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1 **Streptomyces karpasiensis sp. nov., isolated from Northern Cyprus soil**

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11

12 **Subject category:** New Taxa: *Bacteria*

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15 **Running title:** *Streptomyces karpasiensis* sp. nov.

16

17 The GenBank accession number for the 16S rRNA gene sequence of *Streptomyces*
18 *karpasiensis* K413^T is (= KCTC 29096^T = DSM 42068^T) **JQ864430.**

19

20 **Keywords:** *Actinobacteria*, *Streptomyces karpasiensis*, Polyphasic taxonomy

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22

23

24 **Abstract**

25 A novel actinobacteria, designated strain K413^{T}, was isolated from soil collected from Karpaz
26 National Park, Magusa, Northern Cyprus, was characterized to determine its taxonomic
27 position. The isolate was found to have chemical and morphological properties associated
28 with members of the genus *Streptomyces*. Phylogenetic analyses based on an almost-complete
29 16S rRNA gene sequences indicated that isolate was closely related to members of the genus
30 *Streptomyces*, and was shown to form a distinct phyletic line in the *Streptomyces* phylogenetic
31 tree. Strain K413^{T} was most closely related to *Streptomyces marinus* DSM 41970^{T} (98.01 %).
32 Sequence similarities with other strains of the genus *Streptomyces* were lower than 98.0 %.
33 The cell wall of the novel strain contained LL-diaminopimelic acid. The predominant
34 menaquinone was MK-9(H_8) (45.0 %). The polar lipids detected were diphosphatidylglycerol,
35 phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine,
36 phosphatidylinositol. Major fatty acids were *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$ and *anteiso*-$C_{17:0}$. Based on
37 16S rRNA gene sequence analysis, DNA-DNA relatedness, phenotypic characteristics and
38 chemotaxonomic data, strain K413^{T} was identified as member of novel species of the genus
39 *Streptomyces*, for which the name *Streptomyces karpasiensis* sp. nov. (type strain K413^{T} =
40 DSM 42068^{T} = KCTC 29096^{T}) is proposed.

41 **Introduction**

42 The genus *Streptomyces* was proposed by Waksman and Henrici (1943), which contains the
43 largest number of species of any genus in the domain *Bacteria*, contains over 600 species with
44 validly published names (Euzéby, 2012; http://www.bacterio.cict.fr/s/streptomycesa.html).
45 Members of this genus are well known as a rich source of bioactive secondary metabolites,
46 such as antibiotics and industrially and commercially important enzymes, therefore the
47 continued interest in screening novel streptomycetes (Chater *et al*., 2010; Goodfellow &

48 Fiedler, 2010; Rateb *et al*., 2011; Kim *et al*., 2012; Santhanam *et al*., 2013). The cost of
49 discovery of novel secondary metabolites from common *Streptomyces* species becoming
50 increasingly counterproductive. Hence, there is a need to isolate and describe streptomycetes
51 from diverse environments for screening for the discovery of novel bioactive compounds
52 (Busti *et al*., 2006; Lam, 2007; Goodfellow & Fiedler, 2010; Santhanam *et al*., 2013). In our
53 continuing research on culturable actinobacterial biodiversity of diverse habitats, a putatively
54 novel *Streptomyces* strain, K413^T, was isolated from soil sample, Karpaz National Park,
55 Magusa, Northern Cyprus. The present study was undertaken to establish the taxonomic
56 position of isolate K413^T using polyphasic approach.

57 Strain K413^T was isolated from soil sample collected from Karpaz National Park, Magusa,
58 Northern Cyprus, after two weeks incubation at 28 °C on humic acid-vitamin (HV) agar
59 (Hayakawa & Nonomura, 1987), supplemented with cycloheximide (50 µg/ml) and nalidixic
60 acid (10 µg/ml). The strain was isolated as a pure culture and maintained on yeast malt extract
61 agar [International Streptomyces Project medium 2 (ISP 2); Shirling & Gottlieb, 1966] slopes
62 at room temperature and stored in glycerol suspensions (20 %, v/v) at -20 °C.

