Shimia haliotis sp. nov., a bacterium isolated from the gut of an abalone, *Haliotis discus hannai*

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A novel Gram-stain-negative, motile, rod-shaped bacterium, designated strain WM35^T, was isolated from the intestinal tract of an abalone, *Haliotis discus hannai*, which was collected from the northern coast of Jeju in Korea. The cells of the isolate grew optimally at 30 °C, pH 7, and with 3 % (w/v) NaCl. Based on 16S rRNA gene sequence similarity comparisons, strain WM35^T was grouped in the genus *Shimia* and was closely related to the type strains of *Shimia isoporae* (98.7 % similarity) and *Shimia marina* (97.8 % similarity). The major cellular fatty acids were summed feature 8 and $C_{16:0}$ 2-OH. Ubiquinone Q-10 was the predominant respiratory quinone. The polar lipids of strain WM35^T comprised phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, an unidentified aminolipid and an unidentified lipid. The DNA G+C content of the isolate was 53.8 mol%. DNA–DNA hybridization values indicated <16 % genomic relatedness with members of the genus *Shimia*. The physiological, chemical and genotypic analyses indicated that strain WM35^T represents a novel species of the genus *Shimia*, for which the name *Shimia haliotis* sp. nov. is proposed. The type strain is WM35^T (=KACC 17212^T=JCM 18870^T).

The abalone *Haliotis discus hannai*, known as Pacific abalone or Japanese abalone, is distributed throughout the coastal waters of East Asia. *H. discus hannai* is recognized as a valuable marine resource and the aquaculture of this species has been developed widely in Japan, China and Korea (Oakes & Ponte, 1996).

Gut microbes have important roles in the host physiology of abalone and they can affect the efficiency of aquaculture (Sawabe *et al.*, 2007). Infections or blooms of bacterial pathogens can cause mass mortalities in abalone. For example, *Vibrio harveyi* caused the mass mortality of *H. discus hannai* during August 2002 by producing haemolymphatic oedema throughout the major circulatory system (Sawabe *et al.*, 2007). However, the alginate-degrading bacteria that reside in the intestinal tract of abalone, such as *Vibrio halioticoli*, have important roles in algal polysaccharide catabolism (Tanaka *et al.*, 2003). Most of the bacteria isolated from *H. discus hannai* have been identified as alginate-degrading bacteria (Sawabe *et al.*, 1995). Thus, controlling the gut microbes may be an important factor that affects high feeding efficiencies. In a previous study, culture-independent methods were used to show that members of the *Vibrio* group were dominant in the gut microbiota of *H. discus hannai*, while members of the classes *Alphaproteobacteria*, *Epsilonproteobacteria*, *Mollicutes* and *Fusobacteria* were also present (Tanaka *et al.*, 2004).

The genus *Shimia*, which belongs to the family *Rhodobacteraceae* in the class *Alphaproteobacteria*, was first proposed by Choi & Cho (2006) and members are defined as Gram-negative, rod-shaped and strictly aerobic bacteria that contain ubiquinone-10 (Q-10) as the predominant quinone. At the time of writing, the genus *Shimia* comprised two species: *Shimia marina* from a coastal fish farm (Choi & Cho, 2006) and *Shimia isoporae* from a reef-building coral (Chen *et al.*, 2011).

During an investigation of the intestinal bacterial diversity of *H. discus hannai*, a novel bacterial strain was isolated and subjected to taxonomic investigation. Thus, this paper describes a novel strain representative of the genus *Shimia*, designated WM35^T, based on a polyphasic analysis.

To isolate the intestinal bacteria, an abalone sample was collected from the northern coast of Jeju in Korea. The homogenized intestine was serially diluted with 0.22 μm-filtered PBS buffer and spread on marine agar 2216 (MA; Difco) plates. An isolate, designated strain WM35^T, was incubated at 25 °C for 1 week and purified by repeated subculture. All of the physiological, chemical and genotypic

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WM35^T is KC196071.

