Arcobacter lekithochrous sp. nov., isolated from a molluscan hatchery

Ana L. Diéguez,1 Sabela Balboa,1 Thorolf Magnesen2 and Jesús L. Romalde1*  

Abstract

Four bacterial strains, LFT 1.7T, LT2C 2.5, LT4C 2.8 and TM 4.6, were isolated from great scallop (Pecten maximus) larvae and tank seawater in a Norwegian hatchery and characterized by a polyphasic approach including determination of phenotypic, chemotaxonomic and genomic traits. All were Gram-stain-negative, motile rods, oxidase- and catalase-positive and required sea salts for growth. Major fatty acids present were summed feature 3 (C16:1ω7c/C16:1ω6c), summed feature 8 (C18:1ω7c or C18:1ω6c), C16:0, C14:0, summed feature 2 (C14:0 3-OH/iso C16:1 ω7c), C12:0 3-OH and C12:0 3-OH. Strain LFT 1.7T contained menaquinone MK-6 as the sole respiratory quinone. Phylogenetic analysis based on 16S rRNA gene sequences indicated that all strains formed a distinct lineage within the genus Arcobacter with a low similarity to known species (94.77–95.32%). The DNA G+C content was 28.7 mol%. Results of in silico DNA–DNA hybridization and average nucleotide identity confirmed that the isolates constitute a novel species of Arcobacter, for which the name Arcobacter lekithochrous sp. nov. is proposed. The type strain is LFT 1.7T (=CECT 8942T=DSM 100870T).

The genus Arcobacter belongs to the family Campylobacteraceae and the type species is Arcobacter nitrofigilis, initially described as a member of the genus Campylobacter [1] and reclassified by Vandamme et al. [2] as representing a new genus. Arcobacter shows a global distribution considering that it has been isolated from different geographical regions such as Spain, India and Canada [3–5]. In the same way, this genus displays a high diversity of ecological niches: marine environments, waste and drinking water, animal faeces, associated with plants or in oil field communities, among others [6–9].

Some species of Arcobacter cause diarrhoea and bacteremia in humans and abortion or mastitis in animals, although Arcobacter has also been isolated from healthy humans and animals. The route of transmission to humans and animals is not clear, but consumption of contaminated food and water has been postulated as the main means of infection [10]. It is assumed that prevalence has been probably underestimated owing to the difficulty in culturing these bacteria [11]. These facts have led to studies on Arcobacter prevalence in different environments and animals such as molluscs which, being filter-feeding, could act as reservoirs for these bacteria. Indeed, many novel species associated with molluscs have been described within this genus in the last decade [12–14].

The aim of this study was to characterize, using a polyphasic approach, four strains isolated from scallop and seawater samples and to determine their taxonomic position.

The bacterial isolates studied here were obtained in 2011 from larvae and seawater in an experimental hatchery in Norway (60° 30’ 53.77” N 4° 54’ 14.75” W) during a study on the microbiota associated with the reproductive cycle of the great scallop (Pecten maximus). After incubation for 7 days at room temperature on marine agar 2216 (MA; Difco) plates the different colony types were isolated and subjected to preliminary characterization. Bacterial isolates LFT 1.7T (=CECT 8942T=DSM 100870T), LT2C 2.5, LT4C 2.8 and TM 4.6 (=CECT 8943=DSM 100869), with characteristics matching those of the genus Arcobacter, were selected for further characterization.

Isolation of DNA from pure cultures on MA was performed using the ‘Instagene’ matrix (Bio-Rad), following the manufacturer’s recommendations. Amplification of the 16S rRNA gene of the different strains was carried out using primers 27F (5’-AGAGTTTGATCCTGGCTCAG) and 1510R (5’-GTACTTACGACTACT) as previously described.

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Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; ML, maximum-likelihood; MLSA, multilocus sequence analysis.

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number MKCO00000000. The version described in this paper is version MKCO01000000.

One supplementary figure and two supplementary tables are available with the online Supplementary Material.