63 Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and
64 purification of the PCR product were carried out following Chun & Goodfellow (1995). The
65 almost complete (1468 bp) 16S rRNA gene sequence of strain K413^T was determined using
66 an ABI PRISM 3730 XL automatic sequencer. The identification of phylogenetic neighbours
67 and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the
68 EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim *et al*., 2012). Multiple alignment with
69 sequences from closely related species was performed by using the program CLUSTAL W in
70 MEGA5 (Tamura *et al*., 2011). Phylogenetic trees were constructed with the neighbour-
71 joining (Saitou & Nei, 1987), maximum parsimony (Kluge & Farris, 1969) and maximum-
72 likelihood (Felsenstein, 1981) algorithms in MEGA5 (Tamura *et al*., 2011). Evolutionary

73 distances were calculated using model of Jukes & Cantor (1969). Topologies of the resultant
74 trees were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings.

75 The 1468 bp sequence corresponding to the 16S rRNA gene region of isolate strain K413^{T}
76 was compared with sequences deposited in the public databases. Comparison of the almost
77 complete 16S rRNA gene sequence of the isolate with corresponding sequences of
78 phylogenetically related species with validly published names showed that it formed a new
79 phyletic line at the periphery of the *S. marinus* DSM 41970^{T} 16S rRNA gene tree, a result
80 supported by all of the tree-making algorithms and by a bootstrap value of 87 % (Fig. 1). The
81 organism shares a 16S rRNA gene similarity of 98.01 % with *S. marinus* DSM 41970^{T}, a
82 value which corresponds to 29 nt differences at 1457 sites. The highest 16S rRNA sequence
83 similarities between the isolate and type strains of recognized species in the databases were
84 97.75 % (33 nt differences at 1,468 sites) to *S. glycovorans* YIM M 10366^{T}, 97.68 % (34 nt
85 differences at 1,464 sites) to *S. haliclonae* DSM 41968^{T}, 97.55 % (36 nt differences at 1,468
86 sites) to *S. xishensis* YIM M 10378^{T}, 97.34 % (39 nt differences at 1,468 sites) to *S.*
87 *ginglanensis* 172205^{T}, 97.34 % (39 nt differences at 1,467 sites) to *S. panacagri* Gsoil 519^{T},
88 97.14 % (42 nt differences at 1,468 sites) to *S. albus* subsp. *albus* NRRL B-2365^{T}, 97.13 %
89 (42 nt differences at 1,464 sites) to *S. rangoonensis* LMG 20295^{T}, 97.13 % (42 nt differences
90 at 1,463 sites) to *S. gibsonii* NBRC 15415^{T}, 97.12 % (42 nt differences at 1,458 sites) to *S.*
91 *almquisti* NBRC 13015^{T}. Sequence similarities with other strains of the genus *Streptomyces*
92 were lower than 97.0 %.

93 DNA-DNA hybridization experiments were performed with strain K413^{T} and most related
94 type species of *S. marinus* DSM 41970^{T}, *S. glycovorans* YIM M 10336^{T}, *S. xishensis* YIM M
95 10378^{T} and *S. haliclonae* DSM 41968^{T} were performed by the Identification Service at the
96 Deutsche Sammlung von Mikroorganismen und Zelkulturen Braunschweig, Germany. DNA
97 was isolated using a French pressure cell (Thermo Spectronic) and was purifed by

98 chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA-DNA
99 hybridisation was carried out as described by De Ley *et al.* (1970) [incorporating the
100 modifications described by Huss *et al.* (1983)] using a model Cary 100 Bio UV/VIS-
101 spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a
102 temperature controller with *in situ* temperature probe (Varian).

103 The taxonomic integrity of the test strains was supported by DNA relatedness data. Strain
104 K413^T showed DNA relatedness values 45.8 % ± 2.5 % to *S. marinus* DSM 41970^T, 40.1 % ±
105 3.6 % to *S. glycovorans* YIM M 10336^T, 37.4 % ± 1.8 % to *S. haliclonae* DSM 41968^T and
106 30.2 % ± 4.1 % to *S. xishensis* YIM M 10378^T (based on the average of duplicate
107 determinations in 2X SSC and 10 % formamide at 70 °C), below the recommended criterion
108 of 80 % for species delineation of the genus *Streptomyces* (Labeda, 1992).

