

## *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter*

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A Gram-negative bacterium, designated LA31B<sup>T</sup>, was isolated from water collected from a hypersaline lagoon on Laysan Atoll in the north-western Hawaiian Islands. Single cells of LA31B<sup>T</sup> were slightly curved but became helical as their length increased. Preliminary characterization based on 16S rRNA gene sequence analysis showed that LA31B<sup>T</sup> shared 96.0% identity with an *Arcobacter* sp. isolated from a cyanobacterial mat in hypersaline Lake Sinai, and 94% identity with *Arcobacter nitrofigilis*, the type species of the genus *Arcobacter*. A polyphasic taxonomic study was conducted and confirmed the phylogenetic affiliation of strain LA31B<sup>T</sup> to the genus *Arcobacter*. However, LA31B<sup>T</sup> was found to be distinct from all recognized *Arcobacter* species, by a comprehensive biochemical test analysis, whole-cell fatty acid profiling, DNA G+C content (35 mol% in LA31B<sup>T</sup>) and degree of DNA–DNA reassociation. Most notably, LA31B<sup>T</sup> was found to be an obligate halophile, a hitherto undescribed feature among recognized *Arcobacter* species. These data indicate that LA31B<sup>T</sup> should be considered to represent a novel species in the genus *Arcobacter*, for which the name *Arcobacter halophilus* sp. nov. is proposed. This is the first obligately halophilic member of the genus. The type strain is LA31B<sup>T</sup> (= ATCC BAA-1022<sup>T</sup> = CIP 108450<sup>T</sup>).

The currently recognized epsilon subdivision of the *Proteobacteria* represents a taxonomically diverse but phylogenetically distinct group of Gram-negative bacteria that includes the genera *Arcobacter*, *Campylobacter*, *Helicobacter*, *Wolinella*, *Sulfurospirillum*, *Nautilia*, *Caminibacter*, *Sulfurimonas*, *Sulfurovum* and *Thiovulum*, among others (Vandamme *et al.*, 1991; Schumacher *et al.*, 1992; Alain *et al.*, 2002; Miroshnichenko *et al.*, 2002; Inagaki *et al.*, 2003, 2004; Takai *et al.*, 2004). The ecological niches occupied by these bacteria vary considerably, with some species living in association with various animals (including humans) and other species that are free-living and found in environments such as sea water and anaerobic sludge. Most of the aforementioned genera contain species that are either commensals or pathogens (*Campylobacter*, *Helicobacter*, *Wolinella*) or free-living (*Sulfurospirillum*, *Nautilia*, *Caminibacter*, *Thiovulum*) but not both. At present, however, the genus

*Arcobacter* is unique among the  $\epsilon$ -*Proteobacteria* as it contains species found in both animal and environmental sources. Three of the four recognized species (*Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*) have been recovered from humans and livestock, in which they are often associated with reproductive and/or gastrointestinal diseases (Mansfield & Forsythe, 2000). The two remaining species are free-living: a nitrogen-fixing bacterium, *Arcobacter nitrofigilis*, isolated from *Spartina alterniflora* roots in a salt marsh (McClung *et al.*, 1983), and 'Candidatus *Arcobacter sulfidicus*', an obligate microaerophile that oxidizes sulfides (Wirsen *et al.*, 2002). Just as more than one species from humans and livestock has been presented, it is likely that the genus will expand as representatives from other habitats are cultivated and described. Indeed, molecular analyses of bacterial strains isolated from seawater and marine sediment samples have detected 16S rRNA gene sequences that group in the genus and thus might be considered to be *Arcobacter* spp. (Llobet-Brossa *et al.*, 1998; Bowman & McCuaig, 2003; Fera *et al.*, 2004). Identification and classification of *Arcobacter* and related organisms is challenging because they tend to respond

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of LA31B<sup>T</sup> is AF513455.

poorly, if at all, in conventional biochemical and growth tests (e.g. On, 1996).

