Streptomyces hoynatensis sp. nov., isolated from deep marine sediment

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A novel actinobacterium, strain S1412^T, was isolated from a deep sediment sample, collected from the southern Black Sea coast of Turkey, and was examined using a polyphasic approach. The organism had chemical and morphological features typical of the genus *Streptomyces*. The cell wall of the novel strain contained LL-diaminopimelic acid. Whole-cell hydrolysates contained galactose, glucose and traces of xylose. The polar lipid profile of S1412^T consisted of the predominant compound diphosphatidylglycerol, moderate amounts of phosphatidylethanolamine and phosphatidylinositol, and minor amounts of phosphatidylglycerol. Strain S1412^T exhibited an unusual quinone system, with the predominant compounds MK-10(H_8), MK-9(H_8) and MK-10(H_6) and small amounts of MK-9(H_6) and MK-10(H_4). Major fatty acids were iso-$C_{16:0}$, iso-$C_{16:1}$ H and anteiso-$C_{17:0}$. The 16S rRNA gene sequence similarities for strain S1412^T with respect to the most closely related type strains of species of the genus *Streptomyces* were less than 97.0 %. Phenotypic data clearly distinguished the isolate from its closest relatives, *Streptomyces specialis* GW 41-1564^T, *Streptomyces mayteni* YIM 60475^T, *Streptomyces hainanensis* YIM 47672^T, *Streptomyces avicenniae* MCCC1A01535^T and *Streptomyces sedi* YIM 65188^T. Based on chemotaxonomic, phenotypic and genotypic characteristics, strain S1412^T is considered to represent a novel species of the genus *Streptomyces*, for which the name *Streptomyces hoynatensis* sp. nov. is proposed. The type strain is S1412^T (=KCTC 29097^T=DSM 42069^T).

The Black Sea is unique, and it is considered to be the most isolated sea in the world. The Black Sea is the world's largest body of water containing hydrogen sulfide, and its hydrogen sulfide layer begins around 200 m below the surface. The upper layer of marine water supports the unique Black Sea ecosystem. The deeper and more dense layers of water are saturated with hydrogen sulfide that has accumulated over thousands of years as a by-product of decaying organic matter (Eremeev *et al.*, 1998). The marine environment is a rich source of both biological and chemical diversity and there are several reports that marine actinomycetes yield metabolities such as antibiotics and enzymes (Mincer *et al.*, 2002; Stach *et al.*, 2003; Pathomaree *et al.*, 2006; Goodfellow and Fiedler, 2010; Xu *et al.*, 2012). Therefore, the identification of phylogenetically novel streptomycetes from marine environments is important in the search for new bioactive compounds. In our continuing research on the culturable actinobacterial biodiversity of Black Sea deep sediment, a novel putative member of the genus *Streptomyces*, strain S1412^T, was isolated from sediment samples from the Melet River offshore in Turkey. Data from the present taxonomic study indicate that the strain represents a novel species of the genus *Streptomyces*.

Strain S1412^T was isolated from a sediment sample collected by a dredger at a depth of 12 m, along the Melet River offshore (40° 59.650′ N 37° 58.953′ E) from the southern Black Sea coast of Turkey, in August 2010. Sediment samples were subsampled aseptically and stored at −20 °C until use. Strain S1412^T was isolated from the sediment sample by using SM3 medium (l^{-1} distilled water: 10 g glucose, 5 g peptone, 3 g tryptone, 5 gNaCl and 15 g agar; pH 7.2–7.4) (Tan *et al.*, 2006), supplemented with filter-sterilized rifampicin (5 μg ml^{-1}), nalidixic acid (10 μg ml^{-1}) and novobiocin (10 μg ml^{-1}) incubated at 28 °C for 30 days. The strain was maintained on yeast extract-malt extract (ISP medium 2; Shirling & Gottlieb, 1966) agar slopes at room temperature and as 20 % (v/v) glycerol suspensions at −20 °C.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of the PCR product were carried out following the methods of Chun

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S1412^T is JQ582693.