109 Chemotaxonomic analyses were carried out to support the phylogenetic affiliation of strain
110 K413^T to genus *Streptomyces*. The strain was grown in GYM broth (DSMZ Medium 65)
111 under aerobic conditions in flasks on rotary shaker at 160 r.p.m. and 28 ºC for 10 days.
112 Biomass was harvested by centrifugation, washed twice in distilled water and re-centrifuged
113 and freeze-dried. Gram-staining was performed by the non-staining method as described by
114 Buck (1982). Isomers of diaminopimelic acid in whole-cell hydrolysates and sugars were
115 prepared according to Lechevalier & Lechevalier (1970) and analysed by thin layer
116 chromatography (Staneck & Roberts, 1974). Polar lipid and respiratory quinones analyses
117 were carried out by the Identification Service of the DSMZ and Dr. B. J. Tindall, DSMZ,
118 Braunschweig, Germany. Respiratory quinones were extracted from 100 mg of freeze dried
119 cells based on the two stage method described by Tindall (1990a; 1990b). Respiratory
120 quinones were separated into their different classes (menaquinones and ubiquinones) by thin
121 layer chromatography on silica gel (Macherey-Nagel Art. NO. 805 023), using hexane: tert-
122 butylmethylether (9:1 v/v) as solvent. UV absorbing bands corresponding to menaquinones or

123 ubiquinones were removed from the plate and further analysed by HPLC. This step was
124 carried out on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse
125 phase column (Macherey-Nagel, 2 mm x 125 mm, 3 µm, RP18) using methanol as the eluant.
126 Respiratory lipoquinones were detected at 269 nm.

127 A starter collection for the fatty acid analyses was prepared in a flask containing 20 ml N-Z-
128 amine broth (DSM medium 554) which was shaken at 160 rpm at 28 ^{o}C for 5 days. Five ml of
129 the resultant culture was used to inoculate 50 ml of N-Z-amine which was incubated under the
130 same conditions, the biomass harvested by cellulose filtration (pore size 0.45 µm) and the wet
131 cells (200 mg) placed in an extraction tube. Cellular fatty acids were extracted, methylated
132 and separated by gas chromatography using an Agilent Technologies 6890 N instrument,
133 fifted with an autosampler and a 6,783 injector, according to the standard protocol of the
134 Sherlock Microbial identification (MIDI) system (Sasser 1990; Kämpfer & Kroppenstedt,
135 1996), the fatty acid methyl ester peaks were quantified using TSBA 5.0 software. The DNA
136 G+C content of the isolate was determined following the procedure of Gonzalez & Saiz-
137 Jimenez (2005).

138 The cell wall diamino acid of strain K413^{T} was LL-diaminopimelic acid and glycine (Type I;
139 Lechevalier & Lechevalier, 1970), and the whole-cell hydrolysates contained mainly
140 galactose and glucose, with a small amounts of mannose and ribose. Phospholipids of strain
141 K413^{T} were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine,
142 phosphatidylmonomethylethanolamine, phosphatidylinositol, three unidentified
143 phospholipids, two unidentified phosphoglycolipids and two unidentified glycolipids
144 (Supplementary Fig. S1). The predominant menaquinone of strain K413^{T} was MK-9(H_8) (45.0
145 %); MK-9(H_6) (6.0 %) and MK-9(H_4) (2.0 %), in addition an unknown component with a
146 retention time really close to MK-9(H_8) were also detected. The major cellular fatty acids
147 were *anteiso*-$C_{15:0}$ (30.15 %), iso-$C_{16:0}$ (23.90 %) and *anteiso*-$C_{17:0}$ (14.97 %). Comparative

148 cellular fatty acid compositions of strain K413^T and *S. marinus* DSM 41970^T, *S. glycovorans*
149 YIM M 10336^T, *S. haliclonae* DSM 41968^T and *S. xishensis* YIM M 10378^T shown in
150 Supplementary Table S1. The G+C content of the DNA was 71.9 mol %.

151 Cultural characteristics were investigated on media from the International *Streptomyces*
152 Project (ISP) (Shirling & Gottlieb, 1966), modified Bennett's agar (MBA; Jones, 1949),
153 Czapek's and tryptic soy agar (TSA; Difco). The degree of growth, aerial mycelium and
154 pigmentation were recorded after 14 days of incubation at 28 °C. National Bureau of
155 Standards (NBS) Colour Name Charts (Kelly, 1964) was used for determining colour
156 designation and names. Colony morphology and micromorphological properties of strain
157 K413^T was determined by examined gold coated dehydrated specimens of 14-days cultures
158 from ISP 3 (oatmeal) medium using a JEOL JSM 6060 scanning electron microscope. Growth
159 at different temperatures (4, 10, 20, 28, 37, 45 and 50 ºC), and pH 4.0–11.0 (at intervals of 1.0
160 pH unit), and in the presence of NaCl (0–10 %; w/v) was determined on ISP 2. Established
161 methods were used to determine whether the strains degraded Tween 20 and 80 (Nash &
162 Krent, 1991); the remaining degradation tests were examined using methods described by
163 Williams *et al*. (1983). Carbon source utilization was tested using carbon source utilization
164 (ISP 9) medium (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1 %
165 of the tested carbon sources. Nitrogen source utilization was examined using the basal
166 medium recommended by Williams *et al.* (1983) supplemented with a final concentration of
167 0.1 % of the tested nitrogen sources. The type strains *S. marinus* DSM 41970^T, *S. glycovorans*
168 YIM M 10366^T, *S. xishensis* YIM M 10378^T and *S. haliclonae* DSM 41968^T were included
169 for comparison in all tests. Commercially available API-ZYM (Biomérieux) were used
170 following the instructions of the manufacturer for the biochemical characterization of the
171 strain K413^T.