During a molecular and cultivation-based survey of prokaryotes from little-explored aquatic habitats in Hawai'i, Donachie *et al.* (2004a) cultivated aerobic heterotrophs from the hypersaline lagoon on Laysan Atoll, including novel species (Donachie *et al.*, 2004b). Laysan Atoll is already recognized for its unique flora and fauna, and hosts over 40 endemic insect and bird species (Ely & Clapp, 1973; Bakus, 1978). Geographical isolation and high salinity, however, probably keep macrofaunal diversity in the lake low. For example, aquatic vertebrates are absent and the largest invertebrate is an *Artemia* sp., a genus commonly found in saline lakes (Maciolek, 1982; Savage & Knott, 1998). Seven million birds that annually use the island deposit guano in and around the lake, an input that probably affects the composition of the lagoon's microbial flora, just as avian populations in other areas affect local lakes and ponds (Lindeboom, 1984). We report here the isolation and characterization of strain LA31B<sup>T</sup>, a Gram-negative bacterium; its nearest reported neighbour is an undescribed strain isolated from Solar Lake, Sinai, Egypt (GenBank/EMBL/DDBJ accession no. L42994; Teske *et al.*, 1996). Phenotypic and genotypic data place the strain firmly in the genus *Arcobacter* within the  $\epsilon$ -*Proteobacteria*, but it can be distinguished from all recognized arcobacters by its obligate halophilily and fatty acid composition.

### Sample collection and water chemistry

A water sample was collected directly into a sterile 1 litre Nalgene<sup>®</sup> bottle from the surface of the hypersaline lagoon at the centre of Laysan Atoll (25° 46' N, 171° 44' W) in the north-western Hawaiian Islands in October 2000 (Donachie *et al.*, 2004a). Salinity was determined with an AGE model 2100 Minisal salinometer calibrated against International Association for the Physical Sciences of the Ocean standard (Wormley) sea water.

### Enrichment and isolation of bacteria

A 100 µl subsample of water was spread on aspartic acid medium (ASP), based on that of Pochon & Tardieux (1962), containing (per litre) 1 g aspartic acid (monosodium salt), 10 g glycerol, 1 g K<sub>2</sub>HPO<sub>4</sub>, 40 g NaCl, 15 g agar, 40 g NaCl and 1 ml SL8 micronutrient solution (Atlas, 1997). Inoculated plates were incubated aerobically in the dark at 25 °C. A 6 mm diameter, flat, rough, transparent, eroded, shiny colony was observed after 12 days incubation on ASP. This was designated strain LA31B<sup>T</sup> and was transferred to marine agar 2216E (Difco) (MA) for purification. Uniform translucent beige colonies of 0.5–1 mm diameter developed within 48 h. Strain purity was checked by microscopy, including Gram-stain and wet mounts, and consistency of colony characteristics. Strain LA31B<sup>T</sup> was maintained on MA, or on blood agar (BA), the latter containing Oxoid nutrient broth no. 2, 2% agar, 5% whole blood (sheep or horse) and 3.5% (w/v) NaCl. Stock cultures

in marine broth 2216E (Difco) (MB) and 30% (w/v) glycerol were stored frozen at –80 °C or lyophilized.

### Phenotypic analyses

Strain LA31B<sup>T</sup> was grown microaerobically at room temperature (18–22 °C) on BA for 72 h, and was subsequently examined with an extensive, standardized biochemical identification scheme for arcobacters and related bacteria (On *et al.*, 1996). Additional tests for all temperature and atmospheric tolerances were performed on BA as well as on BA without supplemental NaCl. Tolerance by LA31B<sup>T</sup> for NaCl was tested on trypticase soy agar (TSA) containing 0.5–20% (w/v) NaCl.

Nitrate reduction was determined in BA supplemented with 0.1% KNO<sub>3</sub>. Motility was determined in a hanging-drop preparation under a 100× oil-immersion objective using cells grown for 24 h in MB. Fatty acids in whole cells on BA and MB were identified by using the MIDI Sherlock Microbial Identification System (Sasser, 1997). Cells grown for 72 h in MB were prepared for scanning electron microscopy according to the method of Donachie *et al.* (2002). In addition, cells from a 10 day culture on BA were suspended in 10 µl of 1% glutaraldehyde with 0.05% sodium cacodylate (pH 7.4) on a Formvar-coated copper grid. After 30 s the cells were washed twice with sterile distilled water and negatively stained with 1% uranyl acetate. Negatively stained cells were viewed in a Leo 912 energy-filtered transmission electron microscope at 100 kV, and digital images were captured using a Proscan HighSpeed Slow-Scan System Controller.