Two supplementary figures are available with the online version of this paper.

analyses were conducted in triplicate at least. The isolate was stored at −80 °C as a suspension in marine broth 2216 (MB; Difco) containing 40 % (v/v) glycerol.

The maximum, minimum and optimum temperatures for growth were determined in MB by culturing cells at 4, 10, 15, 20, 25, 30, 37, 45, 55 and 65 °C. Growth in the presence of NaCl was tested using different concentrations of NaCl (0, 1, 2, 3, 4, 5, 8, 10, 12 and 15 %, w/v) in a medium that contained all of the ingredients of MB, except that NaCl was added at specific concentrations. The pH tolerance range and optimum for growth were tested at pH 4–11 (at intervals of 1.0 pH unit) by adjusting the pH of MB using 10 mM MES for pH 4–6, 10 mM TAPS for pH 7–8 and 10 mM Na_2HPO_4 for pH 9–11. The growth under each condition was determined by measuring the turbidity of cultures at 600 nm (OD_{600}) using a spectrophotometer (SYNERGY MX; BioTek) after 24 h, 48 h and 7 days of incubation. Growth in anaerobic conditions was examined after 7 days of cultivation at 37 °C on MA plates in an anaerobic chamber filled with a $N_2/CO_2/H_2$ (90 : 5 : 5) atmosphere. Strain WM35^T grew at 15–37 °C, pH 6–9, and with 0–5 % (w/v) NaCl. Optimal growth occurred at 30 °C, pH 7, and with 3 % (w/v) NaCl. Anaerobic growth of the isolate was not observed.

Gram staining was performed using a Gram staining kit (bioMérieux), according to the manufacturer's instructions. Light microscopy (Eclipse 50*i*; Nikon) was used to observe Gram staining and cell morphology. The motility of the isolate was determined in semi-solid MA containing 0.4 % agar (Tittsler & Sandholzer, 1936). Transmission electron microscopy (SUPRA VP55; Zeiss) was used to determine the presence of flagella. Cells of strain WM35^T were Gram-stain-negative, aerobic, rod-shaped (0.6–1.0 μm wide and 0.8–3.2 μm long), and motile with several monopolar flagella (Fig. S1, available in IJSEM Online). Colonies were circular, convex with an entire margin, beige or cream, and 0.6–0.9 mm in diameter after 72 h of culture on MA medium at 30 °C.

A phylogenetic analysis was conducted based on 16S rRNA gene sequences. The 16S rRNA gene of the isolate was amplified by colony PCR using a PCR Premix (*i*NtRon Biotechnology) with two universal bacterial primers: forward

