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

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A novel, Gram-staining-positive, facultatively anaerobic, non-motile and coccus-shaped bacterium, strain WL80^T, was isolated from the gut of an abalone, *Haliotis discus hannai*, collected from the northern coast of Jeju in Korea. Optimal growth occurred at 30 °C, pH 7–8 and with 1 % (w/v) NaCl. Phylogenetic analyses based on the 16S rRNA gene sequence revealed that strain WL80^T fell within the cluster of the genus *Actinomyces*, with highest sequence similarity to the type strains of *Actinomyces radicidentis* (98.8 % similarity) and *Actinomyces urogenitalis* (97.0 % similarity). The major cellular fatty acids were $C_{18:1}\omega9c$ and $C_{16:0}$. Menaquinone-10 (H_4) was the major respiratory quinone. The genomic DNA G+C content of the isolate was 70.4 mol%. DNA–DNA hybridization values with closely related strains indicated less than 7.6 % genomic relatedness. The results of physiological, biochemical, chemotaxonomic and genotypic analyses indicated that strain WL80^T represents a novel species of the genus *Actinomyces*, for which the name *Actinomyces haliotis* sp. nov. is proposed. The type strain is WL80^T (=KACC 17211^T=JCM 18848^T).

The genus *Actinomyces* is the type genus of the order *Actinomycetales* within the phylum *Actinobacteria*. This genus comprises a broad spectrum of anaerobic or facultatively anaerobic, non-spore-forming, non-motile, Gram-staining-positive micro-organisms with a high DNA G+C content (Schaal, 1986). At the time of writing, the genus *Actinomyces* contained 41 recognized species (see http://www.bacterio.net/a/actinomyces.html). Common habitats of members of the genus *Actinomyces* include the mucous membranes of humans and animals, particularly the oral mucosa or urogenital and intestinal tracts (Acevedo *et al.*, 2008). In recent years, several novel species of the genus *Actinomyces* have been isolated from the oral cavity of a dog (Hijazin *et al.*, 2012), a human clinical osteoarticular sample (Renvoise *et al.*, 2010) and a wound swab from a human patient (Funke *et al.*, 2010).

The intestinal microbes of the abalone, *Haliotis discus hannai*, are known to be closely linked to the abalone's physiology (Tanaka *et al.*, 2003) and the efficiency of abalone aquaculture (Sawabe *et al.*, 2007). During an attempt to investigate the intestinal bacterial diversity of *H. discus hannai*, a novel bacterial strain, designated strain WL80^T, was isolated. The new strain was then subjected to taxonomic characterization based on a polyphasic analysis.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WL80^T is KC196072.

The abalone *H. discus hannai* was sampled from the northern coast of Jeju in Korea. For isolation of the intestinal bacteria, the intestinal tract was detached, homogenized and serially diluted with 0.22 μm filtered PBS buffer. Diluted samples (by 10^{-1}, 10^{-2} and 10^{-3}) were inoculated by spreading on brain heart infusion (BHI, Bacto) agar plates and incubated at different temperatures (20, 25 and 30 °C). Strain WL80^T was isolated from a 10^{-2}-diluted sample after incubation at 30 °C for 72 h. The isolate was purified by repeated subculture. The purified isolate was stored at −80 °C as a suspension in BHI containing 40 % (v/v) glycerol. All of the physiological, biochemical, chemotaxonomic and genotypic analyses were repeated at least three times.

Gram-staining, cell morphology and colony appearance were tested with cells grown on BHI agar plates at 30 °C for 48 h. Gram-staining was performed with a Gram stain kit (bioMérieux) according to the manufacturer's instructions. Gram-staining and cell morphology were observed by light microscopy (Eclipse 50*i*, Nikon). Motility of the isolate was assessed according to the methods of Tittsler & Sandholzer (1936). Cells of strain WL80^T were Gram-staining-positive, non-motile and coccus-shaped (0.7–1.1 μm in diameter). The isolate formed ivory coloured, convex, circular colonies, 0.6–1.2 mm in diameter, with entire margins. The minimum, maximum and optimum temperatures for growth were tested in BHI at different temperatures (4, 10, 15, 20, 25, 30, 37, 45, 55 and 65 °C). The pH tolerance

