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Hoeflea halophila sp. nov., a novel bacterium isolated from marine sediment of the East Sea, Korea

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Abstract A Gram-negative, aerobic, motile, straight or curved rod-shaped marine bacterium was isolated from marine sediment of the East Sea, Korea. The isolated strain, JG120-1^T, grows with 0–5 % (w/v) NaCl and at 15–30 °C and pH 6–9. α-galactosidase activity test was positive. Comparative 16S rRNA gene sequence studies showed that this strain belonged to the *Alphaproteobacteria* and was the most closely related to *Hoeflea alexandrii* AM1 V30^T, *Hoeflea phototrophica* DFL-43^T and *Hoeflea marina* LMG 128^T (98.9, 97.9 and 97.0 % 16S rRNA gene sequence similarities, respectively). Strain JG120-1^T was found to possess summed feature 8 (C18:1ω7c/C18:1ω6c, 71.11 %) as the major cellular fatty acid. The major

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The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain JG120-1^T is GU564401

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K.-S. Shin · M.-N. Kim Biological Resources Center, KRIBB, Daejeon 305-806, Republic of Korea ubiquinone was determined to be Q-10. Polar lipids include phosphatidylglycerol, phosphatidylethanolamine, sulfoquinovosyl diacylglycerol, phosphatidylcholine and phosphatidylmonomethylethanolamine. The G+C content of the genomic DNA of strain JG120-1^T was determined to be 57.8 mol %. DNA–DNA relatedness data indicated that strain JG120-1^T represents a distinct species that is separate from *H. phototrophica* DFL-43^T, *H. marina* LMG128^T and *H. alexandrii* AM1 V30^T. On the basis of polyphasic evidences, it is proposed that strain JG120-1^T (= KCTC 23107^T = JCM 16715^T) represents the type strain of a novel species, *Hoeflea halophila* sp. nov.

Keywords *Hoeflea halophila* · Novel species · Marine sediment · Polyphasic taxonomy

Introduction

According to Garrity et al. (2004), the family *Phyllobacteriaceae* of the order *Rhizobiales* of the class *Alphaproteobacteria* consists of eight genera representing various origins and lifestyles: *Phyllobacterium* (Knösel 1984), *Aminobacter* (Urakami et al. 1992), *Aquamicrobium* (Bambauer et al. 1998), *Defluvibacter* (Fritsche et al. 1999), '*Candidatus* Liberobacter' (Jagoueix et al. 1994), *Mesorhizobium* (Jarvis et al. 1997), *Nitratireductor* (Labbé et al. 2004) and *Pseudaminobacter* (Kämpfer et al. 1999). Strain LMG 128 (=Ahrens A43 = ATCC 25654) is Gram-negative,



non-spore-forming, strictly aerobic, short, regular and rod-shaped cells isolated from seawater of the Baltic Sea, off the coast of the Germany and the strain was initially included in the species Agrobacterium ferrugineum (Ahrens et al. 1968). Agrobacterium ferrugineum LMG 128 was reclassified as Hoeflea marina gen. nov., sp. nov. within the family Phyllobacteriaceae, by Peix et al. (2005). Since this genus was proposed in 2005, Hoeflea spp. have been isolated from cultures of marine dinoflagellates and cyanobacterium: H. phototrophica from a culture of Prorocentrum lima (Biebl et al. 2006), H. alexandrii from the toxic dinoflagellate Alexandrium minutum AL1V (Palacios et al. 2006), H. anabaenae from heterocysts of a strain of Anabaena (Stevenson et al. 2011). A Hoeflea-like bacterial strain JG120-1^T was isolated from marine sediment of the East Sea, Korea, which may expand niche diversity of the genus Hoeflea. The aim of the present study was to determine the taxonomic position of strain JG120-1^T by using polyphasic taxonomic characterization including determination of phenotypic and chemotaxonomic properties and phylogenetic analysis of 16S rRNA gene sequences. The isolated strain, JG120-1^T, is considered to represent a novel species of the genus Hoeflea.

