Thioalbus denitrificans gen. nov., sp. nov., a chemolithoautotrophic sulfur-oxidizing gammaproteobacterium, isolated from marine sediment

Soo-Je Park, Vinh Hoa Pham,[†] Man-Young Jung, So-Jeong Kim, Jong-Geol Kim, Dong-Hyun Roh and Sung-Keun Rhee

Correspondence
Sung-Keun Rhee
rhees@chungbuk.ac.kr

Department of Microbiology, Chungbuk National University, 12 Gaeshin-dong, Cheongju 361-763, Republic of Korea

A mesophilic, facultatively anaerobic, autotrophic bacterium, designated strain Su4^T, was isolated from marine sediment. The isolate was able to utilize reduced sulfur compounds including thiosulfate, tetrathionate, sulfur and sulfide but not sulfite as the energy source. Growth occurred under aerobic and denitrifying chemolithoautotrophic conditions in the presence of thiosulfate as an electron donor and bicarbonate as a carbon source. The G+C content of the genomic DNA was 64.5 mol%. Comparative 16S rRNA gene sequence studies showed that strain Su4^T was clearly affiliated with the class *Gammaproteobacteria*. The isolate was Gram-negative-staining and rod-shaped, lacked flagella and grew in artificial seawater medium at 10–40 °C (optimum 28–32 °C) and in 1–5% (w/v) NaCl (optimum 3% NaCl). Strain Su4^T possessed C_{16:0}, C_{16:1ω7c}/iso-C_{15:0} 2-OH and C_{18:1ω7c}/ω9t/ω12t as the major fatty acids. On the basis of phenotypic and phylogenetic analysis, the isolate represents a novel species of a novel genus, for which the name *Thioalbus denitrificans* is proposed. The type strain is Su4^T (=KCTC 5699^T =JCM 15568^T).

Sulfur is a constituent of living organisms and is biogeochemically cycled on the earth. Reduced inorganic sulfur compounds generated by sulfur-reducing bacteria in anoxic environments are oxidized to sulfate by some bacteria and archaea under aerobic conditions (Friedrich *et al.*, 2001). Oxidation of reduced inorganic sulfur compounds mediated by micro-organisms is one of the major reactions in marine sediments (Sorokin, 2003). Sulfur oxidizing bacteria have been isolated from various oxic and anoxic environments (Ito *et al.*, 2004; Kelly *et al.*, 2000; Kuenen & Robertson, 1992; McHatton *et al.*, 1996; Otte *et al.*, 1999; Sorokin & Mityushina, 1998; Sorokin *et al.*, 2000; Sorokin *et al.*, 2007a; Sorokin *et al.*, 2008; Sorokin *et al.*, 2002a; Sorokin *et al.*, 2002b; Sorokin *et al.*, 2006b; Sorokin *et al.*, 2001b) and even under extreme conditions such as those associated with deep-sea hydrothermal vents (Nakagawa & Takai, 2008), solfataras (Kletzin *et al.*, 2004), hypersaline environments (Rijkenberg *et al.*, 2001; Sorokin & Mityushina, 1998; Sorokin

et al., 1996; Sorokin *et al.*, 2000; Sorokin *et al.*, 2001a; Sorokin *et al.*, 2002a; Sorokin *et al.*, 2006a; Sorokin *et al.*, 2002c; Sorokin *et al.*, 2001b) and volcanic environments (King, 2007). Sulfur is oxidized by chemotrophic and phototrophic bacteria which are phylogenetically and physiologically diverse (Friedrich *et al.*, 2001; Friedrich *et al.*, 2005). The cellular processes of inorganic sulfur metabolism in prokaryotes are well established (Dahl & Friedrich, 2007) although the significance of these processes and their contribution to bacterial habitats are poorly described.