range and optimum pH for growth were tested at pH 4–11 (at intervals of 1.0 pH unit), with the pH of BHI adjusted 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. Growth in the presence of different NaCl concentrations (0, 1, 2, 3, 4, 5, 8, 10, 12 and 15 %, w/v) was tested in BHI containing all constituents except NaCl, with NaCl then added to yield the appropriate concentration. The growth under each condition was determined by measuring the turbidity of each culture at OD_{600} using a spectrophotometer (Synergy MX, BioTek) after 24 h, 48 h and 7 days of incubation. Anaerobic growth of the isolate was examined after 7 days of cultivation at 37 °C on BHI agar plates in an anaerobic chamber filled with $N_2/CO_2/H_2$ (90 : 5 : 5). Growth of strain WL80^T was observed at 20–37 °C, pH 6–9 and with 0–4 % (w/v) NaCl. Optimum growth conditions were at 30 °C, pH 7–8 and with 1 % (w/v) NaCl. Anaerobic growth of the isolate was observed. Unless stated otherwise, all tests characterizing strain WL80^T were conducted under optimal growth conditions.

A phylogenetic analysis was performed based on the 16S rRNA gene sequences. The 16S rRNA gene sequence of strain WL80^T was amplified by colony PCR using a PCR Premix (*i*NtRon Biotechnology) with two universal bacterial primers: forward 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), and the assembled sequence of strain WL80^T was compared with other sequences of type strains in the EzTaxon-e server (Kim *et al.*, 2012). The results of the 16S rRNA gene sequence similarity analysis identified strain WL80^T as a member of the genus *Actinomyces* in the family *Actinomycetaceae*. The closest relatives of the isolate were *Actinomyces radicidentis* DSM 15433^T (98.8 % similarity) and *Actinomyces urogenitalis* DSM 15434^T (97.0 % similarity). The phylogenetic relationships between strain WL80^T and closely related species were determined based on 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 (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms based on 1000 bootstrap replicates. On the basis of the 16S rRNA gene sequences, phylogenetic consensus trees indicated that strain WL80^T was a member of a monophyletic branch containing the recognized species of the genus *Actinomyces* (Fig. 1). To perform a more comprehensive characterization of strain WL80^T, we selected *A. radicidentis* DSM 15433^T (Collins *et al.*, 2000) and *A. urogenitalis* DSM 15434^T (Nikolaitchouk *et al.*, 2000) as reference species. The type strains of *A. radicidentis* (DSM 15433^T) and *A. urogenitalis* (DSM 15434^T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

To characterize the biochemical properties of strain WL80^T, enzyme activities, assimilation of sole carbon sources and acid production from various carbohydrates were compared with those of the type strains of *A. radicidentis* and *A. urogenitalis*. The biochemical assays were performed after cultivation of strain WL80^T and the reference strains under optimum growth conditions for 48 h on BHI agar medium. 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. Acid production from carbohydrates was examined using API 50 CH test strips (bioMérieux) with 50 CHB/E medium, according to the manufacturer's instructions. Utilization of various sole carbon sources was determined using GP2 MicroPlates (Biolog) and GN/GP inoculating fluid (Biolog). The enzyme activities were assessed using API ZYM test strips (bioMérieux), according to the manufacturer's instructions. Strain WL80^T could be distinguished from *A. radicidentis* DSM 15433^T and *A. urogenitalis* DSM 15434^T by its ability/inability to produce acid from D-arabinose, L-xylose, methyl β-D-xyloside, L-rhamnose, inositol, D-mannitol, methyl α-D-mannoside, *N*-acetylglucosamine, inulin and L-fucose (based on API 50 CH). It was further distinguished by its ability/inabiliy to assimilate α-cyclodextrin, β-cyclodextrin, glycogen, inulin, Tween 40, Tween 80, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, L-fucose, D-gluconic acid, D-mannitol, melezitose, methyl α-D-galactoside, methyl β-D-galactoside, D-xylose, α-hydroxybutyric acid, D-lactic acid methyl ester, L-asparagine, adenosine-5′-monophosphate, thymidine-5′-monophosphate, uridine-5′-monophosphate and D-L-α-glycerol phosphate (based on Biolog GP2 MicroPlate) and enzyme activity for esterase (C4), valine arylamidase, cystine arylamidase, *N*-acetyl-β-glucosaminidase and α-mannosidase (based on API ZYM). The isolate was catalase-positive and oxidase-negative. The results of the biochemical tests are described in the species description, and the differences in the biochemical characteristics of the isolate and the reference strains are shown in Table 1.