NOTE
by Lane [15]. Amplicons were Sanger-sequenced using the same primers at StabVida Laboratories (Portugal). Sequences from the closest relatives were retrieved from GenBank/EMBL. Sequence data analysis was carried out using the EzTaxon-e server (www.eztaxone.ezbiocloud.net) [16]. Phylogenetic analysis was carried out using the program MEGA 6.0 [17]. Sequences of the 16S rRNA gene of isolates LFT 1.7T, LT2C 2.5, LT4C 2.8 and TM 4.6 showed a similarity of 99.6–100% among themselves and the highest similarity with A. nitrofigilis DSM 7299T in a range from 94.77 to 95.32%. Other closest relatives for these strains were Arcobacter ellisii F79-6T (94.13–95.24%) and Arcobacter venerupis F67-11T (93.17–94.59%). The maximum-likelihood (ML) phylogenetic tree reconstructed with these sequences showed that the isolates formed a robust and independent branch separated from other species of the genus Arcobacter (Fig. 1). It is interesting to note that similarities in 16S rRNA gene sequences among type strains of all described species of the genus Arcobacter range from 91.20 to 99.57%, showing that some species share very low similarities and indicating that they could even belong to a different genus. Therefore, a deep taxonomic study of the genus Arcobacter is required.

The genomic DNA of strain LFT 1.7T for in silico DNA–DNA hybridization (DDH), average nucleotide identification (ANI) and G+C content analysis was obtained using a High Pure PCR Template Preparation kit (Roche). Illumina

**Fig. 1.** Phylogenetic reconstruction based on nucleotide sequences of the 16S rRNA gene in the ML algorithm (model GTR+G+I) showing the position of the novel Arcobacter species. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the nodes. Bar, 0.05 substitutions per nucleotide position.
paired-end sequencing was performed at Sistemas Genómicos (Valencia, Spain). The quality of reads was analysed using Trimmomatic 0.32 [18]. Genome assembly was performed using SPAdes 3.6.1 [19]. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number MKCC00000000. Five housekeeping genes, *atpA*, *hsps70*, *gyrA*, *gyrB* and *rpoB*, were obtained from the whole genome of strain LFT 1.7T and a multilocus sequence analysis (MLSA) was carried out. The phylogenetic tree based on these sequences confirmed the independent position of this group within the genus *Arcobacter* (Fig. S1, available in the online Supplementary Material).

The in silico DDH value was estimated between strain LFT 1.7T and available genomes of *Arcobacter anaeoprophilus* IR1 (JXXG00000000), *Arcobacter butzleri* RM4018T (NC_009850.1), *Arcobacter cibarius* LMG 21996T (JABW00000000), *Arcobacter cryoaerophilus* LLKQ00000000, *Arcobacter defluvii* L (NC_017192), *Arcobacter faecis* AF10784T (JARS00000000), *Arcobacter lanthieri* AF1440T (JARU00000000), *A. nitrofigilis* DSM 7299T (NC_014166), *Arcobacter skirrowii* L405 (LRUX00000000) and *Arcobacter therieus* LMG 24486T (LLKQ00000000) using the genome-to-genome distance calculator (GGDC2.0) [20–22]. The ANI using the BLAST (AN Ib) and MUMmer (ANIm) algorithms was calculated using the software J-species (v1.2.1) as described by Richter and Roselló-Mora [23]. Alternatively, OrthoANI was calculated as described by Lee et al. [24].

The G+C content of strain LFT 1.7T was 28.7 mol%, a value within the range reported for the genus *Arcobacter*. DDH results ranged between 18.30 and 20.50 % whereas OrthoANI analysis revealed a similarity of between 73.07 and 76.29 % with its closest relatives (Table S1). All these values were lower than the thresholds established to demarcate species (95–96 % in OrthoANI and 70 % in DDH).

Total cell fatty acids of the four Norwegian strains and *A. nitrofigilis* CECT 7204T, grown on MA at 25 °C for 48 h, were determined on the MIDI system following the instructions of the manufacturer [25] using the Phospholipid Fatty Acids (PFLAD1) and Environmental Aerobes (TSBA) databases. Results showed that all strains presented similar fatty acid profiles with summed feature 3 (C_{16:1}ω7c/C_{16:1}ω6c), summed feature 8 (C_{18:1}ω7c or C_{18:1}ω6c), C_{16:0}, C_{14:0}, summed feature 2 (C_{14:0} 3-OH/iso-C_{16:1} I), C_{12:0} 3-OH and C_{12:0} as major components, although the percentages of these fatty acids differed among strains (Table 1).

**Analysis of the polar lipids and respiratory quinones of strain LFT 1.7T** were carried out by the Identification Service of the DSMZ (Braunschweig, Germany), following methodologies described by Bligh and Dyer [26] and Tindall et al. [27–29]. Polar lipids present were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol and two unidentified phospholipids, one phosphoaminolipid and one lipid (Fig. 2). The respiratory quinone of strain LFT 1.7T was menaquinone MK-6.

