Actibacter haliotis sp. nov., isolated from the gut of an abalone, *Haliotis discus hannai*, and emended description of the genus *Actibacter*

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A novel strain, designated strain W113^T, was isolated from the gut of an abalone, *Haliotis discus hannai*, which was collected from the northern coast of Jeju in Korea. The isolate was a Gram-staining-negative, facultatively anaerobic, non-motile, rod-shaped bacterium producing yellow-to-orange carotenoid-type pigments. 16S rRNA gene sequence analysis showed that the isolate belonged to the genus *Actibacter* in the family *Flavobacteriaceae* and it shared the highest sequence similarity with the type strain of *Actibacter sediminis* (98.8 % similarity). Optimal growth occurred at 25 °C, at pH 7 and with 2 % (w/v) NaCl. The major cellular fatty acids were iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{15:1}$ G. Menaquinone-6 was the main respiratory quinone. The polar lipids of the isolate were phosphatidylethanolamine, three unidentified amino lipids, and three unidentified lipids. The genomic DNA G+C content was 42.6 mol% and DNA–DNA hybridization values indicated that the strain shared <18 % genomic relatedness with the most closely related species. The results of the phylogenetic, phenotypic and genotypic analyses indicated that strain W113^T represents a novel species in the genus *Actibacter*, for which the name *Actibacter haliotis* sp. nov. is proposed. The type strain is W113^T (=KACC 17209^T=JCM 18868^T).

The microbes present in abalone may affect its physiology (Tanaka *et al.*, 2003) and the efficiency of abalone aquaculture (Sawabe *et al.*, 2007). The abalone *Haliotis discus hannai* is recognized as a valuable marine resource in East Asia. In our previous investigations of the gut microbes of *H. discus hannai*, we isolated and characterized novel strains that belong to diverse taxa: *Shimia haliotis*, *Pseudoruegeria haliotis* (class *Alphaproteobacteria*), *Cloacibacterium haliotis* (family *Flavobacteriaceae*) and *Actinomyces haliotis* (phylum *Actinobacteria*) (Hyun *et al.*, 2013a, b, 2014a, b). During this ongoing study of abalone microbes, we isolated a novel *Actibacter*-like strain, designated strain W113^T. This paper describes the taxonomic characterization of this novel bacterial isolate based on a polyphasic analysis, which suggests that the isolate represents a novel species.

The genus *Actibacter* in the family *Flavobacteriaceae* was first introduced by Kim *et al.* (2008) and it comprises Gram-staining-negative, aerobic, rod-shaped bacteria. At present, the genus *Actibacter* comprises one species, *Actibacter sediminis*, which was isolated from a tidal flat sediment (Kim *et al.*, 2008).

The abalone *H. discus hannai* was sampled from the northern coast of Jeju in Korea. To isolate intestinal bacteria from the abalone, the intestinal tract was detached, homogenized and subjected to serial dilution using sterile PBS buffer. The sample dilutions were inoculated onto marine agar 2216 (MA; Difco) and incubated at 15, 20, 25 and 30 °C. Strain W113^T was isolated from the 10^{-1}-diluted specimen after incubation at 25 °C for 72 h. To obtain a pure isolate, subculture was performed at least five times. The purified isolate was stored at −80 °C as a suspension in marine broth 2216 (MB; Difco) containing 40 % (v/v) glycerol. All of the physiological, biochemical, chemotaxonomic and genotypic analyses were performed according to the proposed minimal standards for the description of new taxa in the family *Flavobacteriaceae* (Bernardet *et al.*, 2002), and all tests were repeated at least three times.

A phylogenetic analysis was performed based on 16S rRNA gene sequences. The 16S rRNA gene sequence of the isolate was amplified using Ex *Taq* PCR premix (Takara) with a universal bacterial primer pair: forward primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain W113^T is KC193210.

One supplementary figure is available with the online Supplementary Material.