Materials and methods

Isolation and culture conditions

A sediment sample collected from the East Sea of Korea was serially diluted with filter-sterilized (0.22 µm pore size, Millipore) natural sea water containing 0.1 % yeast extract (Difco). After incubation of diluted aliquots at 25 °C under aerobic conditions for 2 weeks, the aliquot of the last dilution showing turbidity was spread onto the same medium with agar (1.5 %, w/v) which were incubated and at 25 °C for 2 weeks to obtain pure culture isolates. Single colonies were purified by transferring them onto marine agar 2216 (MA; Difco) and subjecting them to additional incubation at 25 °C for 3 days. Isolated strains were routinely grown in Marine Broth 2216 (MB) (Difco) or on MA for further characterization. The cultures grown in MB were supplemented with an equal volume of glycerol solution (40 %, w/v) before long-term storage at -80 °C. The three reference strains (*H. alexandrii* AM1V30^T, *H. phototrophica* DFL-43^T and *H. marina* LMG128^T) for comparative studies were obtained from the Korean Collection for Type Cultures (KCTC, Republic of Korea). Unless otherwise stated, all reference strains were grown in MB or on MA under their optimal culture conditions (Biebl et al. 2006; Palacios et al. 2006; Peix et al. 2005).

Morphological, physiological and biochemical characterization

The Gram reaction was determined by using a Gramstain kit (Difco) according to the manufacturer's instructions. Cell morphology was examined by light microscopy (Eclipse 80i; Nikon) and transmission electron microscope (EM-109; Carl Zeiss) after negative staining with 1 % (w/v) phosphotungstic acid. Catalase activity was determined by bubble production in 3 % (w/v) H₂O₂ and oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylenediamine. Motility of the cells was examined by the semisolid agar method (Shirling and Gottlieb 1966).

Growth at different temperatures (4, 10, 15, 20, 25, 30, 35 and 40 °C) was assessed after 3 days incubation on MA. Growth at different NaCl concentrations (0, 0.5, 1, 2, 3, 4 and 5 %, w/v) was measured using artificial sea water (ASW; 26.4 g NaCl, 0.67 g KCl, 1.36 g CaCl₂·2H₂O, 6.29 g MgSO₄·7H₂O, 4.66 g MgCl₂·6H2O, 0.18 g Na₂CO₃ per liter of distilled water) modified from Kester et al. (1967) without NaCl supplemented with 0.1 % (w/v) yeast extract. The response to pH (pH 5.5–9.5 at intervals of 0.5 pH units) was determined in MB at 30 °C for 3 days. Three different buffers were used (final concentration, 10 mM): acetate buffer (pH 5.5); phosphate buffer (pH 6.0–8.0); Tris buffer (pH 8.5–9.5).

The utilization of carbon sources was determined in ASW medium containing 0.1 g yeast extract l^{-1} to provide the required growth factors. The following carbon sources were tested at a concentration of 1 g l^{-1} (acids as sodium salts): acetate, arabinose, butyrate, citrate, formate, fumarate, galactose, glucose, glutamate, glycerol, lactate, lactose, lysine, maltose, mannitol, mannose, pyruvate, sucrose, threonine, urea, xylose and yeast extract. An organism was considered to be able to utilize a substrate if it resulted in a significant increase in maximum OD_{600} compared with controls that contained only yeast extract.



Substrate utilization profiles were also determined in this manner for *H. alexandrii* AM1V30^T, *H. phototrophica* DFL-43^T and *H. marina* LMG128^T to allow for direct comparisons to be made.

Extracellular enzyme activities were characterized using the API ZYM kit for strain JG120-1^T and *H. alexandrii* AM1V30^T, *H. phototrophica* DFL-43^T and *H. marina* LMG128^T. Cells for inoculation were harvested from cultures in mid-exponential phase (OD₆₀₀ 0.4–0.5) in ASW medium containing 0.1 g yeast extract 1⁻¹. Strips were inoculated and were then incubated at 30 °C for 24 h prior to reading the results. The sensitivity of the isolates to the following antibiotics was tested using MA plate containing each antibiotic: ampicillin (10 μ g), chloramphenicol (25 μ g), erythromycin (15 μ g), gentamicin (10 μ g), kanamycin (30 μ g), penicillin G (10 μ g), streptomycin (10 μ g) and tetracycline (30 μ g).