Phenotypic characteristics were determined by a set of classical and specific tests recommended for the description of novel species in the family *Campylobacteraceae* [12, 30–32] including: cell morphology and motility, Gram, catalase and oxidase activity, acid production from glucose by oxidation and fermentation, nitrate reduction, Voges–Proskauer, indole, urea, and hydrolysis of indoxyl acetate, casein, starch and gelatine. The pH range for growth was examined in marine broth (MB; Pronadisa) using appropriate biological buffers for adjusting the pH to 3, 4, 5, 6, 7, 8, 9 and 10.

**Values are percentages of the total fatty acids; TR, fatty acids that make up <1 % of the total; ND, not detected.** For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. The cis isomer is indicated by the suffix c. Summed features are groups of two fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 2 includes C_{16:0} 3-OH/iso-C_{16:1} I, summed feature 3 contains C_{16:1}ω7c/C_{16:1}ω6c and summed feature 8 contains C_{18:1}ω7c and/or C_{18:1}ω6c. All data were obtained in the present study.

Table 1. Cellular fatty acid content of the novel strains (*Arcobacter lekithochrous* sp. nov.) and the closest relative *A. nitrofigilis* CECT 7204T

<table>
<thead>
<tr>
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<th><em>A. lekithochrous</em></th>
<th><em>A. nitrofigilis</em></th>
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<tr>
<td></td>
<td>LFT 1.7T</td>
<td>LT2C 2.5</td>
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<tr>
<td>C_{10:0}</td>
<td>TR</td>
<td>TR</td>
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<tr>
<td>C_{12:0}</td>
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<td>2.5</td>
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<tr>
<td>C_{12:0} 3-OH</td>
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<td>C_{14:0}</td>
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<td>7.5</td>
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<tr>
<td>Summed feature 2</td>
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<td>Summed feature 3</td>
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<td>51.1</td>
</tr>
<tr>
<td>C_{16:0}ω7c</td>
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<td>C_{16:0}ω6c</td>
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<tr>
<td>Summed feature 8</td>
<td>18.8</td>
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<tr>
<td>C_{18:0}</td>
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<td>ND</td>
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<tr>
<td>C_{18:0} 10-methyl</td>
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<td>C_{18:0}ω9c</td>
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Tolerance to different saline concentrations of NaCl and sea salts (Sigma-Aldrich) was tested on basal medium agar (BMA) [4 g yeast extract l⁻¹ solidified with 1.5% (w/v) American bacteriological agar (Pronadisa)] supplemented with NaCl (0, 0.5, 1, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 8 and 10%, w/v). Growth at 4, 15, 25, 30 and 37 °C was determined on MA plates, and as such at different gaseous conditions. Growth on different media was tested on trypticase soy agar (TSA) supplemented with 2 and 3% (w/v) NaCl (TSA-2, TSA-3), TSA with 5% sheep blood, commercial blood agar medium (Oxoid) and Campylobacter charcoal deoxycholate agar (CCDA; Oxoid). The ability to grow on MacConkey agar (Sigma-Aldrich), minimal medium M9 (per litre: 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl and glucose to 0.2%, v/v), triple sugar iron agar (TSI; Biolife), nutrient agar (NA) supplemented with sodium deoxycholate (0.1%), Oxgall (1%), glycine (1%), 2,3,5-triphenyl tetrazolium chloride (TTC, 0.04%), fuchsin (0.005%), brilliant green (0.001%), safranin (0.05%) or crystal violet (0.0005%) was determined. To observe growth, all these media were supplemented with 3% (w/v) sea salts. Use of 47 different substances as sole carbon source was tested on BMA containing 50% (v/v) artificial sea water [ASW, per litre: 23.38 g NaCl, 24.65 g MgSO₄.7H₂O, 1.5 g KCl, 2.2 g CaCl₂.2H₂O, 6.1 g Tris-HCl, 1 g NH₄Cl, 0.075 g K₂HPO₄, 0.028 g FeSO₄.7H₂O and 1.8% (w/v) American bacteriological Agar (Pronadisa)] supplemented with different substrates [33]. Alternatively, the API ZYM (bioMérieux) system was used to determine enzymatic activities.

All strains were Gram-stain-negative, motile, oxidase- and catalase-positive rods that were capable of reducing nitrate. They were negative for indole and the Voges–Proskauer reaction and hydrolysis of urea, casein, gelatine and starch. The hydrolysis of indoxyl acetate was negative in all strains except for LT4C 2.8. All strains were halophilic; no growth was observed in the absence of sea salts. NaCl was not sufficient to stimulate growth. These strains were able to grow at temperatures from 15 to 25 °C, except strains TM 4.6 and LT4C 2.8, which were able to grow at 4 °C, but the isolates could not grow at 37 °C. The results obtained with the specific tests for Campylobacteraceae allowed us to differentiate the scallop isolates from other Arcobacter species (Table 2). These isolates were negative in most tests. The four isolates grew on NA with safranin. All strains, except LFT 1.7⁺, were able to grow on NA with fuchsin and TTC and only strain LT4C 2.8 was able to grow on NA supplemented with Oxgall.