Here, we describe the properties of a novel, slightly halophilic, obligately chemolithoautotrophic and facultatively anaerobic, sulfur-oxidizing bacterium, designated strain Su4^T, isolated from marine sediment. Strain Su4^T was isolated from an anaerobic enrichment culture oxidizing sulfur compounds. The inoculum was collected from marine sediment of the East Sea, South Korea. A 3 g sediment sample was collected in a sterile conical tube and serially diluted in filter-sterilized (0.22 µm pore size, Millipore) natural sea water containing 3 mM thiosulfate as a sole electron donor, 6 mM nitrate as an electron acceptor and the following supplements (per l filter-sterilized natural sea water): trace element mixture (1 ×) (Widdel & Bak, 1992), 0.001 g resazurin, 3 mM bicarbonate, 0.1 mM phosphate and vitamin solution (1 ×) (Wolin *et al.*, 1963).

[†]Present address: Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore.

Abbreviations: NJ, neighbour-joining; ML, maximum-likelihood.

The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain Su4^T is EU837269.

A supplementary figure is available with the online version of this paper.

Medium without supplements was dispensed into anaerobic pressure tubes or serum bottles and bubbled with oxygen-free N₂ gas to remove dissolved oxygen. Before autoclaving, the tubes or bottles were closed with butyl-rubber stoppers and capped with aluminium seals. All preparations of enrichment cultures, serial dilutions and inoculations were performed under strictly anaerobic conditions in an anaerobic glove box (Coy Laboratory Products) containing an atmosphere of mixed gases (N₂/CO₂/H₂, 90:5:5, v/v/v). The supplement solutions were filter-sterilized and added to the autoclaved medium. If necessary, the pH was adjusted to 7.5–8.0 by adding 1 M NaOH or HCl. The mixture was anaerobically incubated at 25 °C in tubes or bottles and subcultured every 2–3 weeks with 10% (v/v) transfer. After five subculturing in the natural seawater medium, the culture was spread onto the solid artificial sea water medium (ASWM), containing 3 mM thiosulfate and 1.5% (w/v) agarose, in 20 g l⁻¹ sea salts (Sigma), and incubated at 25 °C for 2 weeks under anaerobic and aerobic conditions. The ASWM broth or agar contained the following supplements (l⁻¹): trace element mixture (1×) (Widdel & Bak, 1992), 0.001 g resazurin, 3 mM bicarbonate, 0.1 mM phosphate and vitamin solution (1×) (Wolin *et al.*, 1963). Cells of the isolated strain were able to grow under aerobic as well as anaerobic conditions but the growth rate was higher under aerobic conditions. The strain was preserved at -80 °C as suspensions in ASWM containing 20% (w/v) glycerol.

For phylogenetic analysis of strain Su4^T, genomic DNA was extracted using a commercial genomic DNA extraction kit (Solgent). The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3'; *E. coli* position

8–27) and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'; *E. coli* position 1492–1510) (Park *et al.*, 2006; Weisburg *et al.*, 1991). The purified PCR products were sequenced by Solgent using primers 27F, 338F, 786R and 1492R (Weisburg *et al.*, 1991). The nearly complete 16S rRNA gene sequence of strain Su4^T was obtained (1417 bp), and then compiled by using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Sequence alignments were performed by using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Nucleotide similarity values of the 16S rRNA gene sequences were calculated by using the EzTaxon program (<http://www.eztaxon.org>) (Chun *et al.*, 2007). Evolutionary distances were calculated by using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were reconstructed based on the neighbour-joining (NJ; Saitou & Nei, 1987) and maximum-likelihood (ML; Felsenstein, 1981) methods by using the MEGA4 (Tamura *et al.*, 2007) and PHYLIP programs (Felsenstein, 1993), respectively. Bootstrap analysis was performed with 1000 resampled datasets by using the built-in programs of the software. In the phylogenetic analysis, strain Su4^T was closely related to members of the unclassified family of the class *Gammaproteobacteria*. The novel strain was most closely related to members of the genera *Thiohalomonas* (91.4–92.8% 16S rRNA gene sequence similarity), *Thioalkalivibrio* (92.2–92.4%), *Thiohalospira* (89.8–91.8%), *Thiorhodospira* (91.2%), *Ectothiorhodospira* (92.0%), *Thioalkalispira* (90.6%) and *Thiohalophilus* (90.9%) (Fig. 1). In the phylogenetic tree based on 16S rRNA gene sequences, strain Su4^T formed a monophyletic clade in the unclassified *Grammaproteobacteria* and was most closely related to *Thiohalomonas denitrificans* HLD 2^T (92.8%) (Fig. 1) (Sorokin *et al.*, 2007a). The

