# *Alishewanella agri* sp. nov., isolated from landfill soil

Min-Soo Kim,[1,2]† Seon Kyung Jo,[3]† Seong Woon Roh[1,2] and Jin-Woo Bae[1,2]

Correspondence
Jin-Woo Bae
baejw@khu.ac.kr

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

[2]University of Science and Technology, Biological Resources Center, KRIBB, Daejeon 305-333, Republic of Korea

[3]Ewha Woman's University, Seoul 120-750, Republic of Korea

Strain BL06$^T$ was isolated from landfill soil in Pohang, Korea. Strain BL06$^T$ is Gram-negative, aerobic, non-motile and rod-shaped. For growth, the NaCl range is 0–6 % (w/v), the temperature range is 10–44 °C and the pH range is 5.5–12.0. Based on the 16S rRNA gene and gyrase B (*gyrB*) gene sequences, phylogenetic analysis showed that strain BL06$^T$ is associated with the genus *Alishewanella* and related closely to the type strains of *Alishewanella* species (98.8 % 16S rRNA gene sequence similarity to *Alishewanella aestuarii*, 98.7 % to *Alishewanella fetalis* and 98.5 % to *Alishewanella jeotgali*). Physiological and biochemical tests verified that strain BL06$^T$ is genotypically and phenotypically different from previously described species in the genus *Alishewanella*. DNA–DNA hybridization experiments showed that relatedness between the genomic DNA of strain BL06$^T$ and type strains of other *Alishewanella* species is <41 %. These findings suggest strongly that the strain represents a novel species, despite high 16S rRNA gene sequence similarity between strain BL06$^T$ and related strains. Therefore, strain BL06$^T$ (=KCTC 22400$^T$=JCM 15597$^T$) is proposed to represent a novel species in the genus *Alishewanella*, named *Alishewanella agri* sp. nov.

The genus *Alishewanella* was first proposed by Vogel *et al.* (2000). *Alishewanella fetalis* was isolated from an autopsied human fetus by U. B. Stolt, Akademiska Sjikhuset, Uppsala, Sweden, in November 1992. In 2008, a second strain of *Alishewanella*, strain B11$^T$, was reported to represent a distinct species. This strain had been isolated from the tidal flat sediment in Yeosu, South Korea, and the novel species was named *Alishewanella aestuarii* (Roh *et al.*, 2009). Recently, *Alishewanella jeotgali* MS1$^T$ was isolated from a traditional fermented food in Korea by Kim *et al.* (2009). In this article, we describe another strain in the genus *Alishewanella*, strain BL06$^T$, isolated from landfill soil from iron manufacture in Pohang, South Korea; strain BL06$^T$ is proposed to represent a novel species named *Alishewanella agri* sp. nov.

In order to obtain a pure culture, cells of strain BL06$^T$ were diluted $10^6$-fold with PBS and cultured on Luria–Bertani agar (LA; BBL). The Gram reaction was performed using a Gram staining kit (bioMérieux). Cell morphology and motility were observed by light microscopy (ECLIPSE 80*i*; Nikon) and transmission electron microscopy (JEM 1010; JEOL) after the cells were incubated for 2 days at 30 °C. The cells were also incubated in an anaerobic chamber for 2 days in order to determine the extent of growth under anaerobic conditions. Cells were grown on LA and incubated for 2 days at 0, 4, 10, 15, 25, 30, 37, 40, 44 or 45 °C in order to determine the optimal growth temperature. The optimal NaCl concentration and pH for growth were determined by growing the strain for 2 days on LA with an NaCl concentration that ranged between 0 and 6 % and a pH that ranged from 4.0 to 13.0. Optimal substrate growth was assessed after culturing cells of strain BL06$^T$ on LA, tryptic soy agar (TSA), R2A agar, marine agar (MA) and nutrient agar (NA), all made with BBL medium. LA was the most suitable substrate for growth and was used for mass production of the strain. All experiments were performed with the strains cultivated and maintained in LA at 30 °C, 2 % NaCl and pH 7.0±0.2, unless stated otherwise. *A. fetalis* CCUG 30811$^T$, *A. aestuarii* B11$^T$ and *A. jeotgali* MS1$^T$ were used as reference strains and were grown under the same culture conditions.

†These authors contributed equally to this work.

Abbreviation: *gyrB*, gyrase B.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and partial *gyrB* gene sequences of strain BL06$^T$ are EU909459 and FJ908754, respectively.