To identify the chemotaxonomic characteristics of strain WL80^T, its cellular fatty acid profile and isoprenoid quinone compositions were compared with those of the reference species. Chemotaxonomic analyses were conducted using the cell biomass of the isolate and the reference strains cultured on BHI agar plates at 30 °C for 48 h. The cellular fatty acids were extracted according to the protocol of the Sherlock Microbial Identification System (MIDI, 1999). The cellular fatty acid compositions of the isolate and the reference species were identified by GC (Agilent 6890, Agilent Technologies) and the Microbial Identification software package (Sherlock version 6.2)

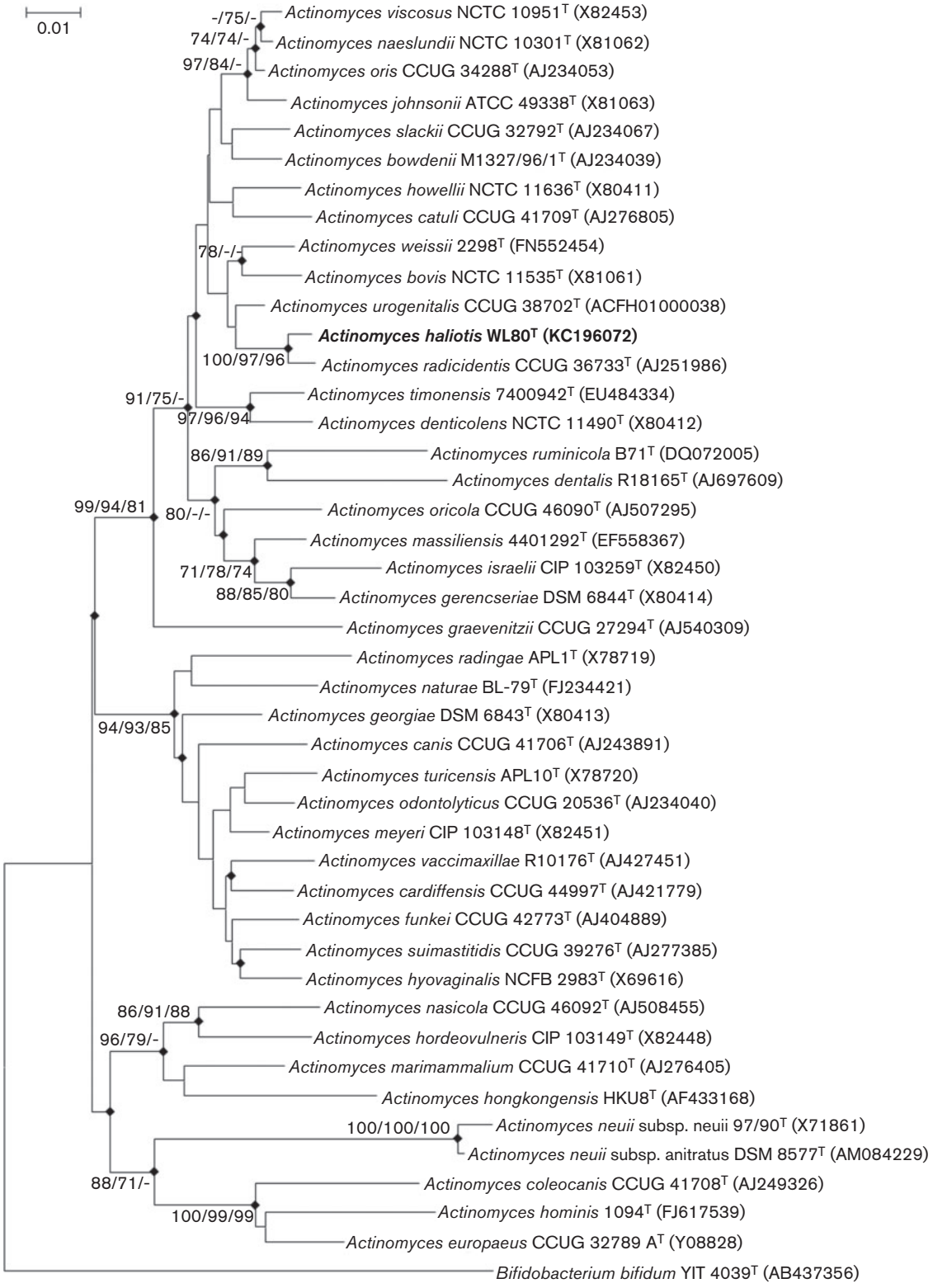

Fig. 1. Phylogenetic consensus tree based on 16S rRNA gene sequences, reconstructed using the neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods, showing the phylogenetic position of strain WL80^T with respect to recognized species of the genus *Actinomyces*. Filled diamonds indicate identical branches generated by all three methods. Numbers at the nodes represent bootstrap values (NJ/MP/ML) as percentages of 1000 replicates. *Bifidobacterium bifidum* YIT 4039^T was used as an outgroup. Bar, 0.01 accumulated changes per nucleotide.

Table 1. Differential characteristics of strain WL80^T and its closest phylogenetic relatives in the genus *Actinomyces*

Strains: 1, WL80^T; 2, *A. radicidentis* DSM 15433^T; 3, *A. urogenitalis* DSM 15434^T. All data were obtained from the current study, except where indicated. +, Positive or weakly positive; –, negative. All strains were positive for the following: catalase activity; acid production from