### DNA isolation and phylogenetic analyses

Genomic DNA was extracted from cultures of strain LA31B<sup>T</sup> grown for 72 h (MB, 30 °C, shaking at 100 r.p.m.) using the G NOME DNA extraction kit (Qbiogene). A ~1.5 kb fragment of the 16S rRNA gene was amplified from genomic DNA in a PCR with *Pfu* DNA polymerase and the primers 27F and 1492R (Lane, 1991). Thermal cycling conditions were as follows: initial denaturation at 94 °C for 3 min followed by 30 cycles of 94 °C for 45 s, 55 °C for 1 min and 72 °C for 90 s. The final extension step was carried out at 72 °C for 7 min, followed by cooling to 4 °C. The PCR product was purified by using a Qiagen PCR purification kit. The purified PCR product was sequenced in both directions in a Beckmann CEQ2000 DNA analyser using the Beckmann sequencing kit and the primers 27F, 519R, 533F and 1492R (Lane, 1991). Nucleotide sequences that had been assembled and edited in SEQMAN (DNASTAR) were aligned in CLUSTAL\_X (Thompson *et al.*, 1997) with 16S rRNA gene sequences of  $\epsilon$ -*Proteobacteria* type strains and affiliated strains retrieved from GenBank. The relationship of LA31B<sup>T</sup> with these bacteria was visualized on the basis of their 16S rRNA nucleotide sequences in a phylogenetic tree constructed from a CLUSTAL\_X alignment using the neighbour-joining method (Saitou & Nei, 1987), corrected for multiple substitutions, and re-rooted in NJPLOT

(Perrière & Gouy, 1996). The tree was rendered in TreeView (Page, 1996) and into Adobe® Illustrator® for publication.