172 Strain K413^T formed a branched white substrate mycelium and white aerial hyphae which
173 differentiated into spiral chains of smooth-surfaced spores on ISP 3 (oatmeal) medium (Fig.
174 2). The organism grew well on ISP 3, 4, 5, and 7, modified Bennett's and TSA agar but
175 moderate grew on ISP 2, ISP 6 and Czapek's agar. No diffusible pigment was detected on any
176 tested media. Melanoid pigments were not produced on ISP 6 or ISP 7 medium. The
177 physiological and biochemical properties are given in Table 1 and the species description.

178 It is evident that strain K413^T can be distinguished chemotaxonomically and phenotypically
179 from its phylogenetic relatives *S. marinus* DSM 41970^T, *S. glycovorans* YIM M 10366^T, *S.*
180 *xishensis* YIM M 10378^T and *S. haliclonae* DSM 41968^T based on 16S rRNA gene sequence,
181 DNA-DNA relatedness, and phenotypic data like positive for L-rhamnose and lactose as sole
182 carbon sources, and glycine, L-hydroxyproline and proline as sole nitrogen sources. It is,
183 therefore, proposed that the organisms be recognized as a novel species, *Streptomyces*
184 *karpasiensis* sp. nov.

185

186 **Description of *Streptomyces karpasiensis* sp. nov.**

187 ***Streptomyces karpasiensis*** (kar.pa.si.en'sis. N.L. masc. adj. *karpasiensis* of or belonging to
188 Karpasia, Magusa, Northern Cyprus, source of the organism).

189 Aerobic, Gram reaction-positive, non-motile, non-acid-alcohol-fast actinomycete which forms
190 branched substrate hyphae and aerial mycelium that differentiates into spiral chains of
191 smooth-surfaced spores. Growth occurs at pH 5.0-10, and at 25-37 ºC, but not at pH 4.0, 11
192 and at temperature of 4, 10, 20 and 45 ºC. Optimum growth occurs on ISP 2 medium at 28ºC
193 and pH 7.2. Arbutin, allantoin and urea are hydrolysed, but not aesculin. Nitrate reduction is
194 negative. Adenine, casein, elastin guanine, hypoxanthine, xanthine, xylan, Tween 20 and 80
195 are not degraded. Utilizes L-arabinose, D-cellobiose, D-galactose, D-mannitol, inulin, L-