16S rRNA gene sequence determination and phylogenetic analysis

Bacterial genomic DNA was extracted using a commercial genomic DNA extraction kit (Cosmo Genetech Co. Ltd, Korea). The 16S rRNA gene was amplified from the chromosomal DNA using the universal bacterial primer 27F (5'-AGAGTTTGATCMTGGCT CAG-3', E. coli position 8-27) and 1492R (5'-TAC GGYTACCTTGTTACGACTT-3', E. coli position 1,492–1,510) (Weisburg et al. 1991) and the purified PCR products were sequenced by Cosmo Genetech Co. Ltd (Korea). The nearly complete 16S rRNA gene sequence (1,419 bp) of strain JG120-1^T was obtained by assembling the sequences with SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Sequence alignments were performed using the CLUSTAL_X program (Thompson et al. 1997). Gaps were edited in the BioEdit program (Hall 1999). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura 1983). Phylogenetic trees were constructed based on the neighborjoining (Saitou and Nei 1987) and the minimum evolution method (Nei et al. 1998) algorithms by using the MEGA5 Program (Tamura et al. 2011). The nucleotide similarity value of the 16S rRNA gene was calculated using the EzTaxon-e program (http:// www.eztaxon.org) (Kim et al. 2012).

Chemotaxonomic characterization

Cellular fatty acids were analyzed in the isolated strain and reference strains (H. phototrophica DFL-43^T, H. marina LMG128^T and H. alexandrii AM1V30^T) were analyzed using cultures grown on MA at 30 °C and pH 7.5 for 3 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI 1999). The fatty acids were analyzed by gas chromatography (Agilent Technologies 6890 N) and were identified using the microbial identification software package (version 6.1). Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum conditions, and re-extracted in acetone. The crude quinone in acetone was then purified using silica gel thin-layer chromatography (TLC) (Merck). Ubiquinone and menaguinone fractions obtained by TLC were dissolved in acetone for HPLC analysis. HPLC analysis was performed according to Hiraishi et al. (1996). Polar lipids of strain JG120-1^T was extracted and was analyzed by two-dimensional ascending TLC (2D-TLC) as described by Minnikin et al. (1984).

The G+C content of the chromosomal DNA was analyzed as described by Gonzalez and Saiz-Jimenez (2002). Chromosomal DNA extracted for 16S rRNA gene amplification was used for determination of the G+C content. RNA in the DNA solution was removed by incubation with a mixture of ribonuclease A and T1 (each, 20 units ml^{-1}) at 30 °C for 1 h. The production of bacteriochlorophyll a and other photopigments by strain JG120-1^T was investigated as described by Biebl et al. (2006).

DNA-DNA hybridization

DNA–DNA hybridization experiments were carried out with strains JG120-1^T, *H. alexandrii* AM1 V30^T and *H. phototrophica* DFL-43^T using the method described by Ezaki et al. (1989). The genomic DNA of strain JG120-1^T and reference strains (*H. alexandrii* AM1V30^T and *H. phototrophica* DFL-43^T) were extracted using a genomic DNA extraction kit (Cosmo, Korea) and used as a probe. Probe DNAs were biotinylated with photobiotin and were hybridized with single-stranded unlabeled chromosomal DNA fragments of test microorganisms. The means



from three independent determinations of DNA–DNA hybridization levels were determined.

Results and discussion

Morphological, physiological and biochemical characteristics

Strain JG120-1^T was Gram-negative, aerobic, and straight or curved rod-shaped. Cells possessed a polar flagellum (Fig. 1) and were motile, which is similar to the rapid motility observed directly for Hoeflea species reported previously (Biebl et al. 2006; Palacios et al. 2006; Peix et al. 2005). Colonies of a diameter of 2-3 mm were beige, circular and convex with regular edges when grown on MA 2216 (Difco) at 30 °C for 3 days. Strain JG120-1^T was able to grow at 15–30 °C, but not at 4 or above 40 °C (optimum, 25-28 °C). Strain JG120-1^T could grow in 0-5 % (w/v) NaCl (optimum 0.5-1 %) and at pH 6.0-9.0 but not at pH values below 5.5 or above 9.5 (optimum, pH 7.5). The physiological characteristics of strain JG120-1^T are summarized in the species description and selective characteristics were compared with those of closely related type strains in Table 1. The overall properties of JG120-1^T are similar to those of other type strains of the genus Hoeflea.