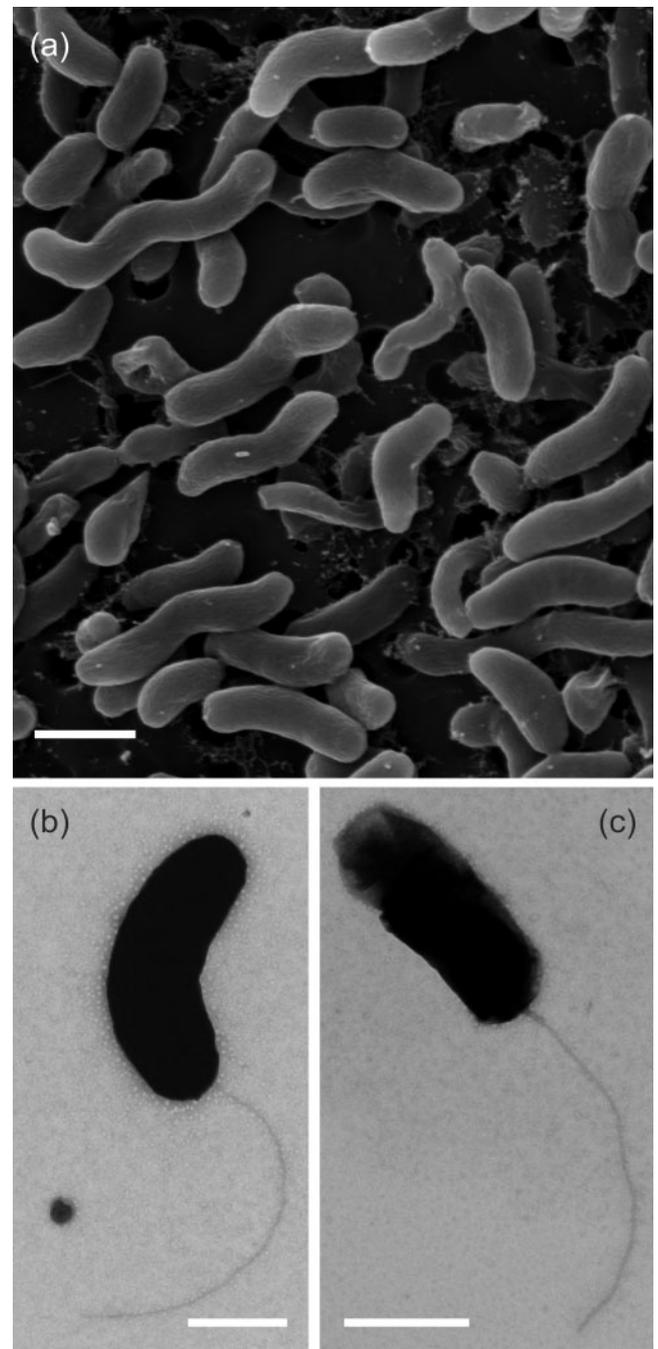
### DNA–DNA reassociation

Genomic DNA was extracted from strain LA31B<sup>T</sup> grown in MB using phenol and chloroform (Marmur, 1961). The G+C content was determined following the method of Sly *et al.* (1986). Genomic DNA was hybridized with that from the type strains of *A. nitrofigilis* NCTC 12251<sup>T</sup>, *A. butzleri* NCTC 12481<sup>T</sup>, *A. cryaerophilus* NCTC 11885<sup>T</sup> and *A. skirrowii* NCTC 12713<sup>T</sup> according to the methods of Huß *et al.* (1983) and Bowman *et al.* (1998). Hybridizations were carried out in 2 × SSC buffer, with renaturation at 64 °C, but owing to the higher G+C content of the DNA in LA31B<sup>T</sup>, the procedure was modified. After shearing by sonication, the DNA was filtered through a sterile 0.2 µm filter and transferred to cuvettes for denaturation within the spectrophotometer (GBC916 spectrophotometer; GBC Instruments) at 95 °C for 10 min. With the cuvette temperature at more than 90 °C, 20 × SSC (pre-heated to ~90 °C) was added to increase the final salt concentration to 2 × SSC. The contents were rapidly mixed by pipette. The cuvette temperature was then set to the optimal renaturation temperature and allowed to equilibrate for 5 min before measurement of renaturation commenced.

### Habitat, cell morphology and phenotypic characteristics

Salinity at the sample collection point was 76 p.p.t. Colonies of LA31B<sup>T</sup> that arose on MA after transfer from ASP were dull-white to cream, low convex, circular, entire, smooth, dull, opaque, often punctiform and rarely exceeded 1 mm in diameter. Colonies developed most rapidly on BA containing 3.5 % NaCl. Cells were curved rods, becoming slightly helical (Fig. 1a). They were also motile with a single polar flagellum (Fig. 1b, c). The comprehensive phenotyping scheme used to characterize strain LA31B<sup>T</sup> was developed principally to identify species of human and veterinary relevance, with some free-living species included for completeness (On *et al.*, 1996). Although the basal media used for the numerous tolerance tests were identified as those giving the most reproducible results for these organisms (On & Holmes, 1991), the same media do not contain sufficient NaCl to support growth of LA31B<sup>T</sup>. The small number of positive results for the tolerance tests included in the scheme highlights the halophilic nature of LA31B<sup>T</sup>, and contributes to the ease by which the strain can be discriminated from other *Arcobacter* species (Table 1). Nevertheless, LA31B<sup>T</sup> also shares typical features of recognized *Arcobacter* species, namely hydrolysis of indoxyl acetate, plus microaerophilic and aerobic growth at room temperature (~18–25 °C). Moreover, LA31B<sup>T</sup> grows anaerobically at room temperature and at 37 °C on BA containing 3.5 % (w/v) NaCl. There is no growth at 42 °C.

LA31B<sup>T</sup> grows well on media containing 2–4 % (w/v) NaCl or 0.1 % potassium nitrate (used for assessing nitrate



**Fig. 1.** (a) Scanning electron micrograph of cells of *Arcobacter halophilus* sp. nov. Bar, 1 µm. (b, c) Negatively stained cells of *A. halophilus* showing single polar flagellum. Bars, 0.5 µm. See text for preparation procedures.

reduction). We suspect the potassium fulfils the physiological requirements otherwise provided by sodium, albeit at a much lower concentration. The strain grows on TSA containing 0.5–13.5 % (w/v) NaCl. In the absence of supplemental NaCl, however, nutrient, charcoal, starch, MacConkey, lecithin, tyrosine, casein and BA media do not

**Table 1.** Characteristics that differentiate *Arcobacter halophilus* sp. nov. LA31B<sup>T</sup> from *A. butzleri*, *A. cryaerophilus*, *A. nitrofigilis* and *A. skirrowii*

1, *A. halophilus* (n=1 strain); 2, *A. nitrofigilis* (n=2); 3, *A. cryaerophilus* (n=19); 4, *A. butzleri* (n=12); 5, *A. skirrowii* (n=9). Percentage of strains testing positive is shown. Data for *Arcobacter* reference species were taken from On *et al.* (1996) and Atabay *et al.* (1998). All strains were oxidase-positive, reduced nitrate and grew in air at 25 °C.

