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

Dong-Wook Hyun, Na-Ri Shin, Min-Soo Kim, Joon Yong Kim, Pil Soo Kim, Sei Joon Oh, Tae Woong Whon and Jin-Woo Bae

Department of Life and Nanopharmaceutical Sciences and Department of Biology, Kyung Hee University, Seoul 130-701, Republic of Korea

A novel Gram-stain-negative, aerobic, non-motile, yellow-pigmented and rod-shaped bacterium, designated strain WB5\(^T\), was isolated from the intestinal tract of an abalone, *Haliotis discus hannai*, collected from the northern coast of Jeju in Korea. The isolate grew optimally at 30 °C, at pH 7 and in the presence of 0.5 % (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain WB5\(^T\) was clustered in the genus *Cloacibacterium* and shared the highest sequence similarity with *C. normanense* (98.2 % similarity). The predominant fatty acids were iso-C\(_{15:0}\) and iso-C\(_{17:0}\) 3-OH. Menaquinone-6 was the major respiratory quinone. The genomic DNA G+C content was 29.6 mol%. The DNA–DNA hybridization values indicated <22 % genomic relatedness with other members of the genus *Cloacibacterium.* The results of physiological, biochemical, chemotaxonomic and genotypic analyses showed that strain WB5\(^T\) represents a novel species of the genus *Cloacibacterium,* for which the name *Cloacibacterium haliotis* sp. nov. is proposed. The type strain is WB5\(^T\) (=KACC 17210\(^T\)=JCM 18869\(^T\)).

The genus *Cloacibacterium* in the family *Flavobacteriaceae* was first proposed by Allen *et al.* (2006) and defined as Gram-negative, rod-shaped bacteria that produce yellow to orange carotenoid-type pigments and contain menaquinone-6 (MK-6) as the major quinone. At the time of writing, the genus *Cloacibacterium* comprises two species: *Cloacibacterium normanense* isolated from municipal wastewater (Allen *et al.*, 2006) and *Cloacibacterium rupense* isolated from freshwater lake sediment (Cao *et al.*, 2010).

The abalone *Haliotis discus hannai,* which is known as Pacific abalone or Japanese abalone, is distributed throughout the coastal waters of East Asia where it is recognized as a valuable marine resource. The aquaculture of this species has been developed widely in East Asia (Oakes & Ponte, 1996). Recent evidence indicates that intestinal microbes in abalone play important roles in the host physiology by affecting the catabolism of algal polysaccharides (Tanaka *et al.*, 2003). Thus, intestinal microbes may affect the efficiency of abalone aquaculture (Sawabe *et al.*, 2007). However, infections or blooms of bacterial pathogens such as *Vibrio harveyi* can lead to mass mortality events in abalone (Sawabe *et al.*, 2007). The present study reports a novel bacterial strain, which was isolated during an investigation of the intestinal bacterial diversity of the abalone *H. discus hannai.* A novel *Cloacibacterium*-like strain was isolated, designated WB5\(^T\), which was subjected to a taxonomic investigation based on a polyphasic analysis.

Strain WB5\(^T\) was isolated from the intestinal tract of an abalone sample collected from the northern coast of Jeju in Korea. Intestinal bacteria were isolated by homogenizing the intestinal tract, preparing serial dilutions in 0.22 μm-filtered PBS, and spreading on trypticase soy agar (TSA; Bacto) plates. Strain WB5\(^T\) was isolated from a 10⁻³ diluted sample after incubation at 25 °C for 72 h. The isolate was transferred repeatedly to obtain a pure culture. 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). The isolate was stored at −80 °C as a suspension in trypticase soy broth (TSB; Bacto) containing 40 % (v/v) glycerol. All of the physiological, biochemical, chemotaxonomic and genotypic analyses were repeated at least three times.