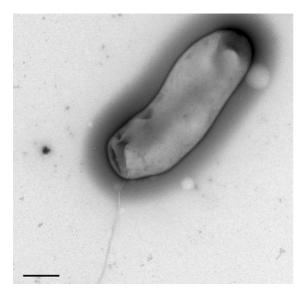


Fig. 1 Transmission electron micrograph of a negatively stained cell of the strain $JG120-1^{T}$. *Bar* 0.5 μ m

Substrate utilization profiles were determined for strain JG120-1^T as well as *H. alexandrii* AM1V30^T, *H. phototrophica* DFL-43^T and *H. marina* LMG128^T to allow for direct comparison (Table 1 and Supplementary table S1). Strain JG120-1^T was able to utilize

Table 1 Selective phenotypic characteristics of strain JG120-1^T and phylogenetically related type strains of the genus *Hoeftea*

Поерей				
Characteristic	1	2	3	4
Salinity range (%)	0–5	0-11.8 ^a	0.5-7 ^b	0-5°
Temperature range (°C)	15-30	10-42 ^a	15–33 ^b	$4-37^{c}$
pH range	6–9	$6-9^{a}$	6–9 ^b	6–8°
Growth at				
4 °C	_	-	-	+
40 °C	-	+	-	-
Extracellular enzyme activ	ity of ^d			
Trypsin	_	-	+	+
α-chemotrypsin	_	-	+	-
Acid phospatase	_	+	+	-
Phosphohydrolase	_	+	_	+
α-galactosidase	+	_	_	-
β -galactosidase	+	_	+	+
Assimilation of				
Butyrate	_	+	+	+
Citrate	+	_	+	+
Galactose	_	+	+	+
Glucose	_	+	+	+
Glutamate	_	+	+	+
Glycerol	_	_	+	+
Lactate	_	_	_	+
Lactose	_	_	_	+
Pyruvate	_	+	+	+
Yeast extract	+	+	_	+
Bacteriochlorophyll a	_	_a	$+^{b}$	_c
DNA G+C content (mol %)	57.8	59.7 ^a	59.3 ^b	53.1°
16S rRNA gene sequence similarity to strain JG120-1 (%)	(100)	98.9	97.9	97.0

Strains: 1 JG120-1^T, 2 *H. alexandrii* AM1V30^T, 3 *H. phototrophica* DFL-43^T, 4 *H. marina* LMG128^T. Characteristics are scored as + positive reaction – negative reaction



^a Data taken form: Palacios et al. (2006)

^b Biebl et al. (2006)

^c Peix et al. (2005)

d Data from API ZYM tests

arabinose, citrate, fumarate, maltose, mannitol, mannose, sucrose and yeast extract. Strain JG120-1^T was not able to utilize butyrate, galactose, glucose, glutamate and pyruvate while other type strains of the genus *Hoeflea* were able to utilize them.

Extracellular enzyme activities were characterized using the API ZYM kit, and the profiles were compared among all type strains in Table 1 and Supplementary table S2. Among the type strains of the genus *Hoeflea*, strain JG120-1^T was unique in that only it possessed α -galactosidase activity. Strain JG120-1^T was susceptible to ampicillin, chloramphenicol, erythromycin, gentamicin, penicillin G, streptomycin, tetracycline and kanamycin.

Phylogenetic characteristics

The almost complete 16S rRNA gene sequence of strain JG120-1^T determined in this study was comprised of 1,419 nucleotides (\sim 93 % of the *Escherichia coli* 16S rRNA sequence). The highest degrees of sequence similarity were found to be with *H. alexandrii* AM1V30^T (98.9 %), *H. phototrophica* DFL-43^T (97.9 %) and *H. marina* LMG128^T

(97.0 %). In the analysis of phylogeny, strain JG120-1^T was determined to clearly belong to the lineage *Hoeflea* of the *Alphaproteobacteria* due to the observation of suitably high bootstrap values (Fig. 2) on the phylogenetic trees of 16S rRNA gene sequences, as constructed based on the neighbor–joining and the minimum evolution method algorithms.