The Norwegian strains showed variable results for the use of carbon sources. All strains used d-mannose, lactic acid, succinic acid, α-aminobutyric acid and malic acid but not d-ribose, arabinose, d-xylene, d-galactose, salicin, d-sorbitol, N-acetyl-d-glucosamine, L-threonine, d-mannitol, myo-inositol, ornithine, putrescine, sarcosine, L-citulline, citric acid or gluconic acid. Results were variable for use of trehalose, L-rhamnose, maltose, cellobiose, lactose, melibiose, myagdalin, L-leucine, L-histidine, lysine, D-fructose, sucrose, glycerol, sodium acetate, propionic acid, pyruvate, L-serine, glutamic acid, D-alanine, L-arginine, aspartic acid, β-hydroxybutyric acid, fumaric acid, D-saccharic acid and glyceric acid. Regarding enzymatic activities tested with the API ZYM system, all strains showed a similar enzymatic profile with the presence of alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase activity. Leucine arylamidase was absent only in the type strain LFT 1.7⁹, and isolate LT4C 2.8 showed lipase (C14) activity (Table S2).

The results from this polyphasic analysis support the identity of the strains representing a novel member of the genus Arcobacter, for which the name Arcobacter lekithochrous sp. nov. is proposed.

**DESCRIPTION OF ARCOBACTER LEKITOCHROUS SP. NOV.**

Arcobacter lekithochrous (le.ki.tho.chro'us, Gr. adj. lekithos pale brown; Gr. n. chroa colour; N.L. masc. adj. lekitho-chorous with pale brown colour).

Cells are Gram-stain-negative motile rods. Colonies on MA are small and slightly brownish. Oxidase- and catalase-positive. Negative for the Voges–Proskauer reaction, production of indole and acid from glucose, as well as for hydrolysis of urea, casein, gelatine and starch. Hydrolysis of indoxyl acetate is negative except for strain LT4C 2.8. All strains can reduce nitrate to nitrite and are sensitive to cefoperazone (30 µg). Growth occurs at 15–25 °C except for

Fig. 2. Polar lipid profile of strain LFT 1.7⁹. DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid; PN, phosphoaminolipid; GL, glycolipid; L, lipid; P, pigment; A+, aminopositive spot.

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strains LT4C 2.8 and TM 4.6, which are able to grow at 4°C. All strains can grow under microaerophilic conditions on MA at 25°C. Halophilic; optimal growth is obtained in media with 3% (w/v) sea salts. No growth is observed without salts. Range of pH for growth is 6-8. Strains LFT 1.7T and LT4C 2.8 can grow at pH 9. No growth on TSA, TSA-2, TSA-3, TSA supplemented with 5% sheep blood, blood agar, CCDA, minimal medium, MacConkey agar, or NA supplemented with glycine, sodium deoxycholate or crystal violet. Only strain LT4C 2.8 can grow on NA supplemented with Oxgall and on TSI agar, but without hydrogen sulphide production. All strains grow on NA supplemented with safranin and, except LFT 1.7T, can also grow on this medium supplemented with TTC or fuchsine. All isolates can use as sole carbon source D-mannose, D-glucose, lactic acid, succinic acid, γ-aminobutyric acid and malic acid, but not D-ribose, arabinose, D-xylene, D-galactose, salicin, D-sorbitol, N-acetyl-D-glucosamine, L-threonine, D-mannitol, myo-inositol, ornithine, putrescine, sarcosine, L-citrulline, citric acid and gluconic acid. Alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase are present. Major fatty acids are summed feature 3 (C16:1ω7c/C16:1ω6c), summed feature 8 (C18:1ω7c and/or C18:1ω6c), C16:0, C14:0, C12:0 3-OH, summed feature 2 (C14:0 3-OH/iso-C16:1 i) and C12:0. Polar lipids are phosphatidylethanolamine, diphosphatidylglycerol, phospatidylglycerol and two unidentified phospholipids, one unidentified phosphomaminolipid and one unidentified lipid. The main respiratory quinone is MK-6.

The type strain is LFT 1.7T (=CECT 8942T=DSM 100870T) isolated from great scallop (Pecten maximus) larvae from Norway (66° 30' 53.77" N 4° 54' 14.75" W).

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**Conflicts of interest**
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**References**


