Egicoccus halophilus gen. nov., sp. nov., a halophilic, alkalitolerant actinobacterium and proposal of Egicoccaceae fam. nov. and Egicoccales ord. nov.

Yong-Guang Zhang,1 Ji-Yue Chen,1,2 Hong-Fei Wang,1,3 Min Xiao,4 Ling-Ling Yang,5 Jian-Wei Guo,1,6 En-Min Zhou,4,5 Yuan-Min Zhang1 and Wen-Jun Li1,4,5

Correspondence
Wen-Jun Li
liwenjun3@mail.sysu.edu.cn or liact@hotmail.com

1Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi 830011, PR China
2Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla 666303, PR China
3College of Life Science, Liaoning Normal University, Dalian 116029, PR China
4State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, College of Ecology and Evolution, Sun Yat-Sen University, Guangzhou 510275, PR China
5Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, PR China
6Key Laboratory of Higher Quality and Efficient Cultivation and Security Control of Crops for Yunnan Province, Honghe University, Mengzi 661100, PR China

A novel Gram-stain-positive, non-motile, moderately halophilic and alkalitolerant actinobacterium, designated EGI 80432T, was isolated from a saline–alkaline soil of Xinjiang province, north-west China. Cells were non-endospore-forming cocci with a diameter of 0.5–0.8 \( \mu \text{m} \). Strain EGI 80432T grew in the presence of 0–9 % (w/v) NaCl (optimum at 3–5 %), and also grew within the pH range 6.0–10.0 (optimum at pH 8.0–9.0) on marine 2216E medium. The peptidoglycan type was A1c. The whole-cell hydrolysates contained glucose, galactose, mannose and three unknown sugars as major sugars. The predominant menaquinone was MK-9(H4). The major fatty acids were C17 : 1\( \omega 8c \), summed feature 3 (C16 : 1\( \omega 7c \)/C16 : 1\( \omega 6c \)), C18 : 1\( \omega 9c \) and iso-C15 : 0. The polar lipids comprised diphosphatidylglycerol, phosphatidylglycerol, one unknown phosphoglycolipid, three unknown phospholipids and four unknown polar lipids. The genomic DNA G + C content was 75.2 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain EGI 80432T clustered within the radius of the class Nitriliruptoria. Levels of sequence similarity between strain EGI 80432T and its phylogenetic neighbours Nitriliruptor alkaliphilus ANL-iso2T and Euzebya tangerina F10T were 94.1 and 88.1 %, respectively. Based on morphological, physiological and chemotaxonomic characteristics and phylogenetic analysis, a novel species of a new genus, Egicoccus halophilus gen. nov., sp. nov., is proposed, within the new family and new order Egicoccaceae fam. nov. and Egicoccales ord. nov. in the class Nitriliruptoria. The type strain of Egicoccus halophilus is EGI 80432T (=CGMCC 1.14988T=KCTC 33612T).

The class Nitriliruptoria was originally proposed by Kurahashi et al. (2010) as the subclass Nitriliruptoridae

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EGI 80432T is KR605110.

One supplementary figure and one supplementary table are available with the online Supplementary Material.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EGI 80432T is KR605110.

One supplementary figure and one supplementary table are available with the online Supplementary Material.
lake sediment and a sea cucumber, respectively. The characteristics that distinguish *Nitriliruptoria* from other classes of the phylum ‘Actinobacteria’ are its phyletic lineage and signature nucleotides of the 16S rRNA gene sequence, Gram-stain-positive rod-shaped cells and cell-wall peptidoglycan type A1γ (Sorokin *et al*., 2009; Kurahashi *et al*., 2010). Beside the orders described above, few taxa have been reported due to limited knowledge on nutrient requirements and the physiology of the class.