A supplementary figure and a supplementary table are available with the online version of this paper.

& Goodfellow (1995). The almost complete (1479 bp) 16S rRNA gene sequence of strain S1412^T was determined using an ABI PRISM 3730 XL automatic sequencer. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim *et al.*, 2012). Multiple alignments with sequences from closely related species were performed using the CLUSTAL W program in the MEGA5 software package (Tamura *et al.*, 2011). Phylogenetic analysis was carried out by using three tree-making algorithms: the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) methods in MEGA software version 5.0 (Tamura *et al.*, 2011). Evolutionary distances were calculated using the model of Jukes & Cantor (1969). Topologies of the resultant trees were evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Cultural characteristics were investigated on media from the International *Streptomyces* Project (ISP; Shirling & Gottlieb, 1966), modified Bennett's agar (MBA; Jones, 1949), Czapek's and tryptic soy agar (TSA; Difco). The degree of growth, aerial mycelium and pigmentation were recorded after incubation for 14 days at 28 °C. The National Bureau of Standards Colour Name Charts (Kelly, 1964) was used for determining colours and names. Colony morphology and micromorphological properties of strain S1412^T were determined by examining gold-coated dehydrated specimens of 21 day cultures from ISP 4 medium using a JEOL JSM 6060 instrument. Growth at different temperatures (4, 10, 20, 28, 37, 45 and 50 °C), at pH 4.0–11.0 (in intervals of 1.0 pH unit), and in the presence of 0–10 % (w/v) NaCl was determined on ISP 2. Anaerobic growth was tested by using Anaerocult A (Merck). Established methods were used to determine whether the strains degraded Tween 40 and 80 (Nash & Krent, 1991); the remaining degradation tests were carried out using methods described by Williams *et al.* (1983). Carbon source utilization was tested using carbon source utilization (ISP 9) medium (Shirling & Gottlieb, 1966) supplemented with a final concentration of 1 % of the carbon sources tested. Nitrogen source utilization was examined using the basal medium recommended by Williams *et al.* (1983) supplemented with a final concentration of 0.1 % (w/v) of the tested nitrogen sources. The antimicrobial ability of strain S1412^T to inhibit the growth of twenty-four micro-organisms such as Gram-positive and Gram-negative bacteria and fungi, was observed using the overlay technique described by Williams *et al.* (1983). Spot-inoculated colonies on MBA plates were inverted over 2 ml chloroform for 40 min. Killed colonies were overlaid with 5–7 ml sloppy modified Bennett's broth inoculated with the test organisms. Zones of inhibition were scored as positive results after 24 h at 37 °C. With the exception of the antimicrobial activity tests, the type strains *Streptomyces specialis* GW 41-1564^T, *Streptomyces avicenniae* MCCC 1A015351^T, *Streptomyces mayteni* YIM 60475^T, *Streptomyces hainanensis* YIM 47672^T and *Streptomyces sedi* YIM 65188^T were included for comparison.

Strain S1412^T was examined for chemical markers considered to be characteristic of strains of the genus *Streptomyces*. The strain was grown in YM broth (1^{-1} distilled water: 3 g yeast extract, 3 g malt extract and 5 g peptone; pH 7.2–7.4,) under aerobic conditions in flasks on a rotary shaker at 160 r.p.m. and 28 °C for 14 days. Biomass was harvested by centrifugation, washed twice in distilled water and recentrifuged and freeze-dried. Standard procedures were used to determine the isomers of diaminopimelic acid and diagnose whole-cell sugars (Hasegawa *et al.*, 1983). Cellular fatty acids were extracted, methylated and separated by gas chromatography using an Agilent Technologies 6890 N instrument, fitted with an autosampler and a 6783 injector, according to the standard protocol of the MIDI Sherlock Microbial Identification System (Sasser, 1990; Kämpfer & Kroppenstedt, 1996); the fatty acid methyl ester peaks were quantified using the TSBA 5.0 database. Polar lipid and respiratory quinone analyses were carried out by the Identification Service of the DSMZ (Braunschweig, Germany). Respiratory quinones were extracted from 100 mg freeze-dried cells based on the two-stage method described by Tindall (1990a, 1990b). Respiratory quinones were separated into their different classes (menaquinones and ubiquinones) by TLC on silica gel (Macherey-Nagel Art. no. 805 023), using hexane/tert-butylmethylether (9 : 1, v/v) as the solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical HPLC (Thermo Separation Products) fitted with a reverse phase column (Macherey-Nagel, 2 mm × 125 mm, 3 μm, RP18) using methanol as the eluant. Respiratory lipoquinones were detected at 269 nm. The DNA G+C content of the isolate was determined by following the procedure of Gonzalez & Saiz-Jimenez (2005).