A supplementary figure and two supplementary tables showing the results of *gyrB* sequence analysis, DNA–DNA hybridization and fatty acid profile of strain BL06$^T$, respectively, are available with the online version of this paper.

Oxidase activity was assessed with 1 % (v/v) *p*-tetramethyl phenylenediamine (bioMérieux). Catalase activity was determined by observing the bubbles produced after 3 % hydrogen peroxide was added to a sample of cells of strain BL06$^T$. Starch hydrolysis was studied in cells cultured on MA after 0.2 % starch and a few drops of iodine had been poured onto the culture medium, as described by Smibert & Krieg (1994). API 20NE and API 50CH test strips (bioMérieux) were used to test enzyme activities and substrate utilization from a sole carbon source, respectively. An API ZYM kit (bioMérieux) was also used to identify the enzyme activities of the strain. Specifically, cells were grown at 30 °C for 4, 24 or 72 h. Chromosomal DNA was extracted and purified using a DNA extraction kit (iNtRON Biotechnology). The G+C content was determined by a fluorimetric method using SYBR green I and a real-time PCR thermocycler (Gonzalez & Saiz-Jimenez, 2002). The physiological and biochemical characteristics of strain BL06$^T$ are compared with those of other strains in Table 1.

Sequencing of the 16S rRNA gene was performed as described previously (Roh *et al.*, 2008). 16S rRNA gene sequences were assembled using SeqMan software (DNASTAR) and aligned using CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997). Phylogenetic relationships between strain BL06$^T$ and representative species were defined by using MEGA4 (Tamura *et al.*, 2007). A consensus phylogenetic tree was generated by the neighbour-joining and maximum-parsimony methods from 1000 random replicates (Fig. 1) (Kluge & Farris, 1969; Saitou & Nei, 1987). 16S rRNA gene sequence similarities between the isolate and type strains of species in the genus *Alishewanella* were calculated by using the PairPro2 pairwise alignment program (http://microbecol.khu.ac.kr) and the EzTaxon server 2.1 (Chun *et al.*, 2007). Similarities between strain BL06$^T$ and the reference strains were 98.8, 98.7 and 98.5 % *for A. aestuarii* B11$^T$, *A. fetalis* CCUG 30811$^T$ and *A. jeotgali* MS1$^T$, respectively.

Amplified gyrase B (*gyrB*) sequence was obtained as described previously (Vogel *et al.*, 2000; Yamamoto &

**Table 1.** Comparison of the characteristics of strain BL06$^T$ with type strains of closely related species

Taxa: 1, strain BL06$^T$; 2, *A. fetalis* CCUG 30811$^T$; 3, *A. aestuarii* B11$^T$; 4, *A. jeotgali* MS1$^T$. Except where indicated, data were obtained in the present study. All strains are positive for reduction of nitrate to nitrite, gelatin hydrolysis, assimilation of maltose, sucrose and starch. All species are negative for indole production, D-glucose fermentation, L-arginine dihydrolase, urease, β-galatosidase and assimilation of potassium gluconate, capric acid, adipic acid, trisodium citrate, phenylacetic acid, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xyloside, D-galactose, D-mannose, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, D-lactose, melibiose, inulin, melezitose, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate and 5-ketogluconate. +, Positive; (+), weakly positive; −, negative.

| Characteristic | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Motility | − | −* | + | + |
| Growth at: | | | | |
| 4 °C | − | −* | − | (+) |
| 10 °C | + | −* | − | + |
| Temperature range (°C) | 10–44 | 25–42* | 18–44 | 4–40 |
| Growth in: | | | | |
| 0 % NaCl | + | −* | + | − |
| 6 % NaCl | + | +* | − | − |
| 8 % NaCl | − | +* | − | − |
| NaCl concentration range (%) | 0–6 | 0–15* | 0–5 | 1–2 |
| Hydrolysis of aesculin | + | + | − | + |
| Assimilation of: | | | | |
| D-Glucose | + | − | − | + |
| Malate | − | + | − | − |
| Glycerol | − | + | − | − |
| D-Fructose | − | + | + | − |
| Inositol | − | + | − | − |
| D-Mannitol | − | + | + | − |
| Aesculin | + | − | − | + |
| Trehalose | − | − | − | + |
| Raffinose | − | − | + | − |
| Glycogen | − | − | − | + |
| DNA G+C content (mol%) | 54.8 | 51.0* | 49.5 | 53.6 |
| Isolation source | Soil | Human fetus | Tidal flat sediment | Fermented food |

*Data from Vogel *et al.* (2000).