Soda deserts are naturally occurring alkaline environments, whose alkalinities are generally caused by the formation of Na₂CO₃ or NaHCO₃ (Horikoshi *et al*., 2011). Diverse prokaryotes have been isolated from these biotopes and characterized because of their specific adaptive mechanisms to the extreme conditions and their potential in biotechnology (Horikoshi, 1999; Horikoshi *et al*., 2011; Sarethy *et al*., 2011). Actinobacteria inhabiting alkaline environments are of great interest for their ability to produce alkaline enzymes and pharmaceutical agents (Horikoshi, 1999; Horikoshi *et al*., 2011; Sarethy *et al*., 2011). Soda desert containing alkaline soil and saline–alkaline soil is widely distributed in Xinjiang province, north-west China. During a programme investigating the diversity of actinobacteria in unexplored soda desert, a novel halophilic, alkali-tolerant strain, designated EGI 80432ᵀ, was isolated that formed a deep phyletic lineage within the class *Nitriliruptoria*. Thus, strain EGI 80432ᵀ was subjected to a polyphasic characterization, and a novel species of a new genus, *Egicoccus halophilus* gen. nov., sp. nov., is proposed. Accordingly, a new family *Egicoccaceae* fam. nov. and a new order *Egicoccales* ord. nov. are proposed.

Saline–alkaline soil was collected in Shihezi district, Xinjiang province, north-west China, during April 2013. The pH and total salts of the sample were 9.1 and 12.0 %, respectively. Two grams of the sample was transferred to an aseptic flask containing 18 ml distilled water and vibrated in a shaker at 150 r.p.m. at 30 °C for 30 min. The soil suspension was then diluted to concentrations of 10⁻³ and 10⁻⁴ (w/v), and 0.1 ml of each was spread on modified marine agar 2216E with pH adjusted to 10.0 with autoclaved 10 M NaOH. After 4 weeks of incubation at 30 °C, strain EGI 80432ᵀ was picked and grown on marine agar 2216E supplemented with 3 % (w/v) NaCl and pH adjusted to 9.0 with NaOH. Salt tolerance was tested on marine agar 2216E supplemented with various concentrations of NaCl (0–20 %, w/v, at intervals of 1 %) and pH adjusted to 9.0 with NaOH. The pH growth range was tested between 4.0 and 12.0, at intervals of 1.0 pH units, in marine broth 2216E supplemented with 3 % (w/v) NaCl by using the buffer system described by Xu *et al.* (2005). Carbon source utilization tests were performed according to the methods described by Shirling & Gottlieb (1966) and nitrogen source utilization tests were analysed as described by Williams *et al.* (1983). Catalase activity was determined using 3 % H₂O₂, and gas production was identified as a positive reaction. Physiological and biochemical characteristics were examined as described previously (Goodfellow, 1971; Williams *et al*., 1983). All the media above were supplemented with 3 % (w/v) NaCl and adjusted to pH 9.0 with NaOH except where otherwise described.

Cells of strain EGI 80432ᵀ were Gram-stain-positive, non-motile, non-endospore- forming cocci with a diameter of 0.5–0.8 μm (Fig. 1). Colonies of the isolate were dry, rough and circular with a diameter of <2 mm after 14 days of incubation on marine agar 2216E at 30 °C. Colonies were light yellow, and no diffusible pigments...
were detected when cultured on marine agar 2216E. Strain EGI 80432\textsuperscript{T} was able to grow at 20–40 °C, with 0–9 % (w/v) NaCl and at pH 7.0–10.8, and optimally at 30 °C, with 3–5 % (w/v) NaCl and at pH 8.0–9.0. Interestingly, the isolate was unable to grow on the medium formulated according to that for marine agar 2216E without NaCl added. These results suggest that the strain is moderately halophilic and alkalitolerant. Other physiological characteristics of strain EGI 80432\textsuperscript{T} are given in Table 1 and the species description below.

Diaminopimelic acid isomers in whole-cell hydrolysates were analysed by TLC as described by Staneck & Roberts (1974). A purified cell-wall preparation was obtained and hydrolysed as described by Schleifer & Kandler (1972) and analysed according to the method of Tang et al. (2009). Cell-wall sugars were determined according to the method used by Tang et al. (2009). Polar lipids were extracted and identified by two-dimensional TLC following the method of Minnikin et al. (1984). Menaquinones were extracted and prepared according to Collins et al. (1977). The purified menaquinones were dissolved in methanol and separated by atmospheric pressure photo-ionization LC-MS. The chromatographic system consisted of an AB SCIEX API 4000+TM LC/MS/MS system and a column oven (ABI). The chromatography and ionization conditions were set as described by Tang et al. (2008). For fatty acid analysis, strain EGI 80432\textsuperscript{T} was cultured in triplicate soy broth medium supplemented with 5 % (w/v) NaCl and pH adjusted to 9.0 with NaOH at 30 °C, and biomass was harvested at the mid-exponential growth phase (4 days). Cellular fatty acid analysis was performed as described by Sasser (1990) according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System. For detection of G+C content, the genomic DNA of EGI 80432\textsuperscript{T} was prepared according to Marmur (1961), and was determined by the HPLC method (Minnikin et al., 1984).

