# *Alishewanella aestuarii* sp. nov., isolated from tidal flat sediment, and emended description of the genus *Alishewanella*

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A Gram-negative strain, B11$^T$, was isolated from tidal flat sediment in Yeosu, Republic of Korea. Strain B11$^T$ did not require NaCl for growth and grew between 18 and 44 °C. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain B11$^T$ was associated with the genus *Alishewanella* and was closely related to the type strain of *Alishewanella fetalis* (98.3 % similarity). Within the phylogenetic tree, the novel isolate shared a branching point with *A. fetalis*. Analysis of 16S rRNA gene sequences and DNA–DNA relatedness, as well as physiological and biochemical tests, indicated genotypic and phenotypic differences between strain B11$^T$ and the type strain of *A. fetalis*. Thus, strain B11$^T$ is proposed as a representative of a novel species, *Alishewanella aestuarii* sp. nov.; the type strain is B11$^T$ (=KCTC 22051$^T$ =DSM 19476$^T$).

The genus *Alishewanella*, proposed by Fonnesbech Vogel *et al.* (2000), belongs to the class *Gammaproteobacteria*. Currently, *Alishewanella fetalis* is the only species within the genus and it was first isolated from an autopsy of a human fetus in Sweden. Here, strain B11$^T$, a representative of another *Alishewanella* species, was isolated from a marine environment and characterized.

Strain B11$^T$ was isolated from tidal flat sediment in Yeosu (34° 47′ 26″ N 127° 34′ 01″ E), Republic of Korea. The strain was isolated on R2A agar (Difco) followed by repeated restreaking to obtain a pure culture. The Gram reaction was determined according to the non-staining method described by Buck (1982). Cell morphology was examined by light microscopy (ECLIPSE 80*i*; Nikon) and electron microscopy. R2A broth was used to examine growth under various NaCl concentrations and temperature conditions. Growth on tryptic soy agar (TSA; Difco), marine agar (MA; Difco), nutrient agar (NA; Difco), Luria agar (LA; Difco) and blood agar containing 5 % sheep blood was also determined. Starch hydrolysis was performed as described by Smibert & Krieg (1994). Catalase activity was determined by observing bubble production in a 3 % (v/v) hydrogen peroxide solution. Enzyme activities and substrate utilization from sole carbon sources were determined using the API 20NE and API ZYM test strips (bioMérieux). Some of these results are given in the species description; Table 1 shows a comparison between the characteristics of strain B11$^T$ and closely related strains.

Chromosomal DNA was extracted and purified using a DNA extraction kit (IntronBiotechnology). The G+C content was determined using HPLC as described by Mesbah & Whitman (1989). The 16S rRNA gene was amplified by PCR from chromosomal DNA using two universal primers for bacteria (Baker *et al.*, 2003). The PCR product was purified and sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer's instructions. Reaction mixtures were analysed using an automated DNA analyser system (PRISM 3730XL DNA analyser; Applied Biosystems). Full-length 16S rRNA gene sequences were assembled using SEQMAN software (DNASTAR). 16S rRNA gene sequences from the novel isolate and related taxa (obtained from the NCBI database) were aligned using the multiple sequence alignment program CLUSTAL_X 1.8 (Thompson *et al.*, 1997). Pairwise 16S rRNA gene sequence similarities were determined using the WATER program in EMBOSS (Rice *et al.*, 2000). Phylogenetic relationships between representative species in the phylogenetic neighbourhood were determined using the programs MEGA3 (Kumar *et al.*, 2004) and PAUP 4.0 (Swofford, 1998). Distance matrices were determined (Kimura, 1980) and used to elaborate dendrograms by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and maximum-

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B11$^T$ is EF660759.

**Table 1.** Differential characteristics of strain B11$^T$ and type strains of closely related species

Strains: 1, *Alishewanella aestuarii* sp. nov. B11$^T$; 2, *A. fetalis* CCUG 30811$^T$ (data from Fonnesbech Vogel *et al.*, 2000); 3, *R. aquimaris* SW-353$^T$ (Yoon *et al.*, 2007); 4, *R. pacifica* CCUG 46544$^T$ (Romanenko *et al.*, 2003); 5, *R. perlucida* BA131$^T$ (Brettar *et al.*, 2006). Data for nitrate reduction and hydrolysis and assimilation tests are from the API 20NE test system. +, Positive; −, negative; (−), weak or negative reaction; NR, not reported; PNPG, *p*-nitrophenyl β-D-galactopyranoside.