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain Su4^T and related taxa. GenBank accession numbers are given in parentheses. Bar, 0.02 substitutions per nucleotide position. Bootstrap values $\geq 50\%$ (based on 1000 replicates) from neighbour-joining (NJ) and maximum-likelihood (ML) methods, respectively, are indicated at branch points; values $< 50\%$ are indicated by a dash.

phylogenetic topology of the NJ and ML trees showed that the clade containing strain Su4^T comprised typical sulfur-oxidizing bacteria. 16S rRNA gene sequence similarity values below the recommended threshold value of 97 % (Wayne *et al.*, 1987) between strain Su4^T and related taxa established strain Su4^T as a distinct genospecies.

Cell morphology and size were determined by phase-contrast microscopy (Eclipse 80i; Nikon). For the preparation of cells of strain Su4^T for scanning electron microscopy (SEM), cells were harvested and immersed in 4 % (v/v) glutaraldehyde and 0.1 M sodium phosphate buffer (pH 7.2) for 24 h at 4 °C, and dehydrated through a graded ethanol series (70–100 %). The specimens were examined with a Zeiss DSM 940 scanning electron microscope (Carl Zeiss). To enable transmission electron microscopic analysis of strain Su4^T, cells were fixed in a 2.5 % paraformaldehyde/1.5 % glutaraldehyde mixture buffered with 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at 4 °C, post-fixed in 1 % osmium tetroxide in the same buffer for 1 h, dehydrated in a graded ethanol series (–100 %), transferred to propylene oxide and embedded in Epon-812 medium (TAAB). Ultra-thin sections generated with an UltraCut E (Leica) ultramicrotome were stained with uranyl acetate and lead citrate and examined with a CM 20 electron microscope 405 (Philips). Gram staining was performed with Gram stain kits (BD) according to the instructions of the manufacturer. Catalase activity was determined by bubble production in 3 % (v/v) hydrogen peroxide solution and oxidase activity was determined using 1 % (w/v) tetramethyl *p*-phenylenediamine (Merck). Cells of strain Su4^T were Gram-negative-staining, oxidase- and catalase-positive, non-motile and rod-shaped and varied in size (0.5–0.8 × 1.2–1.5 µm) (Fig. 2). Elemental sulfur was accumulated when strain Su4^T was grown with thiosulfate as electron donor under anaerobic conditions (Supplementary Fig. S1, available in IJSEM Online). The methyl red/Voges–Proskauer test and tests for indole production and H₂S production were negative. Since anaerobic growth was observed under denitrifying conditions (nitrate as sole electron acceptor and thiosulfate as sole electron donor), strain Su4^T was characterized as a facultative anaerobe. Colonies of strain Su4^T were white, 0.7–1.5 mm in diameter and had entire margins when grown on ASWM agar plates under aerobic conditions for 10 days.

Heterotrophic growth was investigated in ASWM containing potential organic carbon sources; 0.01 % (w/v) each of yeast extract, tryptone, peptone and Casamino acids; 1 mM each of succinate, glucose, glycerol, ethanol, methanol, pyruvate, malate, citrate, propionate and formate; and 0.1 mM each of proline, alanine, aspartic acid and serine were used. Heterotrophic growth was not observed in this study with any of the carbon compounds used. Vitamins also were not required. These results strongly supported the notion that strain Su4^T could grow autotrophically.

Sulfur oxidation was evaluated with 1 mM thiosulfate, 1 mM tetrathionate, 1 mM sulfite, 1 mM sulfide and 1 %

Fig. 2. Electron micrographs of cells of strain Su4^T. Transverse thin sections of single cells (a), scanning electron micrographs of cells (b). Bar, 500 nm.

