Arcobacter haliotis sp. nov., isolated from abalone species Haliotis gigantea

Reiji Tanaka, Ilse Cleenwerck, Yukino Mizutani, Shunpei Iehata, Peter Bossier and Peter Vandamme

Abstract

A Gram-negative, aerobic, polar-flagellated and rod-shaped, sometimes slightly curved bacterium, designated MA5<sup>T</sup>, was isolated from the gut of an abalone of the species Haliotis gigantea collected in Japan. Phylogenetic analyses based on 16S rRNA, gyrB, hsp60 and rpoB gene sequences placed strain MA5<sup>T</sup> in the genus Arcobacter in an independent phylogenetic line. Comparison of the 16S rRNA gene sequence of this strain with those of the type strains of the established Arcobacter species revealed A. nitrofigilis (95.1 %) as nearest neighbour. Strain MA5<sup>T</sup> grew optimally at 25 °C, pH 6.0 to 9.0 and in the presence of 2 to 5 % (w/v) NaCl under both aerobic and microaerobic conditions. The predominant fatty acids found were summed feature 3 (iso-C<sub>15:1</sub> 2-→OH and/or C<sub>16:1</sub>ω7c), C<sub>12:0</sub> 3-→OH and C<sub>18:1</sub>ω7c. Menaquinone-6 (MK-6) and menaquinone-7 (MK-7) were found as the major respiratory quinones. The major polar lipids detected were phosphatidylethanolamine and phosphatidylglycerol. Strain MA5<sup>T</sup> could be differentiated phenotypically from the phylogenetic closest Arcobacter species by its ability to grow on 0.05 % safranin and 0.01 % 2,3,5-triphenyl tetrazolium chloride (TTC), but not on 0.5 % NaCl. The obtained DNA G+C content of strain MA5<sup>T</sup> was 27.9 mol%. Based on the phylogenetic, chemotaxonomic and phenotypic distinctiveness of MA5<sup>T</sup>, this strain is considered to represent a novel species of the genus Arcobacter, for which the name Arcobacter haliotis sp. nov. is proposed. The type strain is MA5<sup>T</sup> (=LMG 28652<sup>T</sup>=JCM 31147<sup>T</sup>).

The genus Arcobacter belongs to the family Campylobacteraceae, which at the time of writing comprised 24 established species, of which 15 have been described in the past 5 years: A. trophiarum [1], A. molluscumorum [2], A. ellisi [3], A. defluvii [4], A. bivalviorum [5], A. venerus [5], A. cloacae [6], A. suis [6], A. anaerophilus [7], A. ebronensis [8], A. aquimarinus [8], A. lanthieri [9], A. pacificus [10], A. facis [11] and A. acticola [12]. Strains of this genus have been isolated from human clinical samples and livestock, where they are often associated with reproductive and/or gastrointestinal diseases [13]. Using culture-independent techniques with species-specific primers or probes, it was found that the genus Arcobacter is widely distributed in natural or artificial environments [14–16]. Many of the recently described Arcobacter species comprise strains that have been obtained from marine environments such as coastal seawater, shellfish, sea sediment, brackish water and roots of salt-marsh plants [2, 3, 5, 6, 10, 12, 17]. In the present study, we report the polyphasic taxonomic characterization of strain MA5<sup>T</sup> which was isolated from the gut of an abalone of the species Haliotis gigantea, collected at a hatchery in Owase, Mie, Japan, in July 2009.

The gut of the collected abalone was aseptically excised and a homogenate sample was prepared using a beads beater (Taisei). Serial 10-fold dilutions of gut homogenate samples were made in 75 % of artificial seawater (10<sup>-1</sup>–10<sup>-7</sup>) (0.1 ml) and spread on marine agar (MA; BD Difco) containing 0.5 % of alginate (YPD medium) [19]. The plates were incubated at 25 °C for 5 days under aerobic conditions. Thirty colonies were randomly picked, subcultivated on MA and subjected to partial 16S rRNA gene sequence analysis. One of the 30 isolates was found to be a member of the genus Arcobacter and was designated strain MA5<sup>T</sup>. Using the above-mentioned cultivation conditions, strain MA5<sup>T</sup> grew very slowly, i.e. at least 72 h was needed to visually notice development of colonies. Other cultivation conditions were tested, such as incubation on blood agar or blood agar containing 2 % of NaCl, at 25 or 30 °C (the latter regarded as suitable for strains of Arcobacter) and under

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Keywords: epsilonproteobacterium; Arcobacter; abalone; Haliotis gigantea; gut.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, apfA, gyrA, gyrB, rpoB and hsp60 gene sequences of strain MA5<sup>T</sup> are AB542077, LC215639, LC215638, LC121818, LC121819 and LC121820, respectively.