| Characteristic | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Motility | + | − | + | + | + |
| Growth at/in: | | | | | |
| 20 °C | + | − | + | + | + |
| 0 % NaCl | + | − | + | + | + |
| 8 % NaCl | − | + | + | + | + |
| Temperature range for growth (°C) | 18–44 | 25–42 | 4–43 | 4–37 | 4–37 |
| Nitrate reduction to nitrite | + | + | + | − | + |
| β-Glucosidase (aesculin hydrolysis) | − | + | + | + | + |
| β-Galactosidase (PNPG hydrolysis) | − | − | NR | + | − |
| Assimilation of: | | | | | |
| Glucose | − | − | + | − | − |
| Arabinose | − | − | − | + | − |
| *N*-Acetylglucosamine | − | − | + | + | + |
| Maltose | + | (−) | + | + | − |
| Trisodium citrate | − | − | − | + | − |
| DNA G+C content (mol%) | 49.5 | 51.0 | 50.5 | 49.6 | 48.9 |
| Isolation source | Tidal flat sediment | Human fetus | Seawater | Seawater | Seawater |

likelihood (Felsenstein, 1981) methods. To evaluate the stability of the trees, a bootstrap analysis was performed using a consensus tree based on 1000 randomly generated trees. The 16S rRNA gene sequence of strain B11$^T$ was compared with sequences of reference strains of species belonging to the class *Gammaproteobacteria*. Fig. 1 indicates that strain B11$^T$ is affiliated phylogenetically with *A. fetalis*; this affiliation is supported by high bootstrap values (100, 98 and 94 % by the neighbour-joining, maximum-parsimony and maximum-likelihood methods, respectively). Similarities between the 16S rRNA gene sequence of strain B11$^T$ and those of *A. fetalis* CCUG 30811$^T$ *Rheinheimera aquimaris* SW-353$^T$ and *Rheinheimera perlucida* BA131$^T$ were 98.3, 96.5 and 96.4 %, respectively. A DNA–DNA hybridization experiment was performed as described previously (Roh *et al.*, 2008); the mean DNA–DNA relatedness value between the isolate and *A. fetalis* CCUG 30811$^T$ was 47.0 %. The 16S rRNA gene sequence similarities and the DNA–DNA relatedness value of less than 70 % (Wayne *et al.*, 1987) indicate that the isolate represents a distinct genospecies.

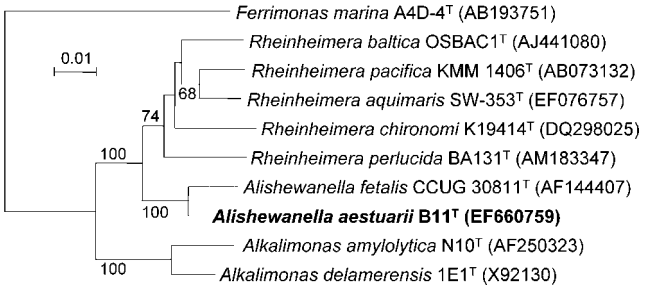


**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences. The position of strain B11$^T$ is shown with respect to strains of related species. The tree was generated using the neighbour-joining method. Numbers at nodes indicate bootstrap values (expressed as percentages of 1000 replications); values greater than 50 % are shown. Bar, 0.01 accumulated changes per nucleotide.

For quantitative analysis of cellular fatty acids, strain B11$^T$ and *A. fetalis* CCUG 30811$^T$ were grown under the same conditions on blood agar plates at 30 °C for 2 days. Cells were harvested and cellular fatty acids were saponified, methylated and extracted as described by the Sherlock Microbial Identification System (MIDI, 1999). Fatty acids were analysed by GC (Hewlett Packard 6890) and identified using the Microbial Identification software package (Sasser, 1990). The predominant cellular fatty acids in strain B11$^T$ were $C_{18:1}\omega7c$, $C_{17:1}\omega8c$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and $C_{17:0}$. Complete fatty acid compositions of the novel strain and *A. fetalis* CCUG 30811$^T$ are shown in Table 2. The fatty acid profiles of strain B11$^T$ and the type strain of *A. fetalis* are similar. In addition to 16S rRNA gene similarity, the major fatty acid components of strain B11$^T$ and its temperature range for growth confirm affiliation of this strain with the genus *Alishewanella*.