(w/v) elemental sulfur under denitrifying conditions. Thiosulfate concentration was determined colorimetrically (Choi *et al.*, 2009; Voroteliak *et al.*, 1993). For the determination of thiosulfate concentration in culture supernatant, the cells of strain Su4^T were pelleted by centrifugation at 13 000 r.p.m. for 7 min. The supernatant (100 µl) was removed and treated with a reagent containing 0.25M KCN (20 µl), 0.2M CuSO₄ (20 µl) and 0.2M ferric nitrate (50 µl) in a glass tube. The tube was vortexed, and OD₄₆₀ was measured with a Nanodrop spectrophotometer ND-1000 (Nanodrop technologies). The sulfide concentration was estimated by using the improved method of Cline (1969): 0.5 ml samples were added to a mixture of 0.25 ml 0.05M zinc acetate and 0.025 ml of diamine reagent (prepared from 4.0 g anhydrous ferric chloride and 1.6 g *N,N*-dimethyl-*p*-phenylene diamine sulfate with 100 ml 6M HCl), with vortex-mixing between additions; 1 ml of distilled water was then added after 20 min and A₆₇₀ was measured after 15 min. Sulfate levels were determined turbidimetrically (Kolmert *et al.*, 2000). Other sulfur-compound assimilation was observed indirectly via the reduction nitrate. Nitrate respiration was determined by measuring the concentration of nitrate remaining in the enrichment culture and the level of nitrite accumulated during denitrification (Strickland & Parsons, 1968). The result showed that strain Su4^T was able to utilize thiosulfate, tetrathionate, sulfide and elementary sulfur but not sulfite. The product of thiosulfate oxidation (4 mM thiosulfate) was sulfate under aerobic (oxygen as

electron acceptor) and denitrifying [nitrate (8–10 mM) as electron acceptor] conditions. After thiosulfate oxidation, the amount of sulfate produced under aerobic conditions was two times greater (Fig. 3). Growth of strain Su4^T was arrested by nitrite accumulation (about 3–4 mM) and nitrate reduction similar to members of the genera *Thiohalomonas* (Sorokin *et al.*, 2007a) and *Thiohalophilus* (Sorokin *et al.*, 2007b).

Growth at different temperatures was assessed after 14 days incubation on ASWM with 4 mM thiosulfate. Strain Su4^T was able to grow at 4–37 °C but not at 0 °C or >40 °C (optimum 28–32 °C). Tolerance of salt was measured on ASWM broth containing 0–20 % (w/v) NaCl and 4 mM thiosulfate. Cultures were incubated at 28 °C for 2 weeks. Cells of strain Su4^T required sodium ions for growth and grew in 1–5 % (w/v) NaCl (optimum 3 % NaCl). Growth at pH 5–9 (intervals of 0.5 pH units) was determined in ASWM at 28 °C for 14 days. The pH was adjusted with 1M HCl or 1M NaOH. Strain Su4^T grew at pH 5.5–8.5 but not at pH values <5 or >9 (optimum pH 7.0–7.5). The physiological characteristics of strain Su4^T are summarized in the species description and selective characteristics were compared with those of closely related genera (Table 1).

Cellular fatty acids of strain Su4^T were analysed using cells grown for 14 days on ASWM containing 4 mM thiosulfate. Cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI, 1999). Fatty acids were analysed by GC (Hewlett Packard 6890) and identified by the Microbial Identification software package. The major cellular fatty acids of strain Su4^T comprised C_{16:0} (32.98 %), C_{16:1}ω7*c*/iso-C_{15:0} 2-OH (32.47 %) and C_{18:1}ω7*c*/ω9*t*/ω12*t* (14.19 %). Other fatty acids detected are shown in Table 2. Strain Su4^T could be differentiated from members of the genera *Thiohalomonas* and *Ectothiorhodospira* by differences

in their fatty acid compositions, particularly the presence or absence of C_{16:1}ω7*c*, C_{18:1}ω7*c* and C_{19:0} Δ8, 9, as shown in Table 2. Chromosomal DNA extracted for 16S rRNA gene amplification was used for determination of G+C content. RNA in the DNA solution was removed by incubation with a mixture of RNase A and T1 (each, 20 units ml⁻¹) at 30 °C for 1 hour. The G+C content of the chromosomal DNA was analysed as described by Mesbah *et al.* (1989) using reversed-phase HPLC. The DNA G+C content of strain Su4^T was 64.5 mol%. Strain Su4^T differed from members of the genera *Thiohalomonas*, *Thiohalospira*, *Thiorhodospira*, *Alkalilimnicola* and *Alkalispirillum* in its DNA G+C content (Table 1).