Chemotaxonomic characteristics

Profiles of major cellular fatty acid of the strain $JG120-1^T$ was composed of summed feature 8 ($C_{18:1}$ $\omega 7c/C_{18:1}$ $\omega 6c$) (71.1%), $C_{18:1}$ $\omega 7c$ 11 methyl (15.1%) and $C_{16:0}$ (5.3%) (Table 2) and its overall profiles of fatty acid were similar to those of H. alexandrii and H. phototrophica (Table 2). However, there were slight differences in the proportion of fatty acids between this study and other experimental results of H. alexandrii AM1V30 T (Palacios et al. 2006), H. phototrophica DFL-43 T (Biebl et al. 2006) and H. marina LMG128 T (Peix et al. 2005) which may be have been caused by their different cultivation, extraction or analytical conditions.

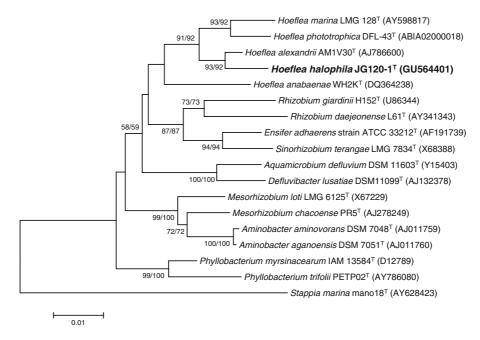


Fig. 2 Phylogenetic consensus tree based on 16S rRNA gene sequences, showing the taxonomic position of strain JG1-102^T with respect to other species in the genus *Hoeflea. Stappia marina* mano18^T served as an outgroup. Numbers at nodes are bootstrap values calculated from neighbor–joining/minimum-

evolution methods probabilities as percentages of 1,000 replications; only values >50 % are shown. GenBank accession numbers are shown in *parentheses*. Bar 0.01 substitutions per nucleotide position



Table 2 Cellular fatty acid compositions of strains JG120-1^T and phylogenetically related type strains of the genus *Hoeflea*

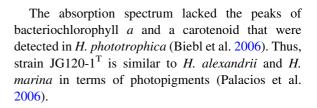
1 7 6	J 1		U	,
Fatty acid	1	2	3	4
Straight-chain fatty acid				
$C_{14:0}$	_	0.9	0.6	-
C _{16:0}	5.3	10.0	7.3	15.6
C _{17:0}	0.9	tr	_	-
C _{17:0 CYCLO}	_	_	_	3.4
C _{18:0}	1.1	2.5	1.3	0.7
Unsaturated fatty acid				
$C_{16:1}\omega 11c$	_	0.6	_	-
$C_{17:1}\omega 8c$	0.6	_	_	-
$C_{17:1}\omega 6c$	_	_	_	-
$C_{18:1}\omega 9c$	0.5	1.7	0.9	-
$C_{18:1}\omega7c$ 11 methyl	15.1	19.9	17.0	11.6
$C_{19:0}\omega 8c$ CYCLO	_	_	_	39.7
$C_{20:1}\omega 7c$	0.6	_	_	-
$C_{20:2}\omega 6,9c$	_	_	_	-
Summed features ^a				
2 (unknown 10.928)	1.6	2.1	2.3	3.8
$3(C_{16:1}\omega7c/C_{16:1}\omega6c)$	2.4	2.5	2.8	2.1
7 ($C_{19:1}\omega7c/.846/19cy$)	_	0.6	1.1	-
8 ($C_{18:1}\omega7c/C_{18:1}\omega6c$)	71.1	58.7	66.7	23.0

Values are percentages of total fatty acid, Fatty acids that occurred at <0.5 % in all four strain are not shown, – not detected, tr trace amount (<0.5 %)

Strains 1 JG120-1^T, 2 *H. alexandrii* AM1V30^T, 3 *H. phototrophica* DFL-43^T, 4 *H. marina* LMG128^T

The major ubiquinone of JG120-1^T was Q-10. All type strains of genus *Hoeflea* produce Q-10 as their single respiratory lipoquinone, a common feature of the majority of *Alphaproteobacteria*. The polar lipids consisted of phosphatidylglycerol (PG), phosphatidylethanolamine (PE), sulfoquinovosyl diacylglycerol (SQDG), phosphatidylcholine (PC) and phosphatidylmonomethylethanolamine (PME) (Supplementary Fig. S1). The presence of PG, SQDG, PC and PME were shared with other type strains of genus *Hoeflea*. Strain JG120-1^T did not contain an aminolipid which all reference strains contained (Stevenson et al. 2011),

The G+C content of genomic DNA of strain $JG120-1^{T}$ was 57.8 mol % which is in the range (53.1–59.7 mol %) of type strains of the genus *Hoeflea*.