In spite of the close 16S rRNA gene sequence similarity between members of the genera *Alishewanella* and

**Table 2.** Fatty acid contents (%) of strain B11$^T$ and *A. fetalis* CCUG 30811$^T$

Data are from this study. Both strains were grown on blood agar at 30 °C for 2 days. Values shown are percentages of total fatty acids. tr, Trace (less than 1.0 %).

| Fatty acid | Strain B11$^T$ | *A. fetalis* CCUG 30811$^T$ |
|---|---|---|
| $C_{11:0}$ 3-OH | 2.2 | 1.7 |
| $C_{12:0}$ | tr | 1.0 |
| $C_{12:0}$ 3-OH | 3.6 | 3.6 |
| $C_{14:0}$ | tr | 2.1 |
| $C_{15:0}$ | 1.2 | 1.9 |
| $C_{15:1}\omega 8c$ | 1.2 | tr |
| $C_{16:0}$ | 11.0 | 14.3 |
| iso-$C_{16:0}$ | 1.3 | tr |
| $C_{17:0}$ | 8.8 | 8.6 |
| $C_{17:1}\omega 6c$ | 1.5 | tr |
| $C_{17:1}\omega 8c$ | 18.0 | 16.0 |
| $C_{18:0}$ | 1.6 | 1.3 |
| iso-$C_{18:0}$ | 1.7 | tr |
| $C_{18:1}\omega 7c$ | 22.6 | 17.5 |
| $C_{18:1}\omega 9c$ | 1.3 | 2.0 |
| Summed feature 1* | 2.3 | 1.8 |
| Summed feature 2* | 3.0 | 3.1 |
| Summed feature 3* | 11.8 | 14.9 |

*Summed features are groups of two or three fatty acids that can not be separated using the MIDI System. Summed feature 1 comprises $C_{13:0}$ 3-OH and/or iso-$C_{15:1}$ H; summed feature 2 comprises unknown fatty acids; summed feature 3 comprises $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH.

*Rheinheimera*, the two genera are divided with a high bootstrap value of 100 % in the phylogenetic tree and the lowest temperature for growth of *Alishewanella* species is significantly higher than that of *Rheinheimera* species. At the time of writing, there are not enough data to support combination of the two genera. It may be possible to amalgamate the two genera if more strains that are closely related to the two genera are isolated, followed by accumulation of the phylogenetic data. In this study, analysis of 16S rRNA gene sequences and DNA–DNA relatedness data, as well as physiological and biochemical tests, have identified genotypic and phenotypic differences between strain B11$^T$ and other species. Taken together, these data clearly differentiate the novel isolate from strains of closely related species. For these reasons, it is concluded that strain B11$^T$ represents a novel species of the genus *Alishewanella*, for which the name *Alishewanella aestuarii* is proposed.

## Emended description of the genus *Alishewanella*

*Alishewanella* (A.li.she.wa.nel'la. L. pron. *alius* the other; N.L. fem. n. *Shewanella* a bacterial genus name; N.L. fem. n. *Alishewanella* the other *Shewanella*).

The description of the genus is based on that given by Fonnesbech Vogel *et al.* (2000), with the following amendments. Cells are Gram-negative, motile or non-motile rods. Oxidase- and catalase-positive. The requirement for NaCl for growth is dependent on the species. Hydrolyse gelatin, but are unable to produce indole, urease, β-galactosidase or arginine dihydrolase. Do not grow at temperatures of 17 °C or less. The DNA G+C content is 49.5–51.0 mol%. The type species is *Alishewanella fetalis*.

## Description of *Alishewanella aestuarii* sp. nov.

*Alishewanella aestuarii* (aes.tu.a'ri.i. L. gen. n. *aestuarii* of a tidal flat).

Cells are Gram-negative and motile with a single polar flagellum. Colonies are transparent and round with a diameter of 0.5–1.0 mm after incubation for 2 days on R2A agar plates at 37 °C. Also grows on TSA, MA, LA and blood agar, but not on NA. Does not require NaCl for growth but is able to grow in up to 5 % NaCl, with optimal growth at 3 % (w/v) NaCl. The temperature range for growth is 18–44 °C (optimum, 37 °C), but no growth occurs at 17 or 45 °C. Catalase- and oxidase-positive. Can reduce nitrate to nitrite and nitrogen. Hydrolyses gelatin, but not starch, aesculin or *p*-nitrophenyl β-D-galactopyranoside. Negative for indole production, glucose fermentation, arginine dihydrolase and urease. Maltose is assimilated. Glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid are not assimilated. Assays using the API ZYM system are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are not observed. Predominant fatty acids are $C_{18:1}\omega 7c$, $C_{17:1}\omega 8c$, summed feature 3 ($C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and $C_{17:0}$.

The type strain is B11$^T$ (=KCTC 22051$^T$ =DSM 19476$^T$), isolated from tidal flat sediment in Yeosu, Republic of Korea. The genomic DNA G+C content of the type strain is 49.5 mol%.

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