Five supplementary figures and one supplementary table are available with the online Supplementary Material.
aerobic or microaerobic conditions (the latter in anaerobic jar system (MGC) with AnaeroPack (MGC)). However, growth could not be improved. Moreover, at 30 °C colony formation could not be detected. Hence, it was decided to routinely cultivate strain MA5T on MA at 25 °C under aerobic conditions.

To determine the phylogenetic position of strain MA5T, partial sequences of the 16S rRNA, gyrA, gyrB, hsp60, atpA and rpoB genes were determined as previously described [17]. Genomic DNA for sequencing and DNA G+C content determination was extracted using a modification [20] of the method described by Wilson [21]. EzTaxon-e server blast analysis [22] revealed that the 16S rRNA gene sequence of strain MA5T (1477 bp) showed the highest pairwise similarity to that of the type strain of *A. nitrofigilis* (L14627, 95.1%). Pairwise 16S rRNA gene sequence similarities to the type strains of all established *Arcobacter* species were calculated using the BioNumerics v7.0 software package (Applied Maths). The type strain of *A. nitrofigilis* was confirmed as nearest neighbour, and less than 94.8% 16S rRNA gene sequence similarity was found towards the type strains of the other *Arcobacter* species. Alignment and phylogenetic analysis based on nearly complete 16S rRNA gene sequences (1477 bp) were performed using MEGA 5 software [23]. Phylogenetic trees were reconstructed using the neighbour-joining, maximum-parsimony and maximum-likelihood methods. Bootstrap values were calculated to estimate the robustness of the tree topologies obtained using the different algorithms. The maximum-parsimony and maximum-likelihood trees (Figs S1 and S2, available in the online Supplementary Material) showed basically the same topology as the neighbour-joining tree (Fig. 1), and placed strain MA5T in a position distinct from the

![Fig. 1](https://www.microbiologyresearch.org/fig1.png)

**Fig. 1.** Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (1477 bp) showing the phylogenetic position of *Arcobacter haliotis* sp. nov. MA5T in the genus *Arcobacter*. Numbers at nodes indicate levels of bootstrap support based on a dataset of 1000 resamplings. Bar, 0.02 substitutions per nucleotide position.
Fig. 2. Neighbour-joining tree based on concatenated *atpA*, *gyrA*, *gyrB*, *rpoB* and *hsp60* gene sequences (3234 bp), showing the phylogenetic relationship position of *Arcobacter haliotis* sp. nov. MA5^T in the genus *Arcobacter*. Numbers at nodes indicate levels of bootstrap support based on a dataset of 1000 resamplings. Bar, 0.02 substitutions per nucleotide position.
established *Arcobacter* species. This position combined with the relatively low level of 16S rRNA gene sequence similarity towards the type strains of the established species of the genus *Arcobacter* implied that this strain represents a novel species in this genus [24, 25].