The cell-wall peptidoglycan of strain EGI 80432\textsuperscript{T} contained meso-diaminopimelic acid, alanine and glutamic acid as major amino acids, which indicated the presence peptidoglycan type A1\textgamma (Schleifer & Kandler, 1972; Sorokin et al., 2009; Kurahashi et al., 2010). Strain EGI 80432\textsuperscript{T} had a complex cell-wall sugar composition, which contained glucose, galactose, mannose and three unknown sugars as major sugars, and fucose, arabinose, xylose, ribose and rhamnose as minor sugars. The predominant menaquinone detected was MK-9(H\textsubscript{4}) (96.3 %), and the minor menaquinone was MK-7 (3.7 %), while the polar lipids were diphasphatidylglycerol, phosphatidylglycerol, one unknown phosphoglycolipid, three unknown phospholipids and four unknown polar lipids (Fig. S1, available in the online Supplementary Material). Strain EGI 80432\textsuperscript{T} contained unsaturated straight-chain fatty acids as the major cellular fatty acids. The main fatty acids (>10 %) of the isolate were C\textsubscript{17} : 1\textit{ω}8\textsubscript{c}, summed feature 3, C\textsubscript{18} : 1\textit{ω}9\textsubscript{c} and iso-C\textsubscript{15} : 0. The detailed fatty acid profile is given in Table S1. The genomic DNA G+C content of strain EGI 80432\textsuperscript{T} was 75.2 mol%.

Genomic DNA extraction and PCR amplification of the 16S rRNA gene were performed as described by Li et al. (2007). Multiple alignments with sequences of the type species belonging to the phylum ‘Actinobacteria’ and

### Table 1. Characteristics that distinguish strain EGI 80432\textsuperscript{T} from its closest phylogenetic neighbours N. alkaliphilus ANL-iso2\textsuperscript{T} and E. tangerina F10\textsuperscript{T}

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Coci</td>
<td>Rods</td>
<td>Rods</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>0.5–0.8</td>
<td>0.4 × 1.5–3.0</td>
<td>0.6–0.8 × 1.5–6.0</td>
</tr>
<tr>
<td>Growth:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH range</td>
<td>6–10</td>
<td>8.2–10.6</td>
<td>7–9</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>8–9</td>
<td>9.0–9.5</td>
<td>7–9</td>
</tr>
<tr>
<td>NaCl range (%) , (w/v)</td>
<td>0–9\textsuperscript{*}</td>
<td>0.1–11.6</td>
<td>0.5–12</td>
</tr>
<tr>
<td>Optimal NaCl (%) , (w/v)</td>
<td>3–5\textsuperscript{*}</td>
<td>1.2–1.7</td>
<td>ND</td>
</tr>
<tr>
<td>Temperature range (℃)</td>
<td>20–40</td>
<td>Mesophilic</td>
<td>25</td>
</tr>
<tr>
<td>Optimal temperature (℃)</td>
<td>30</td>
<td>32</td>
<td>20–28</td>
</tr>
<tr>
<td>Whole-cell sugars</td>
<td>Glc, Gal, Man, three USs</td>
<td>Gal, Glc</td>
<td>Rha, Gal</td>
</tr>
<tr>
<td>Major polar lipids</td>
<td>DPG, PGL, PLs</td>
<td>ND</td>
<td>PG</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>75.2</td>
<td>70.8</td>
<td>68.3</td>
</tr>
</tbody>
</table>

*Data are from parallel tests on marine agar 2216E with pH adjusted to 9.0 and incubated at 30 °C for 4 weeks.*
calculation of levels of sequence similarity were carried out using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012) on the basis of 16S rRNA gene sequence data. Phylogenetic analysis was carried out with three tree-making algorithms, the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1985), and maximum-parsimony (Fitch, 1971) methods, by using the software MEGA 6 (Tamura et al., 2013). The topologies of the resultant trees were evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

An almost-complete 16S rRNA gene sequence (1512 bp) was determined for strain EGI 80432T. Phylogenetic analysis based on the 16S rRNA gene sequences revealed that the novel strain fell within the radius of the class Nitriliruptoria. Levels of sequence similarity between strain EGI 80432T and N. alkaliphilus ANL-iso2T and E. tangerina F10T were 94.1 and 88.1%, respectively. Sequence similarities between strain EGI 80432T and members of other classes of the phylum ‘Actinobacteria’ were below 88.0%.