The 1479 bp sequence corresponding to the 16S rRNA gene region of strain S1412^T was compared with sequences deposited in public databases. Comparison of the almost-complete 16S rRNA gene sequence of the isolate with corresponding sequences of phylogenetically related species with validly published names showed that it formed a branch in the *Streptomyces* 16S rRNA gene tree (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain S1412^T clustered with *S. hainanensis* YIM 47672^T, *S. mayteni* YIM 60475^T, *S. specialis* GW 41-1564^T, *S. sedi* YIM 65188^T and *S. avicenniae* MCCC 1A01535^T. This relationship was supported by all the tree-making algorithms used in this study (data not shown). The highest 16S rRNA gene sequence similarities between the isolate and type strains of recognized species in the databases were 96.80 % (47 nt differences at 1471 sites) to *S. specialis* GW 41-1564^T, 96.66 % (46 nt differences at 1379 sites) to *S. mayteni* YIM 60475^T, 96.43 % (52 nt differences at 1456 sites) to *S. hainanensis* YIM 47672^T, 95.86 % (61 nt differences at 1475 sites) to *S. avicenniae*

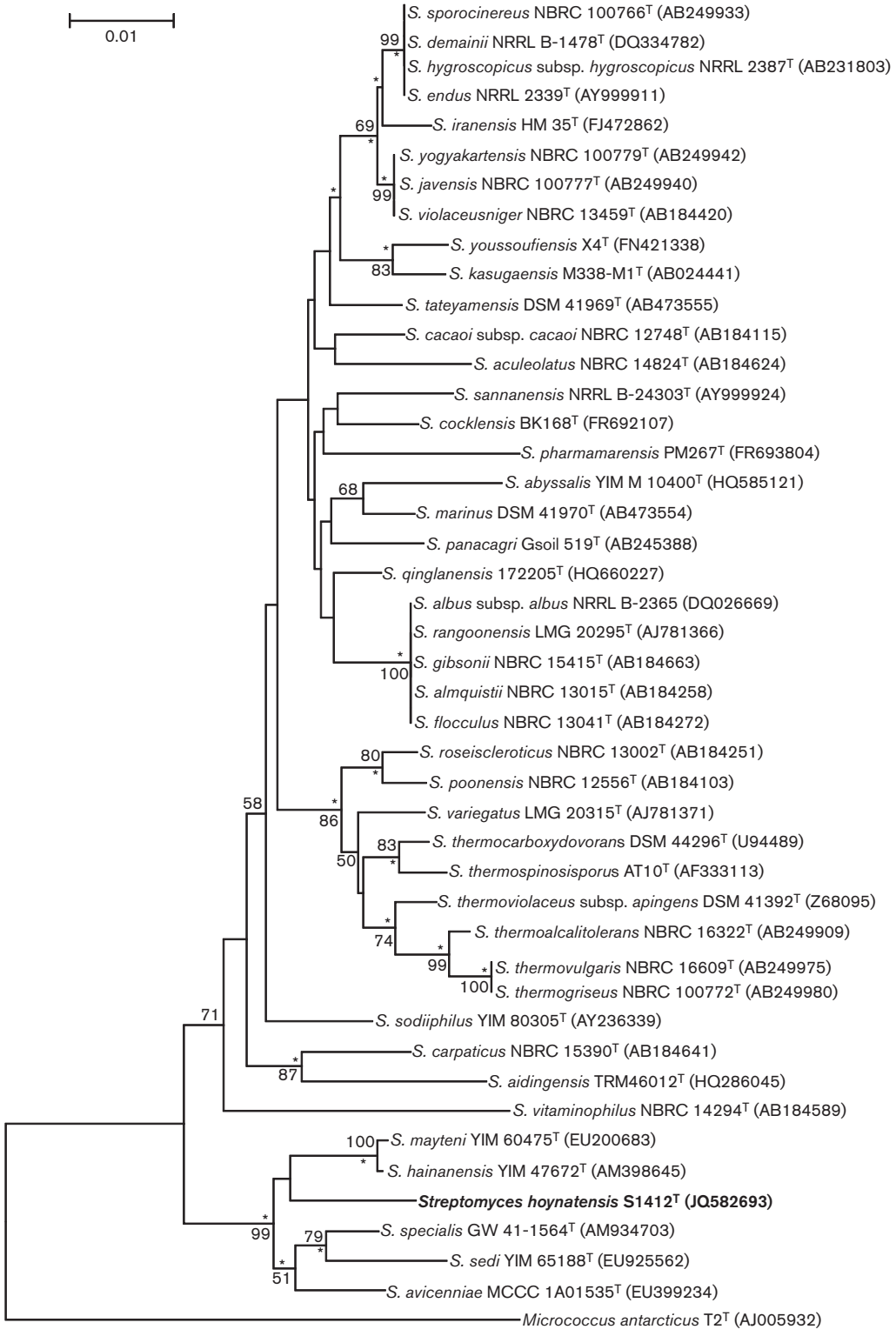

Fig. 1. Neighbour-joining phylogenetic tree (Saitou & Nei, 1987) based on almost-complete 16S rRNA gene sequences showing the position of strain S1412^T in the genus *Streptomyces*. *Micrococcus antarcticus* T2^T (GenBank accession no. AJ005932) was used as an outgroup. Numbers at the nodes indicate the levels of bootstrap support (%); only values $\geqslant$50 % are shown. GenBank accession numbers are given in parentheses. Bar, 0.01 substitutions per site.

MCCC 1A01535^T and 95.58 % (63 nt differences at 1424 sites) to *S. sedi* YIM 65188^T.