The present data demonstrated that the novel, chemolithoautotrophic, sulphur-oxidizing bacterium showed many unique features compared to members of related genera and its phylogenetic position was consistent in all the phylogenetic trees generated by using NJ and ML algorithms. The topology of the maximum-parsimony tree was clearly the same (data not shown). From the phylogenetic analyses, strain Su4^T could not be associated with any known genera and, therefore, appeared to constitute a new genus in the class *Gammaproteobacteria*. Strain Su4^T could also be differentiated physiologically from members of related genera, as shown in Table 1. Members of the reference genera were phototrophic, halophilic and/or alkaliphilic, whereas strain Su4^T was non-alkaliphilic (neutrophilic), non-phototrophic and only slightly halophilic, differentiating it from members of closely related genera in the class *Gammaproteobacteria*. Considering its low 16S rRNA gene sequence similarity (<92 %) to members of closely related taxa, its unique branching position in phylogenetic analyses and its physiological characteristics, it is clear that the isolate cannot be assigned to any previously recognized bacterial genus. Therefore, strain Su4^T represents a novel species of a novel genus, for which the name *Thioalbus denitrificans* gen. nov., sp. nov. is proposed.

Description of *Thioalbus* gen. nov.

Thioalbus [Thi.o.al'bus. Gr. n. *theion* (Latin transliteration *thium*) sulfur; L. masc. adj. *albus* white; N.L. masc. n. *Thioalbus* intended to mean a bacterium which oxidizes thiosulfate and whose colonies are white].

Cells are non-motile, Gram-reaction-negative rods. Obligately chemolithoautotrophic and facultatively anaerobic. Utilizes reduced sulfur compounds as electron donors with oxygen or nitrate as electron acceptors. Slightly halophilic and neutrophilic. Major cellular fatty acids are C_{16:0}, C_{16:1}ω7*c*/iso-C_{15:0} 2-OH and C_{18:1}ω7*c*/ω9*t*/ω12*t*. Cells were isolated from marine sediment. The type species is *Thioalbus denitrificans*, a member of the *Gammaproteobacteria*.

Description of *Thioalbus denitrificans* sp. nov.

Thioalbus denitrificans (de.ni.tri.fi'cans. N.L. part. adj. *denitrificans* from N.L. v. *denitrifico*, denitrifying).

Fig. 3. Growth (◇), thiosulfate oxidation (●) and sulfate production (○) in chemolithoautotrophic cultures of strain Su4^T. Cells were cultivated under aerobic conditions. Error bar represents SD of results from triplicate experiments.

Table 1. Characteristics of strain Su4^T and related genera

Taxa: 1, strain Su4^T (data from this study); 2, *Thiohalomonas* (Sorokin *et al.*, 2007a); 3, *Ectothiorhodospira* (Imhoff, 2005; Ventura *et al.*, 2000); 4, *Thiohalospira* (Sorokin *et al.*, 2008); 5, *Thiorhodospira* (Bryantseva *et al.*, 1999); 6, *Thioalkalivibrio* (Sorokin *et al.*, 2002a; Sorokin *et al.*, 2002c; Sorokin *et al.*, 2001b); 7, *Alkalilimnicola* (Hoeft *et al.*, 2007; Sorokin *et al.*, 2006a; Yakimov *et al.*, 2001); 8, *Alkalispirillum* (Rijkenberg *et al.*, 2001; Sorokin *et al.*, 2006a). +, Positive; –, negative; v, variable; ND, no data available; R, rod; LR, long rod; SR, short rod; VB, vibrioid; SP, spirilla; CLH, chemolithoheterotrophic; CLA, chemolithoautotrophic; PAL, photoautolithotrophic; POH, photo-organoheterotrophic.