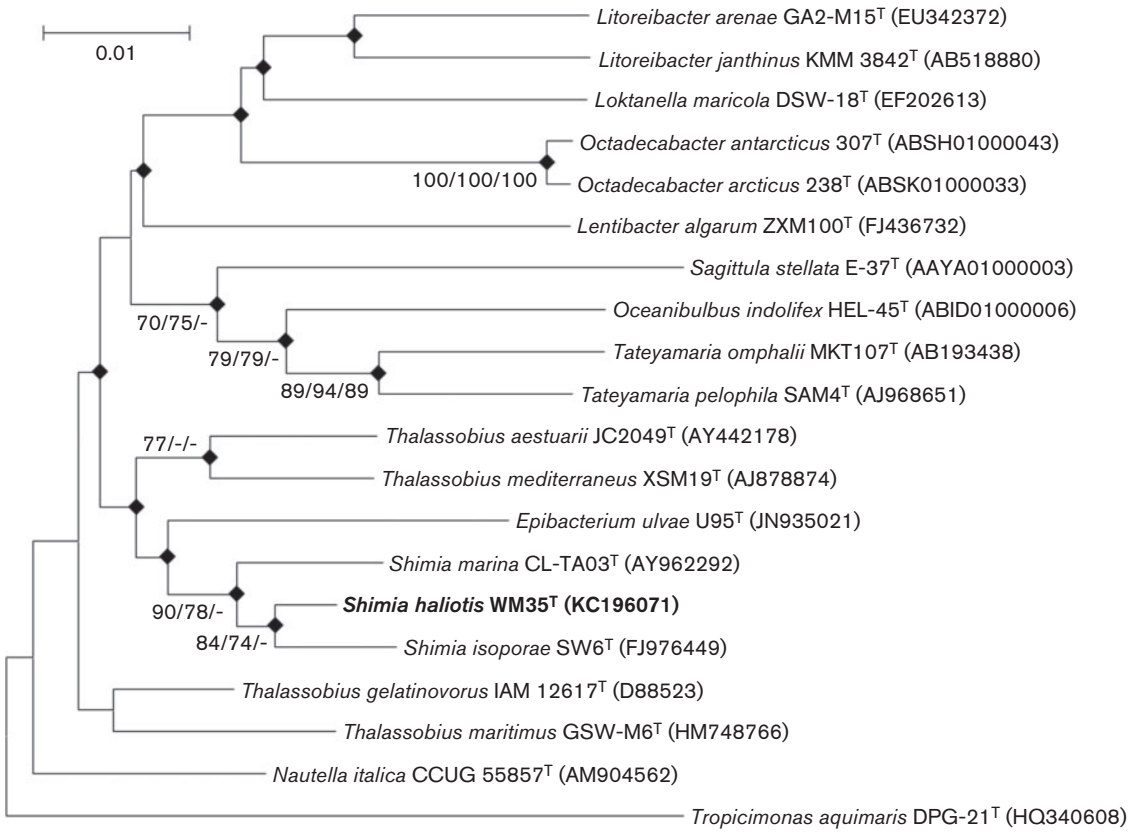

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequences, which was reconstructed using the neighbour-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) methods, showing the phylogenetic position of strain WM35^T with respect to recognized species in the genus *Shimia*. Filled diamonds indicate identical branches generated by all three methods. Numbers at the nodes represent bootstrap values (NJ/MP/ML) as percentages of 1000 replicates. *Tropicimonas aquimaris* DPG-21^T was used as an outgroup. Bar, 0.01 accumulated changes per nucleotide position.

primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-GGYTACCTTGTTACGACTT-3′) (Lane, 1991). The 16S rRNA gene amplicon was sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. The reaction mixtures were analysed using an automated DNA analyser (3730xl DNA Analyser; Applied Biosystems). The 16S rRNA gene sequences were assembled using SeqMan (DNASTAR). The assembled sequence of strain WM35^T was compared with type strains in the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim *et al.*, 2012). Thus, the strain was identified as a member of the genus *Shimia* in the class *Alphaproteobacteria*. The isolate shared the highest 16S rRNA gene sequence similarity with *S. isoporae* SW6^T (98.7 % similarity). The phylogenetic relationships between strain WM35^T and closely related species were determined using the 16S rRNA gene sequences. The sequences were aligned using the multiple sequence alignment program CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic consensus trees were reconstructed based on the aligned sequences using MEGA 5 (Tamura *et al.*, 2011) with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) algorithms based on 1000 bootstrap replicates. The phylogenetic consensus trees based on the 16S rRNA gene sequences indicated that strain WM35^T formed a cluster with other members of the genus *Shimia* (Fig. 1).

To facilitate a more comprehensive characterization of strain WM35^T, the type strains of *S. isoporae* LMG 25377^T (=SW6^T) and *S. marina* KCCM 42117^T (=CL-TA03^T) were obtained from the Belgian Co-ordinated Collections of Microorganisms/Laboratorium voor Microbiologie, Universiteit Gent (BCCM/LMG), and the Korean Culture Center of Microorganisms (KCCM), respectively, and used as reference strains.