The cell wall of strain S1412^T contained LL-diaminopimelic acid. Whole-cell hydrolysates consisted of galactose, glucose and traces of xylose. The polar lipid profile of strain S1412^T consisted of the major compound diphosphatidylglycerol, moderate amounts of phoshphatidylethanolamine and phosphatidylinositol, and minor amounts of phosphatidylglycerol together with one unknown glycolipid, one unknown phosphoglycolipid and four unknown phospholipids [i.e. phospholipid pattern type 2 *sensu* (Lechevalier *et al.*, 1977)] (Fig. S1, available in the online Supplementary Material). Strain S1412^T exhibited a quinone system with predominant compounds: MK-10(H_8) (52.0 %); MK-9(H_8) (19.0 %) and MK-10(H_6) (16.0 %). Small amounts of MK-9(H_6) (2.0 %), MK-10(H_4) (1.0 %) and some unidentified components were also detected. The major fatty acids found were iso-$C_{16:0}$ (54.27 %), iso-$C_{16:1}$ H (13.57 %) and anteiso-$C_{17:0}$ (11.55 %) (Table S1). The G+C content of the DNA was 73.1 mol%.

Morphological observations of a 21 day-old culture of strain S1412^T grown on ISP 4 (inorganic salt starch agar) revealed that strain S1412^T had typical characteristics of members of the genus *Streptomyces*. Aerial and subtrate mycelia were well-developed without fragmentation. The aerial mycelia produced long spore chains and were spiral or ratinaculiaperti (looped at the top) in nature (Fig. 2). The spore surface was smooth. Strain S1412^T grew well on ISP 4, MBA and yeast extract-malt extract agar but poorly on ISP 3, ISP 5, ISP 6, ISP 7, Czapek's, nutrient agar and TSA. No diffusible pigment was detected on any media tested. Melanoid pigments were not produced on ISP 6 or ISP 7 media. White aerial mycelia were produced on ISP 4 but none were formed on the remaining media tested. The physiological and biochemical properties of strain S1412^T are presented in Table 1 and in the species description.