196 rhamnose, lactose, starch, and dextrin as sole carbon sources, but not maltose, D-sorbitol,
197 sucrose. Utilizes glycine, L-phenylalanin, L-valine, L-proline, L-hydroxyproline as sole
198 nitrogen sources, but not L-arginine, L-cysteine, L-methionine, L-serine. The organism is
199 positive for alkaline phosphatase, esterase, esterase-lipase, leucine arylamidase, *α*-
200 chymotrypsin, *α*-mannosidase, *N*-acetyl-*β*-glucosaminidase, and trypsin, but negative for acid
201 phosphatase, *α*-galactosidase, *α*-glucosidase, *β*-glucosidase, α-fucosidase, *β*-galactosidase, *β*-
202 glucuronidase, lipase and naphthol-AS-BI-phosphohydrolase. The predominant menaquinone
203 of the type strain is MK-9(H_8). The polar lipid profile contains diphosphatidylglycerol,
204 phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine,
205 phosphatidylinositol, three unidentified phospholipids, two unidentified phosphoglycolipids
206 and two unidentified glycolipids. The major cellular fatty acids are *anteiso*-$C_{15:0}$, *iso*-$C_{16:0}$ and
207 *anteiso*-$C_{17:0}$. The G+C content of the genomic DNA of the type strain is 71.9 mol %. The
208 type strain, K413^T (= KCTC 29096 ^{T} = DSM 42068 ^{T}) was isolated from a soil sample taken
209 from Karpaz National Park, Magusa, Northern Cyprus.
210
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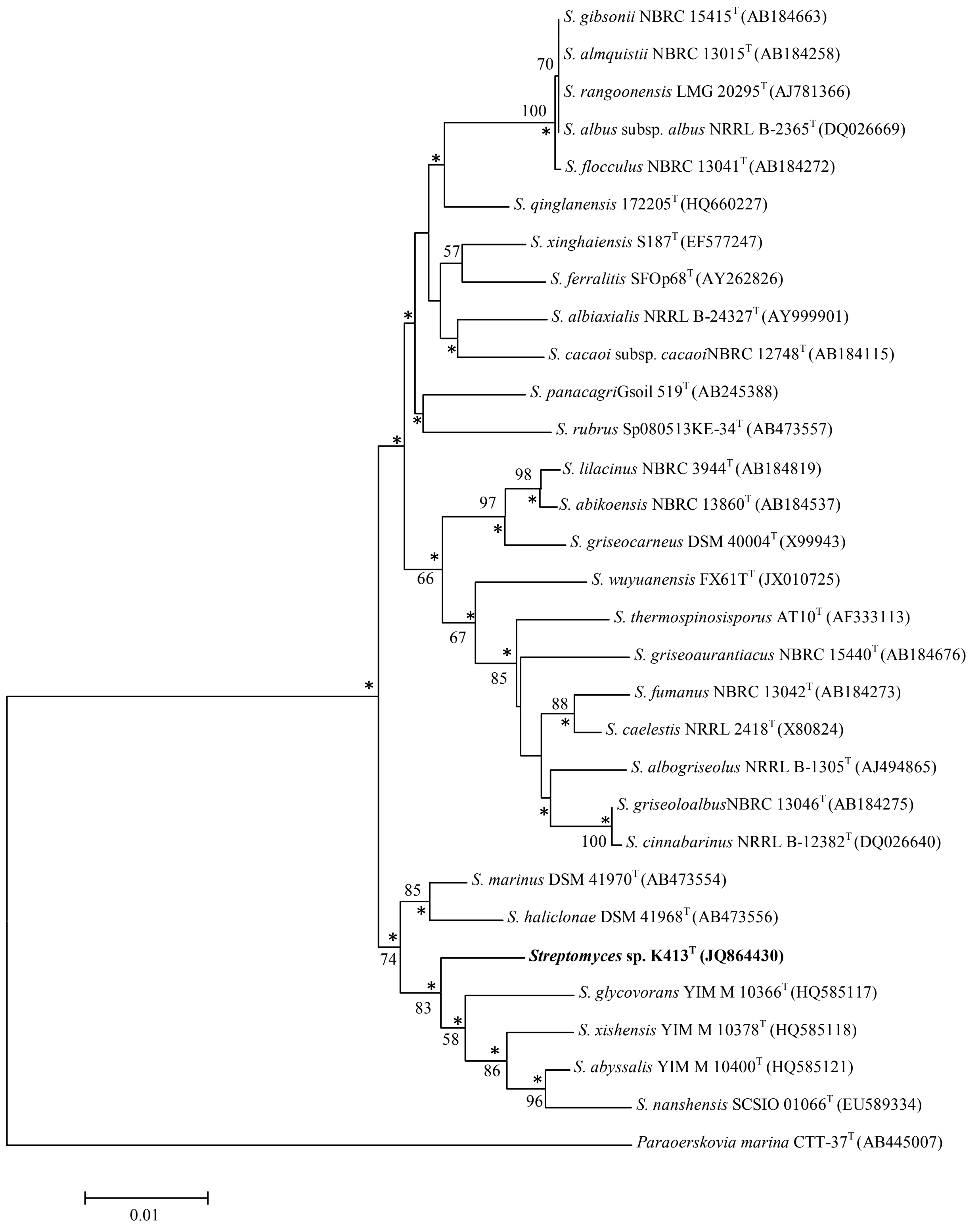
344 **Table 1.** Characteristics that differentiate strains K413^T and related species. Strains: **1,** K413^T; **2,** *S. marinus* DSM 41970^T, **3,**
345 *S. glycovorans* YIM M 10366^T, **4,** *S. haliclonae* DSM 41968^T, **5,** *S. xishensis* YIM M 10378^T. Strains were positive ability of
346 growth at D-mannitol as sole carbon sources (1.0 %) and growth at pH:8, temperature 25 °C, 28 °C and 37 °C, and 1 %, 2 %,
347 3 % NaCl concentrations. But negative ability of growth at L-methionine as sole nitrogen sources (0.1 %) growth at pH:4,
348 temperature 4 °C, 10 °C, and 15 % NaCI concentrations. All data were obtain in this study.