In the phylogenetic tree based on the neighbour-joining algorithm, strain EGI 80432T formed a clade with N. alkaliphilus ANL-iso2T with a high bootstrap value (100%), while E. tangerina F10T clustered in a separate clade (Fig. 2). This relationship was supported by the other tree-making methods used in this study (Fig. 2).

Based on the phenotypic, chemotaxonomic, and phylogenetic data described above, strain EGI 80432T had characteristics typical of the class Nitriliruptoria: peptidoglycan type A1γ, MK-9(H4) as the predominant menaquinone and similar phylogenetic lineage, which suggest that the strain should be assigned to this class. However, strain EGI 80432T also exhibited features distinct from those of N. alkaliphilus ANL-iso2T and E. tangerina F10T (Tables 1 and S1). Compared with the closest phylogenetic neighbour N. alkaliphilus ANL-iso2T, strain EGI 80432T had the following distinct phenotypic characteristics: coccus morphology, and halophilic and alkali tolerant growth features, while its closest neighbour has a rod morphology, and halotolerant and obligately alkaliphilic growth.

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain EGI 80432T. Bootstrap values (expressed as percentages of 1000 replications) of above 50% are shown at branch nodes. Asterisks indicate that the clades were conserved when the maximum-parsimony and maximum-likelihood methods were used to reconstruct the phylogenetic trees. Bar, 2% sequence divergence.
properties (Sorokin et al., 2009). Furthermore, strain EGI 80432T differs greatly from N. alkaliphilus ANL-iso2T by the following chemotaxonomic characteristics: (1) whole-cell sugars (glucose, galactose, mannose and three unknown sugars for strain EGI 80432T), glucose and galactose for N. alkaliphilus ANL-iso2T); and (2) fatty acid profile (Table S1) [C₁₈:1ω9c and iso-C₁₅:0 for strain EGI 80432T, C₁₆:0 and iso-C₁₄:0 as major fatty acids (>10 %) for N. alkaliphilus NBRC 105792T], although both have C₁₇:1ω8c and summed feature 3 as main components (>10 %). The fatty acid profile of N. alkaliphilus NBRC 105792T determined herein differs from previous reports (Sorokin et al., 2009; Kurahashi et al., 2010), which indicates that different media and temperatures for growth greatly influence the fatty acid compositions of this strain. In addition, strain EGI 80432T had distinct signature nucleotides of the 16S rRNA gene sequence from those of N. alkaliphilus ANL-iso2T and E. tangerina F10T at positions 47 : 396 (U–C) and 841 : 845 (G–A), as given in the description of the family. Clearly, strain EGI 80432T should not be affiliated to the order Nitriliruptorales. In comparison with E. tangerina F10T, the new isolate has a coccus morphology and alkali-tolerant growth properties; whereas E. tangerina F10T exhibits a rod morphology and neutrophilic growth properties; furthermore, some chemotaxonomic characteristics differ greatly between strain EGI 80432T and E. tangerina F10T, such as whole-cell sugars, major polar lipids and genomic DNA G+C content (Table 1). Clearly, strain EGI 80432T cannot be assigned to the order Euzelybales. In conclusion, strain EGI 80432T does not fall into any order of the class Nitriliruptoria.

According to the view that higher hierarchical taxa in the class Actinobacteria should be based mainly on 16S rRNA gene sequences. The pattern of 16S rRNA gene sequence components (Table S1) for the new isolate is different from those of N. alkaliphilus ANL-iso2T and E. tangerina F10T. The major fatty acids are C₁₇:1ω8c, iso-C₁₅:0 and summed feature 0. The type strain is EGI 80432T, isolated from Shihezi district, Xinjiang province, northwest China. The G+C content of the genomic DNA of the type strain is 75.2 mol%.

