipid were present in the polar lipid profile of strain TSBY 57T (Fig. S3). Therefore, chemotaxonomic

### Table 2. Cellular fatty acid profiles of strain TSBY 57T and its closest relatives in the genus Epilithonimonas

<table>
<thead>
<tr>
<th>Fatty acid (ω7c)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td><strong>Saturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₁₄ : 0</td>
<td>ND</td>
<td>TR</td>
<td>1.0</td>
<td>TR</td>
</tr>
<tr>
<td>C₁₆ : 0</td>
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<td>6.7</td>
<td>5.9</td>
<td>6.9</td>
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<tr>
<td>C₁₈ : 0</td>
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<td>TR</td>
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<td>ND</td>
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<tr>
<td><strong>Unsaturated</strong></td>
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</tr>
<tr>
<td>C₁₃ : 0</td>
<td>ND</td>
<td>1.9</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C₁₇ : 1ω6c</td>
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<td>ND</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
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<td>TR</td>
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</tr>
<tr>
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<td>TR</td>
<td>TR</td>
<td>TR</td>
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<tr>
<td>** Branched**</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>iso-C₁₃ : 0 3-OH</td>
<td>TR</td>
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<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>iso-C₁₄ : 0 2-OH</td>
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<td>26.6</td>
<td>22.8</td>
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<tr>
<td>anteiso-C₁₅ : 0</td>
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<td>13.5</td>
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<tr>
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<tr>
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<tr>
<td>C₁₅ : 0 2-OH</td>
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<tr>
<td>iso-C₁₅ : 0 3-OH</td>
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<td>3.7</td>
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<td>1.5</td>
<td>1.3</td>
<td>1.7</td>
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<tr>
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<tr>
<td>C₁₇ : 0 2-OH</td>
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<td>1.8</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>iso-C₁₇ : 0 3-OH</td>
<td>8.3</td>
<td>11.8</td>
<td>11.7</td>
<td>15.5</td>
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<tr>
<td><strong>Summed featured 3⁺</strong></td>
<td>22.2</td>
<td>20.3</td>
<td>24.1</td>
<td>25.7</td>
</tr>
</tbody>
</table>

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C₁₆ : 0 3-OH and/or C₁₆ : 1ω7c.
results were in good agreement with the characteristics of other species belonging to the genus *Epilithonimonas*.

Based on the data presented, strain TSBY 57\textsuperscript{T} should be considered to represent a novel species of the genus *Epilithonimonas*, for which the name *Epilithonimonas psychrotolerans* sp. nov. is proposed.

**Description of *Epilithonimonas psychrotolerans* sp. nov.**

*Epilithonimonas psychrotolerans* (psy.chro.to ler.ans. Gr. adj. psychros cold; L. pres. part. tolerans tolerating; N.L. part. adj. psychrotolerans cold-tolerating).

Cells are Gram-stain-negative, non-spore-forming, non-motile rods, approximately 0.4–0.5 μm in width and 0.5–1.7 μm in length (Fig. S1). Strictly aerobic. Oxidase- and catalase-positive. Good growth occurs at 20 °C after 48 h on PYG, TSA, R2A agar, LB agar and nutrient agar, but not on MacConkey agar. Growth within 14 days occurs at 0–30 °C (optimum 20–22 °C), at pH 6–8 (optimum pH 7) and with 0–1.5 % NaCl (optimum 0.5 % NaCl). Colonies are slimy, yellow and circular with entire edges and become mucoid and unidentifiable as single entities after prolonged incubation. Flexirubin-type pigments are produced. Nitrate is not reduced. Indole, H\textsubscript{2}S and acetoin are not produced. Positive for hydrolysis of aesculin, arginine, gelatin and starch (weakly), but negative for citrate utilization. Ornithine decarboxylase and lysine decarboxylase activities are absent. In the API ZYM gallery, alkaline phosphatase, acid phosphatase, arylamidase, \textalpha;-chymotrypsin, \textalpha;-galactosidase, \textbeta;-galactosidase, \textbeta;-glucuronidase, N-acetyl-\textbeta;-glucosaminidase, \textalpha;-mannosidase and \textalpha;-fucosidase are absent. In the API 50CH gallery, acid is produced from l-arabinose, D-xylene, D-glucose, D-fructose, D-mannose, L-ribose, amygdalin, esculin, cellobiose, maltose, lactose (weakly), sucrose, trehalose, inulin, raffinose (weakly), starch, glycerol and gentiobiose. The following compounds are utilized as sole carbon sources in the GN2 microplate: \textalpha;-cyclodextrin, dextrin, glycogen, TWEENs 40 and 80, l-arabinose, cellobiose, D-fructose, gentiobiose, D-glucose, maltose, D-mannose, L-ribose, sucrose, trehalose, turanose (weakly), methyl pyruvate, monomethyl succinate, acetic acid, D-galacturonic acid, \textalpha;-ketovaleric acid, propionic acid (weakly), l-alanine, l-alanyl glycine, l-aspartic acid (weakly), l-glutamic acid, glycyl l-aspartic acid, glycyl l-glutamic acid, L-proline, L-threonine, inosine (weakly), uridine (weakly), thymidine (weakly), glucose 1-phosphate (weakly) and glucose 6-phosphate (weakly). The sole respiratory quinone is MK-6. The predominant polar lipids are phosphatidylethanolamine and one unidentified aminolipid. The most abundant cellular fatty acids (> 7 %) are summed feature 3 (consisting of C\textsubscript{16:0} \textit{1077c} and/or C\textsubscript{16:0} \textit{166c}) and anteiso-C\textsubscript{15:0} \textit{iso-C\textsubscript{15:0}} \textit{iso-C\textsubscript{16:0}} 3-OH and iso-C\textsubscript{17:0} 3-OH.

The type strain is TSBY 57\textsuperscript{T} (=NRRL B-51307\textsuperscript{T}=CCTCC AB 207182\textsuperscript{T}), isolated from alpine permafrost in Tianshan Mountains, Xinjiang Province, China. The DNA G+C content of the type strain is 34.4 ± 0.2 mol%.

**Acknowledgements**

We thank Dr Malla Halpern for providing *E. lactis* H1\textsuperscript{T}, Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures for providing *E. tenax* DSM 16811\textsuperscript{T}, Marine Culture Collection of China for providing *E. ginsengioli* KCTC 32174\textsuperscript{T}, Dr Chengxiang Fang at the China Center for type Culture Collection (CCTCC) for performing the fatty acid analyses, and Songzhen Yang at Guangdong Institute of Microbiology for DNA–DNA hybridization, menaquinone and polar lipids analyses. This study was supported by the National Basic Research Program of China (973 Program) (2013CB429904, 2014CB954203), the Key Program of National Natural Science Foundation of China (31230014) and the Fundamental Research Funds for the Central Universities (lzujby-2013-b05).

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