Characteristic	1	2	3	4	5	6	7	8
Morphology	R	LR, VB	VB	SP	VB, SP	SP	SR, LR	VB
Pigment	—	ND	Red	Yellow	Red	Yellow, brown	—	—
Temperature optimum (°C)	28–32	ND	25–40	32–35	25–30	ND	30–35	35–38
pH optimum	7.0–7.5	7.3–8.2	7.6–10	7.3–8.5	9.0–9.5	9.0–10	9.3–9.5	9.0–10
NaCl range (%)	1–5	5–20	0–20	3–29	0–6	2–23	0–28	0–25
NaCl optimum (%)	3	5–12	1–7	11–20	0.5–1	5–12	3–8	2
Aerobic growth	+	+	v	+	—	+	+	+
Anaerobic growth	+	+	+	v	+	v	+	+
Metabolism	CLA	CLA	PAL, POH, CLA	CLA	PAL	CLA	CLH, CLA	CLH, CLA
DNA G + C content (mol%)	64.5	58–60	61–68	65.6–67	56–57	61–66	66–68	66.2

Table 2. Fatty acid compositions of strain Su4^T and related type species

Taxa: 1, strain Su4^T; 2, *Thiohalomonas denitrificans* HLD 2^T (Sorokin *et al.*, 2007a); 3, *Ectothiorhodospira mobilis* DSM 237^T (Thiemann & Imhoff, 1996). Values shown are percentages of total fatty acids. tr, Trace (<1 %); –, not detected; ECL, equivalent chain length.

Fatty acid	1	2	3
C _{10:0} 3-OH	2.76	tr	—
C _{12:0}	2.53	3.90	—
C _{12:0} 3-OH	2.11	—	—
C _{14:0}	—	—	1.20
anteiso-C _{15:0}	2.32	—	—
C _{16:0}	32.98	25.41	35.70
C _{16:0} 10-methyl	4.40	1.26	—
C _{16:1} ω9c	—	—	2.70
C _{16:1} ω7c	—	27.27	—
C _{16:1} ω5c	—	2.97	—
C _{17:0} cyclo	—	8.38	—
C _{17:1} 11-methyl	—	2.53	—
C _{18:0}	1.71	tr	7.60
C _{18:1} ω9c	2.43	5.10	—
C _{18:1} ω7c	—	11.84	—
C _{18:1} ω5c	—	1.64	—
C _{18:1} 11-methyl	—	1.29	—
C _{19:0} Δ8, 9	—	—	33.80
C _{19:0} cyclo	—	3.26	—
Summed features*			
4	32.47	—	—
7	14.19	—	16.50
Unknown (ECL 16.111)	2.11	—	—

*Summed features represent groups of two or three fatty acids that cannot be separated by a GC with the MIDI system. Summed feature 4 comprises C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, summed feature 7 comprises C_{18:1}ω7c/ω9t/ω12t.

Cells are Gram-reaction-negative, oxidase- and catalase-positive, aerobic, denitrifying, rod-shaped (0.5–0.8 × 1.2–1.5 µm) and have no flagellum. Cells are able to oxidize thiosulfate, tetrathionate, sulfide and elemental sulfur but not sulfite under the denitrifying conditions. Favourable growth occurs aerobically, forming circular colonies of ~0.7–1.5 mm diameter with regular edges within 10 days of incubation at 10–40 °C (optimum 28–32 °C). Elemental sulfur is accumulated via thiosulfate oxidation when cells are grown under anaerobic conditions (Supplementary Fig. S1). Does not use yeast extract, Tryptone, peptone, Casamino acids, succinate, glucose, glycerol, ethanol, methanol, pyruvate, malate, citrate, propionate, formate, proline, alanine, aspartic acid or serine as organic carbon sources or electron donors. Growth factors, such as vitamins, are not required. Neutrophilic, non-phototrophic and slightly halophilic. NaCl is required for growth. Optimum growth occurs at pH 7.0–7.5 and in 3 % (w/v) NaCl. Major cellular fatty acids are C_{16:0}, C_{16:1}ω7c/iso-C_{15:0} 2-OH and C_{18:1}ω7c/ω9t/ω12t.

The type strain, Su4^T (=KCTC 5699^T =JCM 15568^T), was isolated from a sulfur-oxidizing enrichment culture from marine sediment of the East Sea, South Korea. The DNA G + C content of the type strain is 64.5 mol% (as determined by HPLC).

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