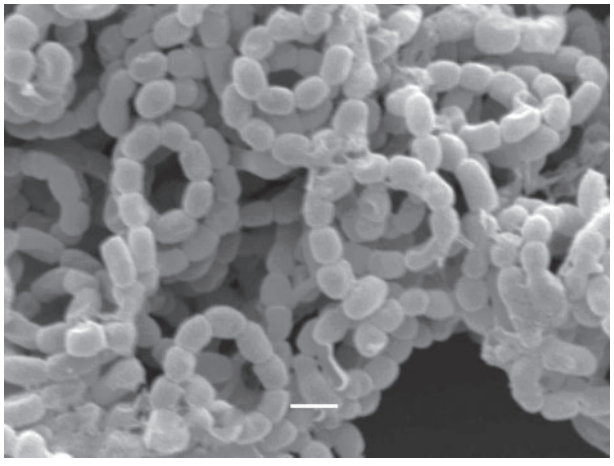

Fig. 2. Scanning electron micrograph of strain S1412^T grown on inorganic salts-starch medium (ISP 4) at 28 °C for 21 days. Bar, 1 μm.

It is evident that strain S1412^T clearly differs from all other members of the genus *Streptomyces*. It shares less than 97.0 % 16S rRNA gene sequence similarity with strains of other species of the genus *Streptomyces*, and it can be distinguished chemotaxonomically and phenotypically from its closest phylogenetic relatives *S. specialis* GW 41-1564^T, *S. mayteni* YIM 60475^T, *S. hainanensis* YIM 47672^T, *S. avicenniae* MCCC 1A01535^T and *S. sedi* YIM 65188^T based on significant quantitative differences in the contents of iso-$C_{16:0}$, and in the quinone system, with MK-10(H_8) derivatives predominating, which is unique among streptomycetes to date. However, unlike the other *Streptomyces* clades, some obvious similarities in chemical characteristics between strain S1412^T and the members of the clade are shown in Table 1, including: the unusual quinone systems (MK-10); diphosphatidylglycerol as the predominant polar lipid, although this was not specified for *S. mayteni* YIM 60475^T (Chen *et al.*, 2009) and *S. hainanensis* YIM 47672^T (Jiang *et al.*, 2007); and iso-$C_{16:0}$ and anteiso-$C_{17:0}$ as the major cellular fatty acids.

It can be concluded that strain S1412^T represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces hoynatensis* sp. nov. is proposed.

Description of *Streptomyces hoynatensis* sp. nov.

Streptomyces hoynatensis (hoy.na.ten′sis. N.L. masc. adj. *hoynatensis* of or pertaining to the sea area of the Hoynat Island in the southern part of the Black Sea, from where the type strain was isolated).