The temperature conditions suitable for growth were determined in TSB at 4, 10, 15, 20, 25, 30, 37, 45, 55 and 65 °C. Growth in the presence of various NaCl concentrations (0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 8, 10 and 12 %, w/v) was tested in a medium that contained all of the ingredients of TSB except NaCl. The maximum, minimum and optimal pH for growth were tested at pH 4–11 (at intervals of 1.0 pH unit) by adjusting the pH of TSB using 10 mM MES for pH 4–6, 10 mM TAPS for pH 7–8 and 10 mM Na\(_2\)HPO\(_4\) for pH 9–11. Growth was determined in each condition by measuring the turbidity of each culture at
600 nm (OD600) using a spectrophotometer (Synergy MX; BioTek) after 24 h, 48 h and 7 days of incubation. Growth was tested in anaerobic conditions after 7 days of cultivation at 37°C on TSA plates in an anaerobic chamber filled with a N2/CO2/H2 (90:5:5) atmosphere. Strain WB5T grew at 15–37°C, at pH 6–8 and with 0–1.5% (w/v) NaCl. Optimal growth occurred at 30°C, at pH 7 and with 0.5% (w/v) NaCl. Anaerobic growth of the isolate was not observed. The Gram staining reaction, cell morphology and colony appearance were examined after incubation at 30°C for 48 h on TSA. Gram staining was performed using a Gram staining kit (bioMérieux), according to the manufacturer’s instructions. Gram staining and the cell morphology were observed by light microscopy (Eclipse 50i; Nikon). The cellular motility of the isolate was determined in semi-solid TSA (containing 0.4% agar) (Tittsler & Sandholzer, 1936) after incubation at 30°C for 7 days. Cells of strain WB5T were Gram-stain-negative, non-motile and rod-shaped (0.2–0.4 μm wide and 0.8–2.4 μm long). The isolate formed round, convex, yellow or orange colonies, with an entire margin that ranged in diameter from 1.0 to 2.0 mm. The presence of flexirubin-type pigments was detected by flooding the cell mass harvested from agar plates with 20% (w/v) KOH (Bernardet et al., 2002). To determine the presence of carotenoid-type pigments, cellular pigments were extracted according to the method of Schmidt et al. (1994) with a modification. The extracted cellular pigments were analysed by measuring the absorption spectrum using a UV–visible spectrophotometer (Synergy MX; BioTek). The KOH test for flexirubin-type pigments was negative for strain WB5T. The absorbance spectrum of the cellular pigments extracted from strain WB5T had a triple-peak signature, which is a characteristic of carotenoid-type pigments (Schmidt et al., 1994).

A phylogenetic analysis was performed based on 16S rRNA gene sequences. The 16S rRNA gene sequence of strain WB5T was amplified by colony PCR using a PCR Premix (iNtRon Biotechnology) with two universal bacterial primers: forward primer 8F (5′-AGAGTTTGATCCTG-GCTCAG-3′) and reverse primer 1492R (5′-GTTACCT-TGTTACGACTT-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 WB5T was compared with those of the type strains in the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012). The closest relative of the isolate was Cloacibacterium normanense NRS1T with a 16S rRNA gene sequence similarity of 98.2%, followed by Cloacibacterium rupense R2A-16T with a 16S rRNA gene sequence similarity of 96.7%. The phylogenetic relationships between strain WB5T 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 the MEGA 5 software package (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 showed that strain WB5T clustered within a branch that contained the other members of the genus Cloacibacterium (Fig. 1).

To characterize strain WB5T more comprehensively, Cloacibacterium normanense NRS1T (=DSM 15886T) and Cloacibacterium rupense R2A-16T (=NBRC 104931T) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and NITE Biological Resource Center (NBRC), respectively, and used as reference strains.

To characterize the biochemical properties of strain WB5T, the enzyme activities, hydrolysis, utilization of various sole carbon sources and acid production from various carbohydrates were compared with those of the type strains of C. normanense and C. rupense. The biochemical tests were conducted after cultivating the isolate and the reference strains on TSA medium in the optimum growth conditions for 48 h. Catalase and oxidase activities were assessed based on 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 determined using API ZYM test strips (bioMérieux), according to the manufacturer’s instructions. In addition, nitrate reduction, indole production, D-glucose fermentation, arginine dihydrolase activity, urease and the hydrolysis of aesculin, gelatin and 4-nitrophenyl-β-D-galactopyranoside (PNPG) were tested using API 20NE test strips (bioMérieux). The hydrolysis of starch and casein were examined by incubating the isolate on TSA medium supplemented with 0.5% soluble starch (JUNSEI) and 5% skimmed milk (Difco), respectively (Benson, 1994). The hydrolysis of Tween 20, 40, 60 and 80 were determined according to the method of Gosczynska & Serfontein (1998). The utilization of various sole carbon sources was determined using GN2 MicroPlates (Biolog) with GN/GP inoculating fluid (Biolog). Acid production from various carbohydrates was tested using API 50 test strips (bioMérieux) with 50 CHB/E medium. Strain WB5T was catalase- and oxidase-positive and differed from C. normanense DSM 15886T and C. rupense NBRC 104931T in terms of the enzyme activity level for lipase (C14), x-galactosidase, β-galactosidase and N-acetyl-β-glucosaminidase (based on API ZYM); indole production and D-glucose fermentation (based on API 20NE); hydrolysis of Tween 40 and 60; the ability to utilize Tween 40, L-arabinose, D-fructose, D-galactose, melibiose, sucrose, acetic acid, D-ketosalic acid, propionic acid, succinic acid, L-alaninamide, L-alanine, L-aspartic acid, L-licine, L-threonine, urocanic acid, glycerol and DL-α-glycerol.
phosphate (based on Biolog GN2 MicroPlate); and the ability to produce acid from the carbohydrates L-arabinose, D-xylose, D-galactose, D-mannitol, N-acetylglucosamine, lactose, sucrose, trehalose, inulin, melezitose and raffinose (based on API 50 CH). The results of the biochemical tests for strain WB5T are described in the species description, while Table 1 shows the differences in the biochemical characteristics of strain WB5T and the reference strains.