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 sequence fragments were assembled using SeqMan 5.0 (DNASTAR) and compared with the sequences of type strains in the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim *et al.*, 2012). This comparison of the 16S rRNA gene sequences identified strain W113^T as a member of the genus *Actibacter* in the family *Flavobacteriaceae*. The closest relatives of the isolate were *Actibacter sediminis* KCTC 12704^T (98.8 % similarity), *Lutimonas vermicola* IMCC1616^T (95.7 % similarity) and *Aestuariicola saemankumensis* SMK-142^T (95.2 % similarity). A phylogenetic tree was reconstructed to determine the phylogenetic relationships between the isolate and closely related species. The sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) and used to reconstruct phylogenetic trees with MEGA 6 (Tamura *et al.*, 2013). Phylogenetic distances were calculated using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms based on 1000 bootstrap replicates. The phylogenetic consensus tree indicated that the isolate formed a cluster with *Actibacter sediminis*, which is the sole species in the genus *Actibacter* (Fig. 1).

Suitable growth conditions for strain W113^T were tested at different temperatures, pH levels, and salinities. The isolate was incubated in MB at 4, 10, 15, 20, 25, 30, 37, 40, 45, 55 and 65 °C and at pH 4–11 (at intervals of 1.0 pH unit). The adjustment of the pH of MB was achieved using 10 mM MES (for pH 4–6), 10 mM TAPS (for pH 7–8) or 10 mM Na_2HPO_4 (for pH 9–11). The isolate was incubated in a medium that comprised all of the components of MB except NaCl, which was then supplemented at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 15 % (w/v) NaCl. The growth under each condition was determined by measuring the turbidity of each culture at 600 nm (OD_{600}) using a spectrophotometer (Synergy MX, BioTek) after 24 h, 48 h and 7 days of culture. A precipitate formed in MB 2216 after autoclaving due to the presence of ferric citrate, which is a component of MB. To avoid false-positive results due to the presence of the precipitate in the medium, the turbidity of the MB was measured after autoclaving without cell inoculation and used as the baseline level. To obtain clear results in the growth test, ranges for growth were confirmed by colony formation on MA plates adjusted to the different conditions. Anaerobic growth of the isolate was tested after 7 days of cultivation at 25 °C on MA plates in an anaerobic chamber filled with a $N_2/CO_2/H_2$ atmosphere (90 : 5 : 5, by vol.). Strain W113^T grew at 15–30 °C, pH 6–8 and in the presence of 1–4 % (w/v) NaCl. The optimal growth conditions were 25 °C, pH 7 and 2 % (w/v) NaCl. Anaerobic growth was observed. Unless stated otherwise, all of the tests used to characterize the isolate were conducted under optimal growth conditions.

Cell morphology, colony appearance and Gram staining were determined using cells of strain W113^T after growth on MA under the optimal growth conditions for 48 h. Gram staining was performed using a Gram staining kit (bioMérieux), according to the manufacturer's instructions. Gram staining and cell morphology were observed under a light microscope (Eclipse 50*i*, Nikon). The motility of the isolate was assessed using two methods: a semi-solid agar method using MA containing 0.4 % agar (Tittsler & Sandholzer, 1936) and a gliding motility method using MB (Bernardet *et al.*, 2002). The presence of flexirubin-type pigments was determined using 20 % (w/v) KOH by flooding the cell mass harvested from agar plates (Bernardet *et al.*, 2002). To determine the presence of carotenoid-type pigments, the cellular pigments were extracted according to the method of Schmidt *et al.* (1994). The extracted cellular pigments were analysed by measuring the absorption spectrum using a UV–visible spectrophotometer (Synergy MX, BioTek). Cells of strain W113^T were non-motile, Gram-staining-negative rods (0.7–1.0 μm wide and 2.2–3.8 μm long), and they formed circular and convex colonies with an entire margin, which were viscous and yellow–orange with a diameter of 0.7–1.3 mm on MA. The isolate was negative for flexirubin-type pigments. The absorbance spectrum of the cellular pigments extracted from strain W113^T had a triple-peak signature, which is characteristic of carotenoid-type pigments (Schmidt *et al.*, 1994).