Characteristics	1	2	3	4	5
Biochemical Tests					
Arbutin Hydrolysis	+	+	+	+	-
Allantoin Hydrolysis	+	-	-	-	+
Aesculin Hydrolysis	-	+	-	-	-
Nitrate Reduction	-	-	+	-	+
Urea	+	-	-	-	+
pH tolerence					
5	+	-	-	+	-
9	+	+	+	+	-
10	+	-	-	-	-
Temperature					
45 °C	-	-	+	-	-
NaCl (%)					
4	+	+	-	+	+
5	+	+	-	+	+
6	-	+	-	+	+
7	-	+	-	+	+
8	-	+	-	+	-
9	-	+	-	+	-
10	-	+	-	+	-
Degradation					
Tween 20 (1 %)	-	-	-	+	+
Tween 80 (1 %)	-	-	-	+	-
Use of Sole C sources (1.0 %)					
D-cellobiose	+	-	-	+	+
D-sorbitol	-	-	-	+	-
D-galactose	+	+	-	+	-
Dextrin	+	+	+	+	-
Inulin	+	+	-	+	+
L-arabinose	+	+	-	-	-
L-rhamnose	+	-	-	-	-
Lactose	+	-	-	-	-
Maltose	-	-	+	-	+
Starch	+	-	-	-	-
Sucrose (Saccharose)	-	+	+	+	+
Use of Sole N sources (0.1 %)					
Glycine	+	-	-	-	-
L-arginine	-	-	+	+	-
L-hydroxyproline	+	-	-	-	-
L-phenylalanin	+	-	+	-	-
L-proline	+	-	-	-	-
L-serine	-	-	+	+	+
L-valine	+	-	+	-	-

349
350 Note: +, positive; -, negative.
351

352



353 **Fig.1.**

354

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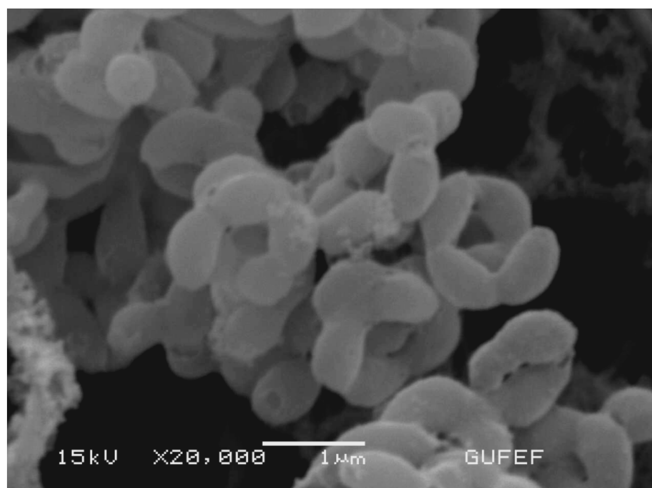

357 **Fig.2.**

358

359 **Legends for Figures**

360 **Fig. 1.** Neighbour-joining tree (Saitou & Nei, 1987) based on almost complete 16 rRNA gene
361 sequences showing the position of strain K413^T amongst its phylogenetic neighbours.
362 Asterisks indicate branches of the tree that were also recovered using the maximum-
363 likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) tree-making
364 algorithms. *Paraoerskovia marina* CTT-37^T (AB445007) was used as an outgroup. Numbers
365 at the nodes indicate the levels of bootstrap support (%); only values ≥ 50% are shown.
366 GenBank accession numbers are given in parentheses. Bar, 0.01 substitutions per site.

367 **Fig. 2.** Scanning electron micrograph of strain K413^T grow on oatmeal medium (ISP medium
368 no. 3) at 28°C for 14 days.

369 **Supplementary Fig. S1.** Molybdophosporic acid stained two-dimensional TLC of polar
370 lipids from strain K413^T.

371 DPG: Diphosphatidylglycerol, PMME: Phosphatidylmonomethylethanolamine PE:
372 Phosphatidylethanolamine, PG: Phosphatidylglycerol, PI: Phosphatidylinositol, PGL1-
373 PGL2: Phosphoglycolipids, GL1-GL2: Glycolipids, PL1-PL3: Phospholipid