glycerol, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, starch, glycogen, gentiobiose, turanose, gluconate and 5-ketogluconate (API 50 CH); substrate assimilation of dextrin, amygdalin, arbutin, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-lactose, lactulose, maltose, maltotriose, D-mannose, melibiose, methyl α-D-glucoside, methyl β-D-glucoside, palatinose, D-psicose, raffinose, D-ribose, salicin, stachyose, sucrose, trehalose, turanose, L-lactic acid, pyruvic acid methyl ester, pyruvic acid, glycerol, adenosine, 2′-deoxyadenosine, inosine, thymidine and uridine (Biolog GP2); and enzyme activity of leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase (API ZYM). All strains were negative for the following: oxidase activity; acid production from erythritol, L-arabinose, D-xylose, D-adonitol, L-sorbose, dulcitol, D-sorbitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol and 2-ketogluconate (API 50 CH); substrate assimilation of mannan, L-arabinose, D-arabitol, D-galacturonic acid, *myo*-inositol, 3-methyl glucose, methyl α-D-mannoside, L-rhamnose, sedoheptulosan, D-sorbitol, D-tagatose, xylitol, acetic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, lactamide, D-malic acid, L-malic acid, succinic acid monomethyl ester, propionic acid, succinamic acid, succinic acid, *N*-acetyl-L-glutamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-glutamic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, D-fructose 6-phosphate, α-D-glucose 1-phosphate and D-glucose 6-phosphate (Biolog GP2); and enzyme activity of alkaline phosphatase, esterase lipase (C8), lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase and α-fucosidase (API ZYM).