DNA-DNA hybridization

The DNA–DNA hybridization levels of strain JG120- $1^{\rm T}$ with *H. alexandrii* AM1V30^T and *H. phototrophica* DFL- $43^{\rm T}$ were 17.5 \pm 0.5 and 12.4 \pm 0.2 %, respectively (Supplementary table S3). In the current bacterial systematics, a species is defined when DNA–DNA relatedness is below 70 % (Wayne et al. 1987). Thus, isolate JG120- $1^{\rm T}$ could be a new species in the genus *Hoeflea*.

Conclusions

The present data demonstrated that the novel isolate, designated strain JG120-1^T is closely related with the genus Hoeflea. The phylogenetic trees of 16S rRNA gene sequences generated by neighbor-joining and minimum evolution algorithms showed that the isolate was differentiated form other genera (Phyllobacterium, Aminobacter, Aquamicrobium, Defluvibacter and Mesorhizobium) of the family Phyllobacteriaceae and that the strain formed a robust cluster within the genus Hoeflea (Fig. 2). Despite the relatively high 16S RNA gene sequence similarity of strain JG120-1^T with the most closely related species H. alexandrii AM1V30^T (98.9 %) and *H. phototrophica* DFL-43^T (97.9 %), the level of DNA-DNA relatedness was sufficiently low (<25 %) to clearly differentiate between the new strain and the two Hoeflea species. Differential features in phenotypic and chemotaxonomic characteristics from other type strains of the genus Hoeflea were also evident. Taken together, strain JG120-1^T should be identified as a novel species, for which we proposed the name Hoeflea halophila sp. nov.

Description of *Hoeflea halophila* sp. nov

Hoeflea halophila (Gr. n. hals halos, salt; N.L. fem. adj. phila (from Gr. fem. adj. phile), friend, loving; N.L. fem. adj. halophila, salt-loving).



^a Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system (version 6.0)

Cells are Gram-negative, aerobic, slightly halotolerant, oxidase- and catalase-positive, straight or curved rod-shaped $(0.7-0.8 \times 2.5 \,\mu\text{m})$, and are motile by means of a single polar flagellum. Favorable growth occurs aerobically with the formation of circular colonies with regular edges within 3 days, with diameters of $\sim 2-3$ mm. Growth occurs at 15-30 °C (optimum, 25–28 °C) and at pH 6.0–9.0 (optimum, pH 7.5) and in 0–5 % (w/v) NaCl (optimum, 0.5–1 %). Na⁺ is not essential for growth. Yeast Extract is required for growth as growth factor. Arabinose, citrate, fumarate, maltose, mannitol, mannose, sucrose and yeast extract are utilized as carbon sources, but acetate, butyrate, formate, galactose, glucose, glutamate, glycerol, lactate, lactose, lysine, pyruvate, threonine, urea and xylose are not. Positive for the following enzyme activities (API ZYM): alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, crystine arylamidase, α -galactosidase, β galactosidase, α -glucosidase and β -glucosidase. Negative for the following enzyme activities (API ZYM): lipase (C14), trypsin, α-chymotrypsin, acid phosphatase, phosphohydrolase, β -glucuronidase, N-acetyl- β glucosaminidase, α -mannosidase and α -fucosidase. Does not produce bacteriochlorophyll a or photopigments. Susceptible to ampicillin, chloramphenicol, erythromycin, gentamicin, penicillin G, streptomycin, tetracycline and kanamycin. Q-10 was detected as the major respiratory quinone. The major cellular fatty acids of JG120-1^T are summed feature 8 ($C_{18:1} \omega 7c$ and/ or $C_{18:1}$ $\omega 6c$). Polar lipids include PG, PE, SQDG, PC and PME. DNA G+C content is 57.8 mol % (as determined by real-time PCR).

The type strain, $JG120-1^{T}$ (= KCTC 23107^T = JCM 16715^T), was isolated from the marine sediment of the East Sea, Korea.

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