Aerobic, Gram-stain-positive, non-motile, non-acid–alcohol-fast actinomycete, which forms branched substrate hyphae and aerial mycelium that differentiates into spiral or ratinaculiaperti spore chains with smooth-surfaced spores. Growth occurs at pH 5.0–10, and at 28–45 °C, but not at pH 4.0 or 11, or at temperatures of 4, 10, 20 and 50 °C. Optimum growth occurs on N-Z-amine medium (DSM medium 554) at 28–37 °C and pH 7.2. NaCl is not required for optimum growth. Anaerobic growth is negative. Arbutin is hydrolysed, but allantoin and urea are not. Nitrate reduction is negative. Tweens 40 and 80 are degraded but adenine, casein, elastin, guanine, hypoxanthine, xanthine and xylan are not. Utilizes D-arabinose, L-arabinose, cellobiose, D-fructose, D-galactose, D-mannose, melezitose, D-ribose, L-rhamnose, lactose, maltose, sucrose, starch, xylose and dextrin as sole carbon sources, but not adonitol, *myo*-inositol, D-sorbitol, xylitol, inulin or sorbose. Utilizes glycine, L-alanine, L-arginine, L-phenylalanine, L-serine, L-valine and L-tyrosine as sole nitrogen sources, but not cysteine, L-histidine, L-proline or L-threonine. Antimicrobial activity is shown against *Bacillus subtilis* NRS-744, *Bacillus cereus* NRRL B-3711, *Staphylococcus aureus* NRRL B-767, *Listeria monocytogenes* ATCC 19117, *Providencia stuartii*, *Aspergillus parasiticus* NRRL-465^T and *Aspergillus flavus* NRRL-1957^T, but not against

Table 1. Phenotypic properties of strain S1412^T and type strains of closely related species

Strains: 1, S1412^T; 2, *S. specialis* GW41-1564^T; 3, *S. mayteni* YIM 60475^T (data from Chen *et al.*, 2009); 4, *S. hainanensis* YIM 47672^T; 5, *S. avicenniae* MCCC 1A01535^T; 6, *S. sedi* YIM 65188^T. All strains were positive for hydrolysis of arbutin, ability to use dextrin (1.0 %) as a sole carbon source and L-arginine (0.1 %) as a sole nitrogen source, and growth at pH 8, 28 °C and with 0 % (w/v) NaCl. All strains were negative for hydrolysis of allantoin, the ability to use inulin and sorbose as sole carbon sources (1.0 %) and L-threonine (0.1 %) as a sole nitrogen source, and growth at pH 4, 11 and 12 and at 4, 10 and 50 °C. All data were obtained in this study except where indicated otherwise. +, Positive; −, negative; ND, not determined; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine.

Characteristic	1	2	3	4	5	6
Isolation source	Marine sediment	Soil	Plant roots	Soil	Rhizosphere	Plant roots
Major menaquinones	MK-10(H_8) (52.0 %), MK-9(H_8) (19.0 %), MK-10(H_6) (16.0 %)	MK-10 (H_4) (58.0 %), MK-10 (H_6) (16.0 %), MK-9 (H_4) (15.0 %)	MK-9 (H_8) (39.2 %), MK-9(H_{10}) (21.3 %), MK-10 (H_0) (18.6 %)	MK-9 (H_4) (45.4 %), MK-10 (H_0) (27.0 %), MK-9 (H_6) (14.0 %)	MK-10 (H_6) (29.0 %), MK-9 (H_6) (16.8 %), MK-9 (H_8) (16.3 %)	MK-11 (H_6) (27.0 %), MK-10 (H_6) (23.0 %), MK-10 (H_8) (10.0 %)
Major polar lipids	DPG	DPG	DPG, PE	DPG, PE	DPG	DPG
Major fatty acids	iso-$C_{16:0}$ (54.3 %), iso-$C_{16:1}$ H (13.6 %), anteiso-$C_{17:0}$ (11.6 %)	iso-$C_{16:0}$ (31.4 %) anteiso-$C_{17:0}$ (30.2 %), anteiso-$C_{17:1}$ (14.3 %)	iso-$C_{16:0}$ (28.1 %), $C_{15:0}$ (16.2 %), anteiso- $C_{17:0}$ (14.8 %)	iso-$C_{16:0}$ (26.6 %), anteiso-$C_{17:0}$ (20.9 %)	iso-$C_{16:0}$ (47.2 %), anteiso-$C_{17:0}$ (9.9 %)	iso-$C_{16:0}$ (41.2 %), anteiso-$C_{17:0}$ (11.1 %)
Biochemical Tests						
Nitrate Reduction	−	−	−	−	−	+
Urea	−	−	−	−	−	+
pH tolerence						
5	+	−	−	−	−	−
6	+	+	+	+	−	−
10	+	+	−	+	+	+
Temperature for growth (°C)						
20	−	−	+	+	+	−
37	+	+	−	−	+	+
45	+	−	−	−	−	−
NaCl tolerance (%, w/v)						
1.0	+	+	+	+	−	+
2.0	+	+	+	+	−	+
3.0	+	−	+	+	−	+
4.0	+	−	−	+	−	+
5.0	−	−	−	+	−	+
6.0	−	−	−	+	−	−
7.0	−	−	−	+	−	−
8.0	−	−	−	+	−	−
9.0	−	−	−	+	−	−
10.0	−	−	−	+	−	−
Carbon source utilization (1.0 %)						
Adonitol	−	+	+	−	+	+
myo-Inositol	−	+	−	−	−	−
D-Arabinose	+	+	−	+	−	−