Characteristic	1	2	3	4	5
Catalase activity	0	100	100	33	100
Urease activity	0	100	0	0	0
Growth in:					
Air at 37 °C	100	50	50	100	100
2% (w/v) NaCl	100	100	84	92	100
4% (w/v) NaCl	100	100	0	0	100
Percentage of strains resistant to:					
Cephalothin (32 mg l <sup>-1</sup> )	0	0	100	100	100
Carbencillin	0	0	100	100	100
Methyl orange	0	0	100	100	100

support growth, despite incubation periods of up to 4 weeks. The strain grew vigorously on BA when 3.5% (w/v) NaCl was added. Obligate halophily is thus the main trait that distinguishes LA31B<sup>T</sup> from the four *Arcobacter* species with validly published names (Table 1). A detailed description of the phenotype of LA31B<sup>T</sup> is given below.

The major fatty acids in whole cells of LA31B<sup>T</sup> are hexadecanoic (C<sub>16:0</sub>), *cis*-9-hexadecenoic acid (C<sub>16:1</sub>ω7*c*) and *cis*-9-octadecanoic acid (C<sub>18:1</sub>ω7*c*) (Table 2). Finster *et al.* (1997) described the potential taxonomic value of the fatty acid composition of *Campylobacter–Helicobacter–Arcobacter* strains. In this respect, a relationship between strain LA31B<sup>T</sup> and *A. nitrofigilis* is supported by the absence from only these two within the genus of C<sub>14:1</sub> *cis* 7 and C<sub>16:1</sub> *trans* 9, whereas only they contain C<sub>12:0</sub> 3-OH. In terms of differentiating LA31B<sup>T</sup>, however, only this strain within the genus lacks C<sub>14:0</sub> 3-OH.