To determine the chemotaxonomic characteristics of strain WB5T, the cellular fatty acid profile and isoprenoid quinone compositions were compared with those of the type strains of *Cloacibacterium normanense* and *C. rupense*. The chemotaxonomic analyses were conducted using cell biomass of the isolate and the reference strains, which were cultured on TSA plates at 30 °C for 48 h. 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 strains were identified by gas chromatography (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 (>10% of the total) of the isolate were iso-C₁₅:₀ (34.5%) and iso-C₁₇:₀ 3-OH (14.5%). There were no major qualitative differences in the fatty acid profiles of strain WB5T and the reference strains, but quantitative differences were detected. The complete fatty acid profiles of the isolate and the reference strains are shown in Table 2. Isoprenoid quinones were extracted according to the method of Collins & Jones (1981a). The isoprenoid quinone extracts were purified by one-dimensional thin-layer chromatography on a silica gel 60 F254 plate (Merck) and identified by high-performance liquid chromatography (Collins & Jones, 1981b) using a reverse-phase Hydrosphere C18 (150 × 2.0 mm) column. The major respiratory quinone in strain WB5T was MK-6, which is also the main quinone in the two recognized species of the genus *Cloacibacterium* (Allen et al., 2006; Cao et al., 2010).

To determine the genotypic characteristics of the isolate, the genomic DNA G+C content was measured and DNA–DNA hybridizations were performed. For the genotypic analyses, genomic DNA was extracted from strain WB5T, *C. normanense* DSM 15886T and *C. rupense* NBRC 104931T, as described by Rochelle et al. (1992). The DNA G+C content of the isolate was estimated by a fluorimetric method with SYBR Gold I using the CFX96 Real-Time PCR.
Table 1. Differences in the characteristics of strain WB5\textsuperscript{T} and its closest phylogenetic relatives in the genus Cloacibacterium

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature for growth (°C)</td>
<td>15–37</td>
<td>18–36*</td>
<td>18–37†</td>
</tr>
<tr>
<td>Range</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Optimum</td>
<td>30</td>
<td>30*</td>
<td>37†</td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Enzyme activities (API ZYM)</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lipase (C14)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>α-Galactosidase, β-galactosidase, N-acetyl-β-glucosaminidase</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Indole production</td>
<td>+</td>
<td>(–)†</td>
<td>(+)†</td>
</tr>
<tr>
<td>D-Glucose fermentation</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tween 40, Tween 60</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tween 80</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Utilization of (Biolog GN2 MicroPlate):</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tween 40, D-fructose, glycerol, DL-2-glycerol phosphate</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Acetic acid, α-ketovaleric acid, propionic acid, L-alanine, L-threonine, urocanic acid</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Tween 80, melibiose, L-1-alaminamide, L-leucine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Arabinoose, sucrose, succinic acid</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Acid production from (API 50 CH):</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>D-Galactose, lactose, sucrose, raffinose</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Inulin, melezitose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>L-Arabinose, D-mannitol, N-acetylgulosamine, trehalose, D-xylene</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>29.6</td>
<td>31.0</td>
<td>33.3</td>
</tr>
<tr>
<td>Isolation source</td>
<td>Gut of abalone</td>
<td>Municipal wastewater</td>
<td>Lake sediment</td>
</tr>
</tbody>
</table>

*Data from Allen et al. (2006).
†Data from Cao et al. (2010).