Support for the novel species status of strain MA5<sup>T</sup> was provided by phylogenetic analysis based on concatenated sequences of the housekeeping genes gyrA, gyrB, hsp60, atpA and rpoB that were previously proven suitable for this purpose [17]. The partial sequences of gyrA (686 bp), gyrB (665 bp), hsp60 (595 bp), atpA (622 bp) and rpoB (666 bp) of strain MA5<sup>T</sup> were concatenated and compared to sequences of strains of established *Arcobacter* species, including their type strains (Table S1). Alignment and phylogenetic analyses were performed using MEGA 5 software. Phylogenetic trees were reconstructed using the neighbour-joining (Fig. 2), maximum-parsimony and maximum-likelihood methods (Figs S3 and S4). Bootstrap values were calculated using an 6890N gas chromatograph (Agilent Technologies). Cultivation of the strains, harvesting of the cells, fatty acid extraction and analysis of the fatty acid methyl esters were performed according to the recommendations of the Sherlock Microbial Identification System (MID). Fatty acids were extracted from cultures grown on MA in a CO<sub>2</sub> enriched atmosphere (Oxoid CO<sub>2</sub> Gen Sachet system, Oxoid) at 25 °C for 3 days. They were identified using the TSBA identification library version 5.0. Summed feature 3 (iso-C<sub>15</sub>:0 2-OH and/or C<sub>16</sub>:1 ω7<sup>c</sup>, 53.4 %), C<sub>12</sub>:0 3-OH (11.5 %) and C<sub>18</sub>:1 ω7<sup>c</sup> (9.2 %) were found as the dominant fatty acids of strain MA5<sup>T</sup>. Compared to *A. nitrofigilis* LMG 7604<sup>T</sup> (Table 1), the fatty acid content of strain MA5<sup>T</sup> was found to contain more summed feature 3 (iso-C<sub>15</sub>:0 2-OH and/or C<sub>16</sub>:1 ω7<sup>c</sup>) and C<sub>12</sub>:0 3-OH and less C<sub>16</sub>:0 and C<sub>18</sub>:1 ω7<sup>c</sup>.

Respiratory quinones were analysed by the Identification Service of DSMZ, Braunschweig, Germany. Lyophilized cells of strain MA5<sup>T</sup> and *A. nitrofigilis* LMG 7604<sup>T</sup> were used for the extraction of respiratory quinones using the two-stage method described by Tindall [29, 30]. Respiratory lipoquinones were separated into their different classes by thin layer chromatography and further analysed using a LDC Analytical HPLC (Thermo Separation Products) fitted with a reverse-phase column (125x2 mm, 3 μm, RP18; Macherey-Nagel) and methanol:heptane 9:1 (v/v) as the eluent. They were detected by UV absorbance at 269 nm. For both strains, the predominant lipoquinones were MK-6 (35 %) and MK-7 (65 %), which is in line with previous observations for species of the genus *Arcobacter* [10].

Polar lipids of strain MA5<sup>T</sup> were extracted from lyophilized bacterial cells, separated using two-dimensional TLC and detected by spraying with the reagents molybdatephosphoric acid, ninhydrin, molybdenum blue, α-naphthol and Dragendorff’s reagent as previously described [31]. The major polar lipids detected were phosphatidyethanolamine and phosphatidylglycerol (Fig. S5), which are commonly found as major polar lipids in other members of *Arcobacter* [7, 10]. One unknown lipid was observed (Fig. S5).

Phenotypic characterization was performed following the proposed minimal standards for describing new species of the family *Campylobacteraceae* [32]. Cell size was measured using light microscopy (Eclipse 50i system; Nikon). Cell morphology was examined through light microscopy and transmission electron microscopy (HT-7700; Hitachi; Fig. 3). Gliding motility was investigated as described by Bowman [33]. Light microscopy revealed highly motile, rod-shaped (sometimes slightly curved) cells with a size of approximately 0.5–0.8 μm in diameter and 0.8–2.0 μm in length. Electron microscopy revealed a single polar flagellum at one end. Gliding motility was not observed.

Growth at 4, 10, 20, 25, 30, 35, 37 and 40 °C was measured in marine broth (MB; BD Difco). The pH range for growth was investigated in MB adjusted to pH 4 to 12 using Good’s buffers (Kishida Chemical). Both these tests were performed

### Table 1. Cellular fatty acid content (%) of *Arcobacter haliotis* sp. nov. MA5<sup>T</sup> and *Arcobacter nitrofigilis* LMG 7604<sup>T</sup>.