Biochemical characteristics, i.e. enzyme activities, hydrolytic activities, utilization of various sole carbon sources and acid production from various carbohydrates, were characterized for strain W113^T based on comparisons with the reference strain *Actibacter sediminis* KCTC 12704^T. The biochemical tests were conducted using the cell masses of the isolate and the reference strain after cultivation on MA under optimal growth conditions for 48 h. Catalase and oxidase activities were assessed on the basis of bubble production with 3 % (v/v) hydrogen peroxide solution and indophenol blue production with 1 % (w/v) tetramethyl-*p*-phenylenediamine (bioMérieux), respectively. Enzyme activities were tested using API ZYM and API 20NE test strips (bioMérieux), according to the manufacturer's instructions with the minor modification that 2 % (w/v) NaCl in demineralized water was used as the inoculation fluid. DNase and lipase activities were tested based on the formation of a clearance zone on 1.5 % (w/v) NaCl-supplemented DNase test agar (Difco) and 2 % (w/v) NaCl-supplemented Spirit blue agar (Difco) with lipase reagent, respectively. Hydrolysis of various substrates was tested using MA medium supplemented with each test substrate: 0.5 % (w/v) soluble starch (Junsei) (Benson, 1994), 5 % (w/v) skimmed milk (Difco) for casein (Benson, 1994), 1 % (w/v) Tweens 20, 40, 60 and 80 (Goszczynska & Serfontein, 1998), and 0.5 g tyrosine l^{-1} and 1 % (w/v) CM-cellulose (Sigma) (Teather & Wood, 1982). The hydrolytic activities were determined based on the formation of clearance zones around colonies (starch, casein, tyrosine and CM-cellulose), or turbid zones around colonies