Detection System (Bio-Rad) (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA extracted from Bacteroides thetaetiaotai–micron VPI 5482\textsuperscript{T}, Escherichia coli K-12 and Ruegeria pomeroyi DSS-3\textsuperscript{T} were used as calibration references in the analysis. The DNA G+C content of strain WB5\textsuperscript{T} was 29.6 mol%. The distinct species status of the isolate and the reference strains were demonstrated by DNA–DNA hybridization using a genome-probing microarray (Bae et al., 2005;
Table 2. Cellular fatty acid profiles of strain WB5\textsuperscript{T} and the type strains of closely related species of the genus Cloacibacterium

<table>
<thead>
<tr>
<th>Fatty acid (%)</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{16:0}</td>
<td>3.6</td>
<td>TR</td>
<td>1.4</td>
</tr>
<tr>
<td>C\textsubscript{18:0}</td>
<td>2.7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Unsaturated acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{18:1\textit{v}9c}</td>
<td>1.6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Branched acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-C\textsubscript{13:0}</td>
<td>3.1</td>
<td>4.2</td>
<td>5.4</td>
</tr>
<tr>
<td>anteiso-C\textsubscript{13:0}</td>
<td>TR</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>iso-C\textsubscript{14:0}</td>
<td>1.1</td>
<td>3.3</td>
<td>2.2</td>
</tr>
<tr>
<td>iso-C\textsubscript{15:1\textit{F}}</td>
<td>4.9</td>
<td>6.0</td>
<td>10.8</td>
</tr>
<tr>
<td>anteiso-C\textsubscript{15:1\textit{A}}</td>
<td>3.1</td>
<td>9.9</td>
<td>5.1</td>
</tr>
<tr>
<td>iso-C\textsubscript{16:0}</td>
<td>34.5</td>
<td>42.9</td>
<td>41.7</td>
</tr>
<tr>
<td>anteiso-C\textsubscript{16:0}</td>
<td>8.9</td>
<td>14.0</td>
<td>5.5</td>
</tr>
<tr>
<td>iso-C\textsubscript{16:1\textit{B}}</td>
<td>–</td>
<td>1.3</td>
<td>2.3</td>
</tr>
<tr>
<td><strong>Hydroxy acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C\textsubscript{12:0} 3-OH</td>
<td>TR</td>
<td>1.1</td>
<td>4.8</td>
</tr>
<tr>
<td>iso-C\textsubscript{15:0} 3-OH</td>
<td>8.0</td>
<td>3.3</td>
<td>4.2</td>
</tr>
<tr>
<td>C\textsubscript{15:0} 2-OH</td>
<td>1.5</td>
<td>–</td>
<td>1.3</td>
</tr>
<tr>
<td>iso-C\textsubscript{16:0} 3-OH</td>
<td>2.5</td>
<td>1.9</td>
<td>4.6</td>
</tr>
<tr>
<td>C\textsubscript{18:1\textit{v}9c}</td>
<td>1.6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>iso-C\textsubscript{17:0} 3-OH</td>
<td>14.5</td>
<td>6.5</td>
<td>4.9</td>
</tr>
<tr>
<td>C\textsubscript{17:0} 2-OH</td>
<td>1.1</td>
<td>0.8</td>
<td>–</td>
</tr>
<tr>
<td><strong>Summed features</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5.1</td>
<td>–</td>
<td>4.5</td>
</tr>
<tr>
<td>8</td>
<td>1.9</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Summed features represent groups of two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 3 comprises C\textsubscript{16:1\textit{v}7c} and/or C\textsubscript{16:1\textit{v}6c}; summed feature 8 comprises C\textsubscript{18:1\textit{v}5c} and/or C\textsubscript{18:1\textit{v}12c}.

Chang et al. (2008). The genetic relatedness values of strain WB5\textsuperscript{T} with respect to C. normanense DSM 15886\textsuperscript{T} and C. rupense NBRC 104931\textsuperscript{T} were 6.9 ± 0.8 % (reciprocal 20.5 ± 5.6 %) and 10.9 ± 2.0 % (reciprocal 21.7 ± 3.9 %), respectively (Table 3). These results suggest that strain WB5\textsuperscript{T} is novel at the species level (Wayne et al., 1987).

Based on differences in phylogenetic, phenotypic and genotypic features compared with strains of reference species, we suggest that strain WB5\textsuperscript{T} represents a novel species in the genus Cloacibacterium, for which the name Cloacibacterium haliotis sp. nov. is proposed.

**Description of Cloacibacterium haliotis sp. nov.**

Cloacibacterium haliotis (ha.li’o.tis. N.L. gen. n. haliotis of Haliotis, systematic name of the genus of abalone, referring to the isolation of the type strain from Haliotis discus hannai).
Acknowledgements

This work was supported by grants from the Mid-career Researcher Program through the National Research Foundation of Korea (2012-0008806) and the National Institute of Biological Resources (NIBR) funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR no. 2013-02-001). We thank Professor J. P. Euzéby (École Nationale Vétérinaire, France) for etymological advice and Mr Hae-Won Lee and Dr Seong Woon Roh (Jeju Center, Korea Basic Science Institute, Korea) for analysing the isoprenoid quinones.

References