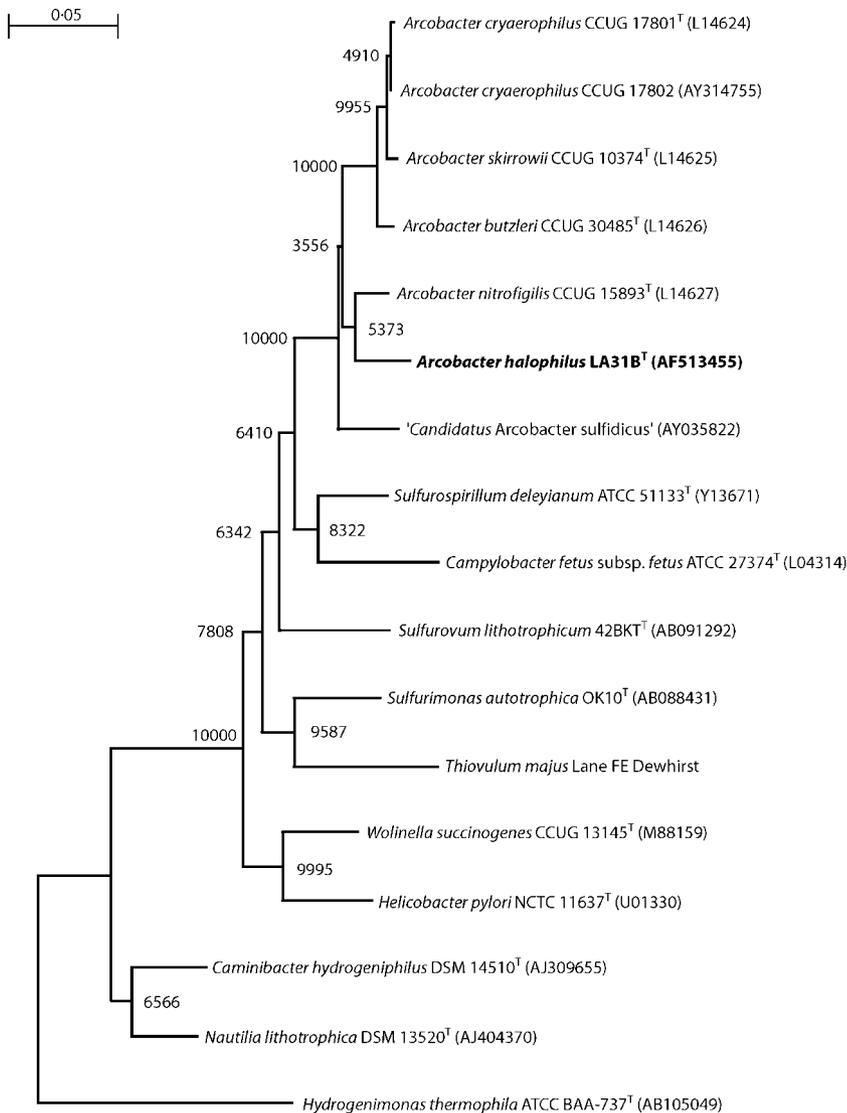
### Genomic analyses

The nucleotide sequence of the 16S rRNA gene in LA31B<sup>T</sup> shares 94% identity over 1402 bases with that of *A. nitrofigilis* NCTC 12251<sup>T</sup>, its nearest neighbour on this basis, and only ~91–93% with the other recognized *arcobacters*. The nucleotide sequence of the 16S rRNA gene of strain LA31B<sup>T</sup> falls firmly within the genus *Arcobacter* (Fig. 2). The G+C content of strain LA31B<sup>T</sup> (35 mol%) also differentiates it from recognized *arcobacters*, namely *A. butzleri* NCTC 12481<sup>T</sup> and *A. cryaerophilus* NCTC 11885<sup>T</sup> (28 mol%), and *A. nitrofigilis* NCTC 12251<sup>T</sup> and *A. skirrowii* NCTC 12713<sup>T</sup> (29 mol%). The same type strains of *A. nitrofigilis* and *A. skirrowii* showed only 4% DNA–DNA reassociation with LA31B<sup>T</sup>, while *A. butzleri* and *A. cryaerophilus* showed 10 and 12% DNA–DNA reassociation, respectively (these values are means of three to four replicate analyses). These data suggest that LA31B<sup>T</sup> cannot

**Table 2.** Fatty acid composition of *Arcobacter halophilus* sp. nov. and recognized members of the genus *Arcobacter*

Strains: 1, *A. halophilus* on MA, 30 °C; 2, *A. halophilus* on BA, 37 °C; 3, *A. nitrofigilis*; 4, *A. butzleri*; 5, *A. skirrowii*; 6, *A. cryaerophilus* subgroup 1; 7, *A. cryaerophilus* subgroup 2. –, Not determined or less than 1%. Data for 3–7 are from Vandamme *et al.* (1992).

Fatty acid	1	2	3	4	5	6	7
C <sub>12:0</sub>	8.51	8.20	7.2	6.8	9.1	3.7	7.7
C <sub>14:0</sub>	5.40	5.04	4.8	4.8	2.4	1.8	1.7
C <sub>15:0</sub>	–	0.32	–	–	–	–	–
C <sub>15:0</sub> anteiso	–	0.31	–	–	–	–	–
C <sub>16:0</sub>	25.77	19.31	32.0	19.6	22.2	18.7	17.8
C <sub>18:0</sub>	4.14	0.43	–	–	–	–	–
Total saturated fatty acids	43.82	33.61	44.0	31.2	33.7	24.2	27.2
C <sub>14:1</sub> <i>cis</i> 7	–	–	–	4.9	1.4	9.2	9.2
C <sub>16:1</sub> iso I, or C <sub>14:0</sub> 3OH or 12:0 alde	2.39	2.07	–	–	–	–	–
C <sub>16:1</sub> ω5 <i>c</i>	–	0.42	–	–	–	–	–
C <sub>16:1</sub> ω7 <i>c</i>	25.90	37.92	30.9	19.2	22.8	45.2	19.0
C <sub>16:1</sub> <i>trans</i> 9	–	–	–	13.8	7.8	–	18.8
C <sub>18:1</sub> ω7 <i>c</i>	25.59	21.72	12.8	11.6	19.7	10.6	10.8
Total unsaturated fatty acids	53.88	62.13	43.7	44.6	50.3	55.8	48.6
C <sub>12:0</sub> 3OH	2.30	2.68	5.5	–	–	–	–
C <sub>14:0</sub> 3OH	–	–	5.3	17.4	9.2	9.4	14.3
C <sub>19</sub> cyclo ω10 <i>c</i>	–	0.52	–	–	–	–	–
Named (%)	100	98.94	98.5	98.1	94.6	98.6	99.3