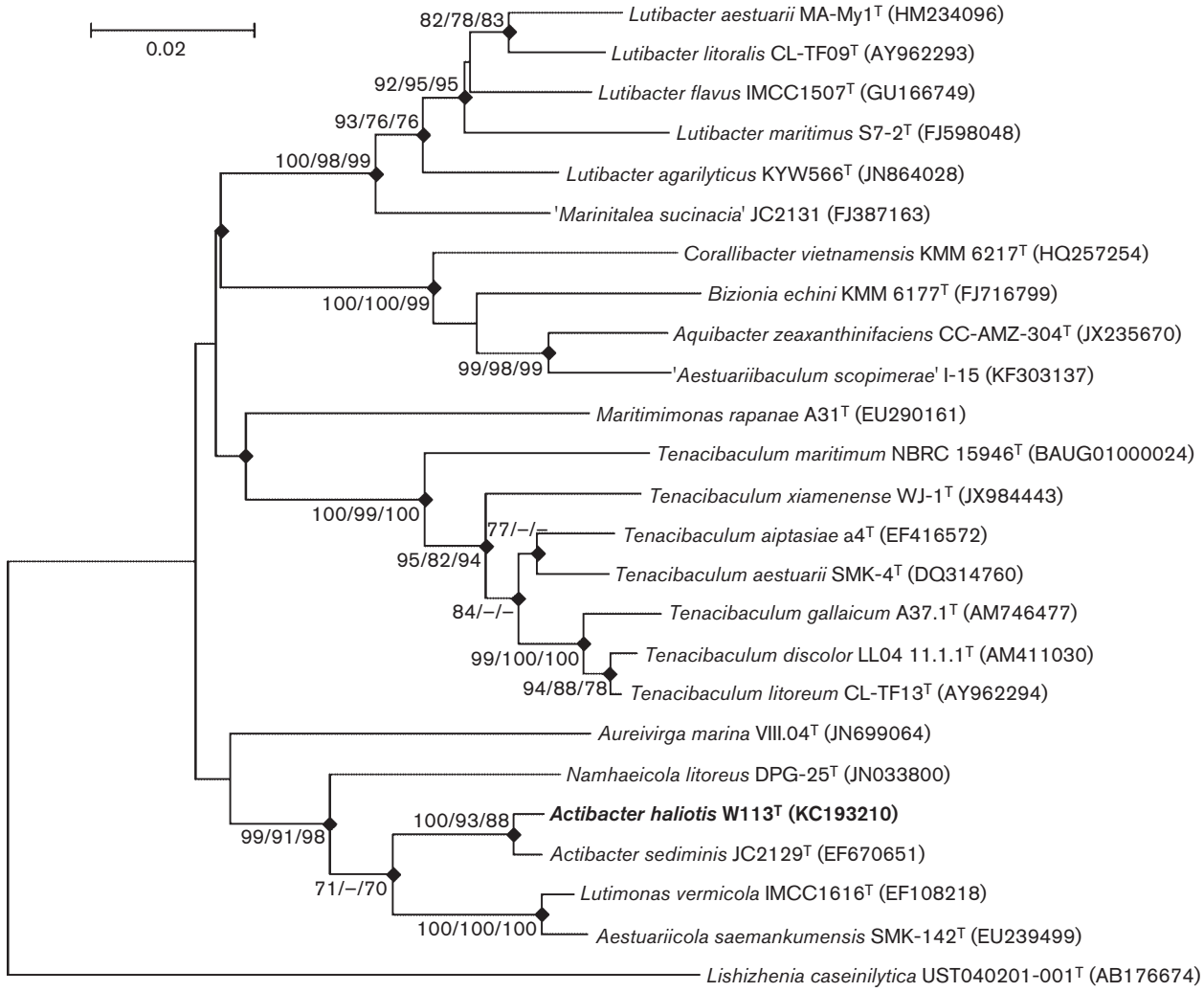

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, which shows the phylogenetic positions of strain W113^T and closely related species. *Lishizhenia caseinilytica* UST040201-001^T was used as an outgroup. Filled diamonds represent identical branches generated using all three algorithms: neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML). Numbers at nodes indicate bootstrap values (NJ/MP/ML) as percentages of 1000 replicates. Values <70 % are not shown at branch points. Bar, 0.02 accumulated changes per nucleotide position.

(Tweens 20, 40, 60 and 80). Citrate hydrolysis and H_2S production were determined using 1.5 % (w/v) NaCl-supplemented Simmons' citrate agar (Difco) and 1.5 % (w/v) NaCl-supplemented Klinger iron agar (BBL), respectively. Chitin hydrolysis was assessed using the methods of Leisner *et al.* (2008) and Teather & Wood (1982). The utilization of sole carbon sources was tested using GN2 MicroPlates (Biolog) with GN/GP inoculating fluid (Biolog) supplemented with 2 % (w/v) NaCl, according to the manufacturer's instructions. Acid production from various carbohydrates was determined using API 50 test strips (bioMérieux) after culture in 50 CHB/E medium supplemented with 2 % (w/v) NaCl. Strain W113^T was positive for catalase and oxidase, but it differed from *Actibacter sediminis* in its biochemical features. The differences in the biochemical characteristics between the novel isolate and the reference strain are listed in Table 1. The results of the biochemical tests for the novel isolate are detailed in the species description.

Chemotaxonomic characteristics, i.e. cellular fatty acid profile, isoprenoid quinones, and polar lipid composition, were characterized for strain W113^T based on comparisons with the reference strain. The chemotaxonomic analyses were performed using cell masses of the isolate and the reference strain after cultivation on MA under optimal growth conditions for 48 h. The cellular fatty acids were saponified, methylated and extracted, according to the protocol for the Sherlock Microbial Identification System (MIDI, 1999). The cellular fatty acid profiles of the isolate and the reference strain were determined using GC (Agilent

Table 1. Differential characteristics of strain W113^T and *Actibacter sediminis* KCTC 12704^T

Strains: 1, W113^T; 2, *Actibacter sediminis* KCTC 12704^T. All data were obtained from the current study, except where indicated. +, Positive or weakly positive; −, negative.

Characteristic	1	2
Temperature for growth (°C)		
Range	15–30	5–37 (5–45*)
Optimum	25	30 (37*)
pH for growth		
Range	6–8	5–8
Optimum	7	7 (6*)
NaCl concentration for growth (%, w/v)		
Range	1–4	1–8 (1–10*)
Optimum	2	1–3
Anaerobic growth	+	−
Enzyme activities (API ZYM/20NE)		
Cystine arylamidase, trypsin, β-glucuronidase, urease	+	−
α-Chymotrypsin	+	− (+*)
Acid production from (API 50 CHB):		
D-Mannitol, amygdalin	+	−
D-Arabinose, L-arabinose, L-xylose, L-fucose	−	+
Utilization of (Biolog GN2 microplate):		
D-Arabitol, α-lactose, D-mannitol, D-psicose, raffinose, pyruvic acid methyl ester, D-alanine, L-serine, uridine, thymidine, 2,3-butanediol, glycerol	+	−
Succinic acid, L-phenylalanine, L-proline	−	+
Lipase activity (Spirit blue agar)	+	−
DNA G+C content (mol%)	42.6	43–45*
Isolation source	Abalone	Tidal flat*

*Data from Kim *et al.* (2008).