Characteristic	1	2	3
Acid production (API 50 CH)			
L-Xylose, inulin	+	+	–
N-Acetylglucosamine	–	+	+
D-Arabinose, D-mannitol, L-fucose, D-L-α-glycerol phosphate	–	+	–
Methyl β-D-xyloside, L-rhamnose, inositol, methyl α-D-mannoside	–	–	+
Substrate assimilation (Biolog GP2)			
D-Gluconic acid, methyl α-D-galactoside, D-lactic acid methyl ester	+	+	–
Tween 40, Tween 80, melezitose, thymidine-5′-monophosphate	+	–	+
N-Acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, D-mannitol, methyl β-D-galactoside	–	+	+
α-Cyclodextrin, β-cyclodextrin, glycogen, adenosine-5′-monophosphate, uridine-5′-monophosphate	+	–	–
Inulin, L-fucose, α-hydroxybutyric acid, L-asparagine, D-L-α-glycerol phosphate	–	+	–
D-Xylose	–	–	+
Enzyme activity (API ZYM)			
Valine arylamidase	+	–	+
Esterase (C4)	–	+	+(–*)
Cystine arylamidase, *N*-acetyl-β-glucosaminidase, α-mannosidase	–	–	+

*Data from Nikolaitchouk *et al.* (2000).

based on the TSBA6 database (Sasser, 1990). The major cellular fatty acids (>10 % of the total) in the isolate were $C_{18:1}\omega9c$ (55.3 %) and $C_{16:0}$ (24.3 %). Strain WL80^T shared two major cellular fatty acids, $C_{18:1}$ $\omega9c$ and $C_{16:0}$, with the two reference species. However, strain WL80^T differed in the proportion of $C_{18:0}$ (1.2 %) from *A. urogenitalis*, for which $C_{18:0}$ was a major fatty acid (10.3 %). The complete fatty acid profiles of the isolate and the reference species are presented in Table 2. The isoprenoid quinones of the isolate and the reference strains were extracted according to the method of Collins & Jones (1981a). The isoprenoid quinone extracts were purified by one-dimensional TLC on a silica gel 60 F_{254} plate (Merck) and identified by HPLC (Collins & Jones, 1981b) using a reversed-phase Hydrosphere C18 (150 × 2.0 mm) column. For more comprehensive analysis of isoprenoid quinones, liquid chromatography (Ultimate 3000, Dionex) was also used with an ion trap-mass spectrometer equipped with an electrospray ionization probe (HCT, Bruker) according to the protocol described by Taguchi *et al.* (2005). The predominant quinone of strains WL80^T and *A. radicidentis* DSM 15433^T was menaquinone-10 (H_4) [MK-10 (H_4)] but that of *A. urogenitalis* DSM 15434^T was menaquinone-10 (MK-10).

The DNA G+C content was measured and DNA–DNA hybridization with the reference species was performed to determine the genotypic characteristics of the isolate. Genomic DNA of strain WL80^T, *A. radicidentis* DSM 15433^T and *A. urogenitalis* DSM 15434^T was extracted 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

Table 2. Cellular fatty acid contents of strain WL80^T and the type strains of closely related species in the genus *Actinomyces*

Strains: 1, WL80^T; 2, *A. radicidentis* DSM 15433^T; 3, *A. urogenitalis* DSM 15434^T. All data were obtained from the current study. TR, Trace level (<0.5 %); –, not detected.

Fatty acid (%)	1	2	3
Saturated acids			
$C_{10:0}$	0.6	TR	1.0
$C_{12:0}$	1.4	1.0	1.1
$C_{14:0}$	1.6	1.4	1.6
$C_{16:0}$	24.3	37.7	42.1
$C_{18:0}$	1.2	3.9	10.3
Unsaturated acids			
$C_{16:1}\omega9c$	3.9	1.4	1.4
$C_{17:1}\omega9c$	1.3	TR	–
iso-$C_{17:1}\omega5c$	0.9	TR	TR
$C_{18:1}\omega9c$	55.3	49.5	39.5
iso-$C_{19:1}$ I	1.3	1.5	0.7
Hydroxy acids			
$C_{16:0}$ 3-OH	TR	TR	0.8
Summed features*			
3	0.8	0.5	TR
4	–	TR	–
7	5.8	1.2	–
8	0.9	TR	TR
9	TR	–	TR

*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 7 comprises $C_{19:1}\omega6c$ and/or unknown ECL 18.446; summed feature 8 comprises $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; summed feature 9 comprises iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$.