Catalase and oxidase activities were tested based on bubble production with 3 % (v/v) hydrogen peroxide solution and indophenol blue production with 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux), respectively. The assimilation of sole carbon sources was determined using GN2 MicroPlates (Biolog) and GN/GP inoculating fluid (Biolog) supplemented with 2 % (w/v) NaCl for strain WM35^T, *S. isoporae* SW6^T and *S. marina* CL-TA03^T. Enzyme activities were assessed using API ZYM test strips (bioMérieux), according to the manufacturer's instructions. Nitrate reduction, indole production, D-glucose fermentation, the activity of arginine dihydrolase and urease, and the hydrolysis of aesculin, gelatin and 4-nitrophenyl-β-D-galactopyranoside (PNPG) were tested

Table 1. Differential characteristics of strain WM35^T and its closest phylogenetic relatives in the genus *Shimia*

Strains: 1, WM35^T; 2, *S. isoporae* LMG 25377^T; 3, *S. marina* KCCM 42117^T. All data were obtained from the current study except where indicated. +, Positive or weakly positive; −, negative.

Characteristic	1	2	3
Temperature range for growth (°C)	15–37	20–30[a]*	15–35[b]
Temperature optimum (°C)	30	25–30[a]	30–35[b]
NaCl range for growth (%, w/v)	0–5	1–5[a]	1–7[b]
pH range for growth	6–9	7–11[a]	6–10[b]
Assimilation of (Biolog GN2 MicroPlate):			
α-D-Glucose, melibiose, acetic acid, citric acid, propionic acid, L-asparagine, L-ornithine	+	−	−
D-Fructose, α-ketoglutaric acid, succinamic acid, L-glutamic acid, L-threonine	−	+	−
i-Erythritol, L-fucose, D-psicose, glucuronamide, L-leucine	−	−	+
Pyruvic acid methyl ester, succinic acid monomethyl ester, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketobutyric acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, glycyl L-glutamic acid, L-proline, L-serine, thymidine	+	+	−
Enzyme activities (API ZYM)			
Valine arylamidase, α-galactosidase, β-galactosidase	+	+	−
α-Chymotrypsin	+	−	−
α-Glucosidase	−	+	−
Hydrolysis of (API 20 NE):			−
Aesculin, urea	−	+ (−[a])	−
Gelatin	+	−	−
PNPG	+	+	−
Hydrolysis of (conventional tests):			
Tweens 40, 60 and 80	−	+ (−[a])	+
Reduction of nitrates to nitrites	+	+ (−[a])	−
DNA G+C content (mol%)	53.8	54.9[a]	57.2[b]

*Data from: *a*, Chen *et al.* (2011); *b*, Choi & Cho (2006).

using API 20NE test strips (bioMérieux). Hydrolysis of starch and casein were examined by incubating the novel isolate on MA medium supplemented with 0.5 % soluble starch (JUNSEI) and 5 % skimmed milk (Difco), respectively (Benson, 1994). Hydrolysis of Tweens 20, 40, 60 and 80 was determined according to the method of Goszczynska & Serfontein (1998). Strain WM35^T was catalase-positive and oxidase-positive but differed from *S. isoporae* SW6^T and *S. marina* CL-TA03^T because it could assimilate α-D-glucose, melibiose, acetic acid, citric acid, propionic acid, L-asparagine and L-ornithine (based on Biolog GN2 MicroPlate). The enzyme activity levels of strain WM35^T differed from the reference type strains with respect to valine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase and α-glucosidase (based on API ZYM), while strain WM35^T could also reduce nitrates to nitrites and hydrolyse gelatin and PNPG (based on API 20NE). Tween 20 was hydrolysed, whereas starch, casein and Tweens 40, 60 and 80 were not hydrolysed. The results of the biochemical tests are described in the species description, while the differences in the biochemical characteristics of the isolate and the reference species are presented in Table 1.