6890 gas chromatograph, Agilent Technologies) and the Microbial Identification software package (Sherlock version 6.2) with the TSBA6 database (Sasser, 1990). The major cellular fatty acids (>10 % of the total) of the isolate were iso-$C_{15:0}$ (17.6 %), anteiso-$C_{15:0}$ (16.2 %) and iso-$C_{15:1}$ G (12.4 %). The fatty acids iso-$C_{15:0}$ and iso-$C_{15:1}$ G were also present in the reference strain, although anteiso-$C_{15:0}$ was not predominant in the reference strain (2.7 %). The complete fatty acid profiles of the test strains are presented in Table 2. Isoprenoid quinones were extracted from strain W113^T according to the method of Collins & Jones (1981a). The isoprenoid quinone extract was purified by one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) and identified by HPLC (Collins & Jones, 1981b) using a reverse-phase Hydrosphere C18 (150 × 2.0 mm) column. A more comprehensive analysis of the isoprenoid quinones was performed by liquid chromatography (Ultimate 3000, Dionex) using an ion trap mass spectrometer, which was equipped with an electrospray ionization probe (HCT, Bruker), according to the method of Kaiser *et al.* (2012). The sole respiratory quinone in strain W113^T was menaquinone-6 (MK-6), which is either the sole or major respiratory quinone in all members of the family *Flavobacteriaceae* (Bernardet, 2011). MK-7, which was detected in *Actibacter sediminis* KCTC 12704^T (Kim *et al.*, 2008), was not detected in the novel isolate. Polar lipids were extracted from strain W113^T and the reference strain according to the method of Xin *et al.* (2000), and separated by two-dimensional TLC on a silica gel 60 F_{254} plate (Merck). During two-dimensional separation, chloroform/methanol/water (65 : 25 : 4, by vol.) was used in the primary dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.) in the secondary dimension. Four spray reagents were used to detect the polar lipids (Tindall, 1990): 5 % ethanolic molybdatophosphoric acid for total lipids, ninhydrin for amino-group-containing lipids, Zinzadze's reagent for phospholipids and α-naphthol for glycolipids. To identify the phospholipids in the isolate, one-dimensional TLC with chloroform/methanol/acetic acid/water (50 : 6 : 6 : 1, by vol.) was performed using four standard compounds (Sigma), as follows: phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine (PE) and diphosphatidylglycerol. The polar lipids of strain W113^T comprised PE, three unidentified amino lipids and three unidentified lipids, which was a similar profile to the type strain of *Actibacter sediminis*, except for the number of unidentified lipids (Fig. S1, available in the online Supplementary Material).

Genotypic characteristics, i.e. the DNA G+C content and DNA–DNA hybridization, were characterized for strain

Table 2. Cellular fatty acid contents of strain W113^T and *Actibacter sediminis* KCTC 12704^T

Strains: 1, W113^T; 2, *Actibacter sediminis* KCTC 12704^T. All data were obtained from the current study. tr, Trace level (<1.0 %); –, not detected

Fatty acid (%)	1	2
Saturated acids		
$C_{16:0}$	1.4	1.9
$C_{18:0}$	1.3	1.3
Unsaturated acids		
$C_{15:1}\omega6c$	3.8	6.3
$C_{17:1}\omega6c$	tr	2.0
Branched acids		
iso-$C_{13:0}$	4.3	7.1
iso-$C_{15:1}$ G	12.4	17.7
iso-$C_{15:0}$	17.6	28.5
anteiso-$C_{15:0}$	16.2	2.4
iso-$C_{15:0}$ 3-OH	3.7	5.9
$C_{15:0}$ 2-OH	tr	1.2
$C_{15:0}$ 3-OH	tr	1.3
iso-$C_{17:0}$	1.4	tr
anteiso-$C_{17:0}$	8.3	–
iso-$C_{17:0}$ 3-OH	6.5	9.9
anteiso-$C_{19:0}$	2.9	–
Summed features*		
3	6.7	5.3
4	1.2	–
9	3.4	2.9

*Summed features refer to groups of two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 3 comprises $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 4 comprises iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B; summed feature 9 comprises iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$.