**Fig. 2.** Phylogenetic tree showing the relationship of *Arcobacter halophilus* sp. nov. LA31B<sup>T</sup> with representatives of the  $\epsilon$ -Proteobacteria, on the basis of 1133 nucleotides from the 16S rRNA gene. See text for a description of the alignment and presentation procedures. The  $\epsilon$ -proteobacterium *Hydrogenimonas thermophila* ATCC BAA-737<sup>T</sup> was used as the outgroup. Numbers at nodes are bootstrap values based on 10 000 replicates. Bar, 0.05 nucleotide substitutions per site.

be considered to represent any of the recognized *Arcobacter* species (Wayne *et al.*, 1987).

In light of the differences described here between LA31B<sup>T</sup> and recognized *Arcobacter* species, we propose that LA31B<sup>T</sup> is the type strain of a novel species within the genus, for which the name *Arcobacter halophilus* sp. nov. is proposed. We believe this is the first documented report of an obligately halophilic *Arcobacter* species.

#### Description of *Arcobacter halophilus* sp. nov.

*Arcobacter halophilus* (ha.lo'phi.us. Gr. n. *hals halos* salt; Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt-loving).

Cells are slightly curved rods, becoming helical as cells lengthen, 0.4–0.5  $\mu$ m wide and 1.5–2.5  $\mu$ m long. Cells stain Gram-negative. After 72 h incubation at 18–22 °C under microaerobic conditions on 5% blood agar containing 3.5% NaCl, colonies are smooth, off-white, circular with entire margins, convex, non-swarming with a sticky

consistency and ~1–2 mm in diameter. Cells are motile by a single polar flagellum. Does not grow, or grows only poorly, on media that contain less than 2% NaCl or less than 0.1% KNO<sub>3</sub>. Oxidase is produced. Indoxyl acetate is hydrolysed. Nitrate is reduced. Does not produce catalase, urease, alkaline phosphatase or DNase. Hippurate is not hydrolysed. Haemolysis is not observed. Hydrogen sulfide is not produced in triple-sugar iron agar. When tested on 5% blood agar containing 3.5% NaCl, growth is observed under aerobic and microaerobic conditions at room temperature and at 37 °C, and under anaerobic conditions at 37 °C. Growth is not observed on 5% blood agar containing 3.5% NaCl under microaerobic conditions at 42 °C. Grows microaerobically on 5% BA containing 2–4% (w/v) NaCl. Growth is not observed under microaerobic conditions on nutrient, charcoal, minimal, MacConkey, potato starch, lecithin, tyrosine or casein media. Growth is not observed under microaerobic conditions on media containing 0.04% triphenyl-tetrazolium chloride, 1% glycine, 0.1% potassium permanganate, 0.001% sodium

arsenite, 0.02–0.05 % safranin, 32 mg nalidixic acid l<sup>-1</sup>, 32 mg cephalothin l<sup>-1</sup>, 32 mg carbenicillin l<sup>-1</sup>, 64 mg cefoperazone l<sup>-1</sup>, 0.032 % methyl orange, 0.1 % sodium deoxycholate, 0.005 % basic fuchsin, 0.0005 % crystal violet, 0.01 % janus green, 0.1 % sodium fluoride or 0.2 % pyronin. Major fatty acids in whole cells grown for 72 h on BA or MA are C<sub>16:1</sub>ω7c, C<sub>18:1</sub>ω7c and C<sub>16:0</sub>; C<sub>14:0</sub> 3-OH is absent.

The type strain is LA31B<sup>T</sup> (=ATCC BAA-1022<sup>T</sup>=CIP 108450<sup>T</sup>).

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