To facilitate the chemical characterization of strain WM35^T, its cellular fatty acid profile, isoprenoid quinone composition and polar lipid profile were compared with those of *S. isoporae* LMG 25377^T and *S. marina* KCCM 42117^T. Chemical analyses were conducted using the cell biomass of the isolate and the reference strains, which were cultured on MA plates at 30 °C for 48 h. The cellular fatty acids were extracted according to the protocol for the Sherlock Microbial Identification System (MIDI, 1999). The cellular fatty acid compositions of the isolate and the reference species were identified by gas chromatography (Agilent 6890 gas chromatograph; Agilent Technologies) and the Microbial Identification software package (Sherlock version 6.2) based on the TSBA6 database (Sasser, 1990). The major cellular fatty acids (>5 % of the total) in the novel isolate were summed feature 8 (71.4 %) and $C_{16:0}$ 2-OH (9.1 %), which were the same as those in the members of the genus *Shimia*. The complete fatty acid profiles of the isolate and the reference strains are presented in Table 2. The isoprenoid quinones were extracted according to the method of Collins & Jones (1981a). The extracts were purified by one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) and identified by HPLC (Ultimate 3000, Dionex) (Collins & Jones, 1981b) using a reversed-phase Hydrosphere C18 (150 × 2.0 mm, YMC) column. The main quinone of strain WM35^T was ubiquinone-10 (Q-10), which is also the dominant quinone in the two recognized species of the genus *Shimia* (Chen *et al.*, 2011; Choi & Cho, 2006). The polar lipid extract was separated by two-dimensional TLC on a silica gel 60 F_{254} plate (Merck). Two solvents were used for separation: chloroform/methanol/water (65 : 25 : 4, by vol.) for the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.) for the second dimension. Four spray reagents were applied to detect the polar lipids (Tindall, 1990): 10 % ethanolic molybdatophosphoric acid reagent for total lipids, ninhydrin reagent for amino-containing lipids, Zinzadze reagent for phospholipids and α-naphthol reagent for glycolipids. The phospholipids were identified by one-dimensional TLC using four standard compounds (Sigma): phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphoethanolamine (PE) and diphosphatidylglycerol (DPG). The polar lipids of strain WM35^T comprised PC, PG, DPG, an unidentified aminolipid (AL1) and an unidentified lipid (L1). The isolate shared PC, PG, DPG, an unidentified aminolipid (AL1) and an unidentified lipid (L1) with the two reference species of the genus *Shimia* (Fig. S2).

The DNA G+C content of strain WM35^T was measured and DNA–DNA hybridization with the two reference strains was conducted to determine the genotypic characteristics of the isolate. Genomic DNA was extracted from

Table 2. Cellular fatty acid contents (%) of strain WM35^T and the type strains of closely related species in the genus *Shimia*

Strains: 1, WM35^T; 2, *S. isoporae* LMG 25377^T; 3, *S. marina* KCCM 42117^T. All data were obtained from the current study. TR, Trace level (<0.5 %); −, not detected.

Fatty acid	1	2	3
Saturated acids			
$C_{10:0}$	TR	TR	TR
$C_{12:0}$	0.6	1.0	1.9
$C_{14:0}$	TR	TR	0.8
$C_{16:0}$	4.5	4.7	1.8
$C_{18:0}$	2.2	0.9	1.2
Unsaturated acids			
$C_{17:1}\omega 8c$	TR	−	TR
$C_{17:1}\omega 7c$	−	0.5	−
$C_{18:1}\omega 9c$	TR	TR	−
11-Methyl $C_{18:1}\omega 7c$	2.7	6.4	13.7
$C_{20:1}\omega 7c$	tr	−	TR
Hydroxy acids			
$C_{10:0}$ 3-OH	3.4	3.8	2.2
$C_{12:0}$ 2-OH	−	1.6	−
$C_{12:0}$ 3-OH	2.0	−	1.2
$C_{16:0}$ 2-OH	9.1	20.0	9.0
$C_{18:1}$ 2-OH	1.6	2.4	4.5
$C_{18:0}$ 2-OH	TR	−	0.5
Summed features*			
3	TR	TR	−
5	−	TR	−
7	−	0.7	0.8
8	71.4	56.2	61.4

*Summed features represent groups of two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 3 comprised $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$; summed feature 5 comprised $C_{18:2}\omega 6$, 9*c* and/or anteiso-$C_{18:0}$; summed feature 7 comprised $C_{19:1}\omega 6c$ and/or unknown ECL 18.446; summed feature 8 comprised $C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$.