<table>
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<td>4.0</td>
<td>24.1</td>
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<td>11.5</td>
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<td>C&lt;sub&gt;14&lt;/sub&gt;:1 ω7&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>19.5</td>
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<tr>
<td>Summed feature 2*</td>
<td>7.7</td>
<td>3.9</td>
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<tr>
<td>Summed feature 3†</td>
<td>53.4</td>
<td>34.8</td>
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</table>

*C<sub>14</sub>:0 3-OH* and/or iso-C<sub>15</sub>:1 i. | *iso-C<sub>15</sub>:0 2-OH* and/or C<sub>16</sub>:1 ω7<sup>c</sup>.
under aerobic and microaerobic conditions, the latter in an anaerobic jar system (MGc) with AnaeroPack (MGc). Growth was determined after incubation for 7 days. Growth in the absence of NaCl and in the presence of 0.5, 1, 2, 3, 4, 5, 6 and 8 % (w/v) NaCl was investigated in yeast (1 %, w/v)/peptone (5 %, w/v) broth. Growth on 0.01, 0.04, 2, 3 and 5 % of 2,3,5-triphenyl tetrazolium chloride (TTC), 0.05 % safranin and 0.1 % sodium deoxycholate were tested on MA. H2S production and nitrate reduction were tested by API Campy (bioMérieux). Hydrolysis of acetoin was tested using VP reagents (BD Difco). Susceptibility to cefoperazone was tested on MA containing 16, 32 and 64 mg l⁻¹ of cefoperazone. Catalase activity was determined by verifying the production of oxygen bubbles after mixing cells with 3 % (v/v) H2O2. Oxidase activity was determined by verifying oxidation of 1 % (w/v) N,N,N′,N′-tetramethyl-p-phenylenediamine solution (Wako). Enzyme activities (including hydrolysis of hirpurate) were tested with API Campy (bioMérieux) according to the manufacturer’s protocols. Hydrolysis of alginate, aesculin, starch, gelatin, casein, chitin, cellulose and DNA was investigated using MA containing 1 % (w/v) of the substrate. Hydrolysis of Tween 80 was investigated as described previously [34], except that artificial seawater was used to prepare the media. Utilization of substrate as sole carbon and energy sources were tested as described previously [35]. Acid production from carbohydrates were tested as described by Leifson [36]. Physiological and biochemical properties of the strain MA5T are given in the species description and in Table 2. The strain can be differentiated from the phylogenetic closest Arcobacter species by its ability to grow on 0.05 % safranin and 0.01 % TTC, and its inability to grow at 30 °C or grow on 0.5 % NaCl.

In conclusion, the phylogenetic, chemotaxonomic and phenotypic data provided in this study demonstrate that strain MA5T represents a novel species within the genus Arcobacter, for which the name Arcobacter haliosis sp. nov. is proposed. The type strain is MA5T (=LMG 28652T=JCM 31147T).

Table 2. Characteristics that differentiate Arcobacter haliosis sp. nov. MA5T from the phylogenetic closest species of the genus Arcobacter

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<td>27.9</td>
<td>28.4</td>
<td>28.0</td>
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<td>NT</td>
<td>24.6</td>
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</table>
DESCRIPTION OF ARCOBACTER HALIOTIS SP. NOV.

Arcobacter haliotis (ha.li.o’tis. N.L. gen. haliotis named after the scientific name of the abalone Haliotis).

Cells are Gram-stain-negative, rod-shaped (sometimes slightly curved), approximately 0.5–0.8 μm in diameter and 0.8–2.0 μm in length, polar-flagellated and highly motile, but not showing gliding motility. Colonies are white in colour and 1.0 mm in diameter after incubation for 3 days at 25°C on MA. Growth under aerobic and microaerobic conditions occurs from 10 to 25°C, but not at 4, 30, 37 and 42°C, and only weakly at 10°C. The optimal growth temperature is 25°C. The optimal pH for growth is between 6 and 8. Growth occurs from 2 to 5% (w/v) NaCl. Grows on MA, 0.01% TTC, 0.05% safranin and 0.1% sodium deoxycholate. No growth occurs on CAT between 6 and 8. Growth occurs from 2 to 5% (w/v) NaCl. Time of the type strain is 27.9 mol%.

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ACKNOWLEDGMENTS

We thank Mr Atsushi Hamabe at the Owase Aquaculture Center for kindly providing abalone samples.

CONFLICTS OF INTEREST

The authors IC and PV are employed by an organization that commercially offers both taxonomic services as well as biological materials to the scientific community. This may be perceived as a potential conflict of interest.

References


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