Table 1. cont.

Characteristic	1	2	3	4	5	6
L-Arabinose	+	−	−	−	+	+
Cellobiose	+	−	+	+	+	+
D-Fructose	+	−	+	−	+	+
D-Sorbitol	−	+	−	−	+	−
D-Galactose	+	−	+	−	+	+
D-Mannose	+	−	+	−	+	−
Melezitose	+	−	−	−	+	+
D-Ribose	+	+	+	−	−	−
L-Rhamnose	+	−	−	−	+	−
Lactose	+	+	−	−	+	−
Maltose	+	−	+	−	+	+
Sucrose	+	+	+	−	+	+
Starch	+	+	+	+	+	−
Xylitol	−	+	−	−	−	−
Xylose	+	−	−	−	+	−
Nitrogen source utilization (0.1 %)						
Glycine	+	−	−	−	−	−
Cysteine	−	−	−	−	−	+
L-Alanine	+	−	−	−	+	+
L-Histidine	−	−	+	−	−	+
L-Phenylalanine	+	−	−	−	−	−
L-Proline	−	−	+	−	−	+
L-Serine	+	−	−	−	+	+
L-Valine	+	−	−	−	+	−
L-Tyrosine	+	+	−	−	+	+
Degradation of:						
Adenine (0.5 %)	−	−	ND	−	+	−
Casein (1 %)	−	−	ND	−	+	−
Elastin (0.3 %)	−	−	ND	+	+	−
Guanine (0.5 %)	−	−	ND	−	+	−
Hypoxanthine (0.4 %)	−	−	+	+	+	−
Tween 40 (1 %)	+	+	ND	+	+	+
Tween 80 (1 %)	+	+	ND	+	+	+
Xanthine (0.4 %)	−	−	ND	−	−	−
Xylan (0.4 %)	−	−	ND	−	−	−

Aspergillus niger, *Candida albicans* ATCC 10231^T, *Candida utilis* NRRL Y-900, *Escherichia coli* ATCC 25922, *Escherichia coli* MC4100, *Citrobacter freundii* NRRL B-2643, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter aerogenes* NRRL B-3567, *Enterobacter aerogenes* NRRL B-427, *Pseudomonas aeruginosa* NRRL B-2679, *Proteus vulgaris* NRRL B-123, *Bacillus subtilis* NRRL B-209, *Bacillus licheniformis* NRRL B-1001, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 33862, *Staphylococcus aureus* ATCC 29213 or *Micrococcus luteus* NRRL B-1018. The predominant menaquinones are MK-10(H_8), MK-9(H_8) and MK-10(H_6) with small amounts of MK-9(H_6) and MK-10(H_4). The polar lipid profile contains diphosphatidylglycerol, phosphatidylinositol, phoshphatidylethanolamine, phosphatidylglycerol, one unknown glycolipid, one unknown phosphoglycolipid and four unknown phospholipids. The major cellular fatty acids are iso-$C_{16:0}$, iso-$C_{16:1}$ H and anteiso-$C_{17:0}$.

The type strain, S1412^T (=KCTC 29097^T=DSM 42069^T) was isolated from a sediment from the southern Black Sea coast in Turkey. The G+C content of the genomic DNA of the type strain is 73.1 mol%.

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