Table 3. DNA–DNA hybridization values for strain WM35^T and the type strains of closely related species in the genus *Shimia* (based on reciprocal analyses)

Data are mean ± standard deviation (SD) of 6 determinations from this study.

Species	DNA–DNA hybridization (%) with:		
	1	2	3
1. Strain WM35^T	100.0 ± 0	9.5 ± 0.5	15.9 ± 0.6
2. *S. isoporae* LMG 25377^T	7.7 ± 0.5	100.0 ± 0	14.0 ± 0.4
3. *S. marina* KCCM 42117^T	12.6 ± 0.3	16.6 ± 0.9	100.0 ± 0

strain WM35^T, *S. isoporae* LMG 25377^T and *S. marina* KCCM 42117^T for the genotypic analyses, as described by Rochelle *et al.* (1992). The DNA G+C content of the novel isolate was estimated by a fluorimetric method with SYBR Gold I using the CFX96 Real-Time PCR Detection System (Bio-Rad) (Gonzalez & Saiz-Jimenez, 2002). The genomic DNA from *Bacteroides thetaiotaomicron* VPI-5482^T, *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3^T and *Bacteroides fragilis* NCTC 9343^T were used as calibration references in the analysis. The DNA G+C content of strain WM35^T was 53.8 mol%. To clarify the genetic relatedness between the isolate and the type strains of *S. isoporae* and *S. marina*, DNA–DNA hybridization was performed using a genome-probing microarray (Bae *et al.*, 2005; Chang *et al.*, 2008). For strain WM35^T, the levels of genetic relatedness to and *S. marina* KCCM 42117^T and *S. isoporae* LMG 25377^T were 12.6 ± 0.3 % (mean ± SD of 6 determinations) and 7.7 ± 0.5 %, respectively, while the values were 15.9 ± 0.6 % and 9.5 ± 0.5 %, respectively, in the reciprocal experiments (using strain WM35^T as the probe) (Table 3). The DNA–DNA hybridization values show that strain WM35^T represents a distinct genomic species (Wayne *et al.*, 1987).

Based on the physiological, biochemical and genotypic analyses, it is suggested that strain WM35^T represents a novel species in the genus *Shimia*, for which the name *Shimia haliotis* sp. nov. is proposed.

Description of *Shimia haliotis* sp. nov.

Shimia haliotis (ha.li.o′tis. N.L. gen. n. *haliotis* of Haliotis, the systematic name of a genus of abalone, isolated from *Haliotis discus hannai*).

Cells are Gram-stain-negative, obligately aerobic, motile, rod-shaped (0.6–1.0 × 0.8–3.2 μm), catalase-positive and oxidase-positive. Colonies on MA medium are circular, convex with an entire margin, beige or cream coloured, and 0.6–0.9 mm in diameter after 3 days at 30 °C. Growth occurs at 15–37 °C (optimum 30 °C), pH 6–9 (optimum pH 7), and with 0–5 % (w/v) NaCl (optimum 3 %). Assimilates α-D-glucose, melibiose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, citric acid, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketobutyric acid, DL-lactic acid, propionic acid, succinic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, glycyl L-glutamic acid, L-ornithine, L-proline, L-serine and thymidine (Biolog GN2). Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase and β-galactosidase (API ZYM). Reduces nitrate to nitrite and hydrolyses gelatin, PNPG (API 20 NE) and Tween 20. The major fatty acids are summed feature 8 and $C_{16:0}$ 2-OH. The predominant ubiquinone is Q-10. The polar lipids comprise phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, an unidentified aminolipid and an unidentified lipid.

The type strain, WM35^T (=KACC 17212^T=JCM 18870^T), was isolated from the intestinal tract of an abalone, *Haliotis discus hannai*, which was collected from the northern coast of Jeju in Korea. The DNA G+C content of the type strain is 53.8 mol%.

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