Description of Egicoccus halophilus sp. nov.

Egicoccus halophilus (hal.o’phi.lus. Gr. n. hals salt; Gr. adj. philos loving; N.L. masc. adj. halophilus salt-loving).

Has the following characteristics in addition to those given for the genus. Cell are non-endospore-forming cocci with a diameter of 0.5–0.8 μm. Colonies are dry, rough and circular when cultured on marine agar 2216E at 30 °C for 14 days. Colonies are light yellow. Growth occurs at 20–40 °C (optimum at 30 °C), at pH 6.0–10.0 (optimum at pH 8.0–9.0) and with 0–9 % (w/v) NaCl (optimum at 3–5 %) on marine 2216E. No growth occurs on marine 2216E medium without NaCl added. Utilizes cellobiose, D-fructose, D-galactose, D-glucose, glycerol, lactose, maltose, D-mannose, melibiose, D-sorbitol, sucrose, trehalose and D-xylitol, but not D-arabinose, L-arabinose, dulcitol, myo-inositol, D-mannitol, L-rhamnose, raffinose, D-ribose, D-xylitol, sodium acetate or sodium citrate as sole carbon source for growth. L-Arginine, L-glutamic acid, L-lysine and L-lysine can be utilized as sole nitrogen source for growth, but not L-alanine, L-aspartic acid, L-cysteine, L-glutamine, L-histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-tryptophan, L-valine or L-hypoxanthine. Positive for nitrate reduction and catalase activity, but negative for H₂S production, and coagulation and peptonization of skimmed milk. Cells can hydrolyse cellulose and Tweens 20, 40, 60 and 80, but not casein, starch or tryptophan. Whole-cell hydrolysates contain glucose, galactose, mannose and three unknown sugars as major sugars. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, one unknown phosphoglycolipid, three unknown phospholipids and four unknown polar lipids. The major fatty acids are C₁₁:0, C₁₂:0, C₁₃:0, C₁₄:0, C₁₅:0, C₁₆:0, C₁₇:0, C₁₇:1ω8c, iso-C₁₅:0 and summed feature 0.

The type strain is EGI 80432T (=CMCC 1.14988 = KCTC 33612T), isolated from a saline–alkaline soil collected from Shihezi district, Xinjiang province, northwest China. The G+C content of the genomic DNA of the type strain is 75.2 mol%.

Description of Egicoccales fam. nov.

Egicoccales (E.gi.co.cae.ca’les. N.L. masc. n. Egicoccus type genus of the family; -ae ending to denote a family; N.L. fem. pl. n. Egicocaceae the family of the genus Egicoccus).

The description is the same as that for the genus Egicoccus. Segregation of these organisms within a new family is justified by their distinct phylogenetic lineage based on 16S rRNA gene sequences. The pattern of 16S rRNA gene sequence signature nucleotides of members of the family consists of 47 : 396 (U–C) and 841 : 845 (G–A).

Description of Egicoccales ord. nov.

Egicoccales (E.gi.co.cae’les. N.L. masc. n. Egicoccus type genus of the family; -ales ending to denote an order; N.L. fem. pl. n. Egicoccales the order of the genus Egicoccus).

The description is the same as that for the genus Egicoccus. Separation of these organisms within a new order is...
justified by their distinct phyletic lineage based on 16S rRNA gene sequences. The pattern of 16S rRNA gene sequence signature nucleotides of members of the order is as for the family Egicoccaceae. The order contains the family Egicoccaceae. Egicoccus is the type genus.

Acknowledgements

We are grateful to Dr Moriyuki Hamada (NBRC, Japan) for providing the reference type strain. This research was supported by the joint fund of the National Natural Science Foundation of China (NSFC) (Nos. U1403101 and 31400009), the West Light Foundation of the Chinese Academy of Sciences (RCY201203) and Xinjiang Uygur Autonomous Region Natural Science Foundation (2014211A074). W.-J. L. was also supported by the ‘Hundred Talents Program’ of the Chinese Academy of Sciences, the High-level Talents Program of Xinjiang Autonomous Region and Guangdong Province Higher Vocational Colleges & Schools Pearl River Scholar Funded Scheme (2014).

References


http://jms.microbiologyresearch.org

535