Detection System (Bio-Rad) (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA from *Bacteroides thetaiotaomicron* VPI-5482^T, *Escherichia coli* K-12, *Ruegeria pomeroyi* DSS-3^T and *Bacteroides fragilis* NCTC 9343^T was used as calibration references in the analysis. The DNA G+C content of strain WL80^T was 70.4 mol%, which was in the range for members of the genus *Actinomyces*. To clarify the genetic relatedness between the isolate and the type strains of *A. radicidentis* and *A. urogenitalis*, DNA–DNA hybridization was conducted using a genome-probing microarray (Bae *et al.*, 2005; Chang *et al.*, 2008). The DNA–DNA relatedness values were calculated from the signal-to-noise ratio of the genomic probes (Loy *et al.*, 2005). The DNA–DNA relatedness values between strain WL80^T and the type strains of *A. radicidentis* and *A. urogenitalis* were 7.6±0.4 % (5.0±0.9 % reciprocal) and 6.3±0.4 % (3.7±0.9 % reciprocal), respectively (Table 3). This value was below the accepted threshold of 70 %. Thus, strain WL80^T was considered to be a distinct genomic species (Wayne *et al.*, 1987).

Based on the physiological, biochemical, chemotaxonomic and genotypic analyses, it is suggested that strain WL80^T represents a novel species in the genus *Actinomyces*, for which the name *Actinomyces haliotis* sp. nov. is proposed.

Description of *Actinomyces haliotis* sp. nov.

Actinomyces 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-staining-positive, facultatively anaerobic, non-motile and coccus-shaped (0.7–1.1 μm in diameter), catalase-positive and oxidase-negative. Colonies on BHI agar medium are ivory coloured, circular, convex with entire margins, and 0.6–1.2 mm in diameter after 2 days at 30 °C. Grows at 20–37 °C (optimum 30 °C), at pH 6–9 (optimum pH 7–8) and with 0–4 % (w/v) NaCl (optimum 1 %). Acid is produced from glycerol, D-ribose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, gentiobiose, turanose, gluconate and 5-ketogluconate (API 50 CH). Assimilates substrates including α-cyclodextrin, β-cyclodextrin, dextrin, glycogen, Tween 40, Tween 80, amygdalin, arbutin, cellobiose, D-fructose, D-galactose, gentiobiose, D-gluconic acid, α-D-glucose, α-lactose, lactulose, maltose, maltotriose, D-mannose, melezitose, melibiose, methyl α-D-galactoside, methyl α-D-glucoside, methyl β-D-glucoside, palatinose (isomaltulose), D-psicose, raffinose, D-ribose, salicin, stachyose, sucrose, trehalose, turanose, D-lactic acid methyl ester, L-lactic acid, pyruvic acid methyl ester, pyruvic acid, glycerol, adenosine, 2′-deoxyadenosine, inosine, thymidine, uridine, adenosine-5′-monophosphate, thymidine-5′-monophosphate

Table 3. DNA–DNA hybridization values for strain WL80^T and the type strains of closely related species in the genus *Actinomyces* (based on reciprocal analyses)

Strain	DNA–DNA hybridization (%) with:		
	1	2	3
1. Strain WL80^T	100.0±0	5.0±0.9	3.7±0.9
2. *A. radicidentis* DSM 15433^T	7.6±0.4	100.0±0	10.8±1.8
3. *A. urogenitalis* DSM 15434^T	6.3±0.4	5.9±0.5	100.0±0

and uridine-5′-monophosphate (Biolog GP2). Positive for leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase (API ZYM). The major fatty acids are $C_{18:1}\omega9c$ and $C_{16:0}$. The predominant respiratory quinone is MK-10 (H_4).

The type strain, WL80^T (=KACC 17211^T=JCM 18848^T), was isolated from the intestinal tract of an abalone, *Haliotis discus hannai*, collected from the northern coast of Jeju in Korea. The DNA G+C content of the type strain is 70.4 mol%.

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