W113^T based on comparisons with the reference strain. Genomic DNA was extracted from strain W113^T and *Actibacter sediminis* KCTC 12704^T according to the method of Rochelle *et al.* (1992). The DNA G+C content of the isolate was estimated by a fluorimetric method with SYBR Gold I (Invitrogen) using the CFX96 Real-Time PCR Detection System (Bio-Rad) (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA extracts 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 W113^T was 42.6 mol%. To clarify the genetic relatedness between the isolate and the reference strain, DNA–DNA hybridization was performed using a genome-probing microarray (Bae *et al.*, 2005; Chang *et al.*, 2008). The DNA–DNA relatedness values were calculated based on the signal-to-noise ratios of the genomic probes (Loy *et al.*, 2005). The DNA–DNA relatedness between strain W113^T and the type strain of *Actibacter sediminis* was 17.1±2.8 % (6.9±3.3 % reciprocal). Values were calculated to include the results of a control (0.04±0.02 %). These results indicate that strain W113^T is a distinct genomic species (Wayne *et al.*, 1987).

The physiological, biochemical, chemotaxonomic and genotypic analyses conducted in this study suggest that strain W113^T represents a novel species within the genus *Actibacter*, for which the name *Actibacter haliotis* sp. nov. is proposed.

Emended description of genus *Actibacter*

The description of the genus is as given previously by Kim *et al.* (2008) but with the following amendments. Cells are aerobic or facultatively anaerobic. The major polar lipids are phosphatidylethanolamine and three unidentified amino lipids. Unidentified lipids also occur.

Description of *Actibacter haliotis* sp. nov.

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

Cells are Gram-staining-negative, facultatively anaerobic, non-motile, rod-shaped (0.7–1.0 μm × 2.2–3.8 μm), catalase-positive and oxidase-positive. On MA, colonies are circular and convex with an entire margin, viscous and yellow–orange, with a diameter of 0.7–1.3 mm after 48 h of incubation at 25 °C. Yellow–orange carotenoid-type pigments are produced, whereas flexirubin-type pigments are not. Growth occurs at 15–30 °C (optimum 25 °C), at pH 6–8 (optimum pH 7) and with 1–4 % (w/v) NaCl (optimum 2 %). Sodium ions are an absolute requirement for growth. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, cystine arylamidase, trypsin, β-glucuronidase, α-chymotrypsin (API ZYM), urease, D-glucose fermentation (API 20NE), and lipase (Spirit blue agar). Aesculin, *p*-nitrophenyl-β-D-galactopyranoside (API 20NE), Tween 20, Tween 40, Tween 60, Tween 80 and starch are hydrolysed. Acid is produced from D-ribose, D-xylose, methyl β-D-xyloside, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, starch, glycogen, gentiobiose, turanose, D-tagatose, 5-ketogluconate, D-mannitol and amygdalin (API 50 CHB). Utilizes dextrin, glycogen, *N*-acetyl-D-glucosamine, cellobiose, D-fructose, gentiobiose, α-D-glucose, maltose, D-mannose, methyl β-D-glucoside, sucrose, trehalose, turanose, succinic acid monomethyl ester, acetic acid, α-ketobutyric acid, DL-lactic acid, L-alaninamide, L-alanine, L-alanylglycine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-ornithine, L-threonine, DL-α-glycerol phosphate, D-arabitol, α-lactose, D-mannitol, D-psicose, raffinose, pyruvic acid methyl ester, D-alanine, L-serine, uridine, thymidine, 2,3-butanediol and glycerol (Biolog GN2). The major fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$ and iso-$C_{15:1}$ G. The sole ubiquinone is MK-6.

The polar lipids comprise phosphatidylethanolamine, three unidentified amino lipids and three unidentified lipids.

The type strain, W113^T (=KACC 17209^T=JCM 18868^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 42.6 mol%.

Acknowledgements

This work was supported by grants from the Mid-career Researcher Program through the National Research Foundation of Korea (2011-0028854) and the National Institute of Biological Resources funded by the Ministry of Environment (MOE) of Korea (NIBR no. 2013-02-001). We thank Professor J. P. Euzéby (École Nationale Vétérinaire, France) for etymological advice and Dr Seong Woon Roh (Jeju Center, Korea Basic Science Institute, Korea) for analysing the isoprenoid quinones.

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