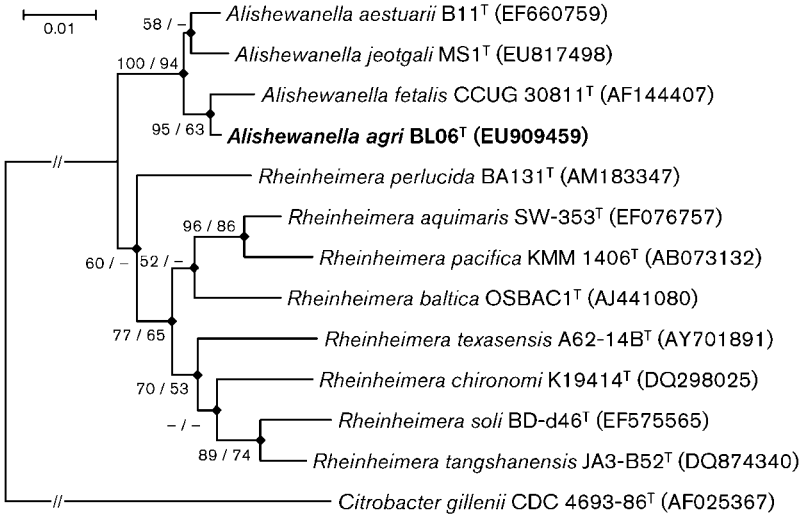


**Fig. 1.** Phylogenetic consensus tree based on 16S rRNA gene sequences. In the tree, the position of strain BL06$^T$ belongs in the genus *Alishewanella*. The tree was configured using the neighbour-joining and maximum-parsimony methods; ◆ indicates generic branches that were present in phylogenetic trees generated by both methods. Numbers at nodes denote bootstrap values as a percentage of 1000 replications. Bootstrap values >50 % are presented at branch points. Bar, 0.01 expected changes per nucleotide.

Harayama, 1995). The amplified fragments from strains BL06$^T$, B11$^T$ and MS1$^T$ were sequenced as described above for the 16S rRNA gene. Phylogenetic analysis was performed by the neighbour-joining method with 1000 random replicates. *gyrB* gene sequence similarities to other species within the genus *Alishewanella* were <95.6 % (91.4 % to strain CCUG 30811$^T$ and 95.6 % to strains B11$^T$ and MS1$^T$). Although no precise *gyrB* gene sequence-similarity threshold is available at the species level for the genus *Alishewanella*, sequence similarities between the genus *Alishewanella* and close genera were <75 % and similarities between the isolate and other *Alishewanella* species were >91.4 % (Vogel *et al.*, 2000). Moreover, a species threshold for *gyrB* gene sequence similarity was given as >90 % in the genus *Shewanella*, as described by Venkateswaran *et al.* (1999). In conclusion, analysis of the *gyrB* gene sequence allowed us to differentiate strain BL06$^T$ from reference strains belonging to the genus *Alishewanella*. However, study of the *gyrB* gene is less appropriate to differentiate phylogenetic relationships of isolates at the species level than the 16S rRNA gene, because *gyrB* gene sequence similarities are generally lower than 16S rRNA gene sequence similarities (Ochman & Wilson, 1987). The phylogenetic positions occupied by the members of the genus *Alishewanella*, based on *gyrB* nucleotide sequences, are given in Supplementary Fig. S1 (available in IJSEM Online).

DNA–DNA hybridization experiments (Ezaki *et al.*, 1989; Hirayama *et al.*, 1996) tested the relatedness between the genomic DNA of strain BL06$^T$ and close relatives, tested by a reciprocal method. This revealed a relatedness of 41 % with *A. fetalis* CCUG 30811$^T$, 36 % with *A. aestuarii* B11$^T$ and 26 % with *A. jeotgali* MS1$^T$. These findings suggest strongly that the strain represents a novel species, because there was <70 % relatedness with other species in the genus (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987). The DNA–DNA hybridization results are given in Supplementary Table S1 (available in IJSEM Online).

Strain BL06$^T$and reference strains were grown on blood agar containing 5 % sheep blood for 2 days at 30 °C. The cells were harvested, and saponification, methylation, and extraction were performed as described by the Sherlock Microbial Identification Systems (MIDI, 1999). Fatty acids were analysed by using GC (Hewlett Packard 6890) and identified with the Microbial Identification software package (Sasser, 1990). The dominant cellular fatty acids of strain BL06$^T$ were $C_{16:0}$ (10.1 %), $C_{17:1}\omega8c$ (20.2 %), $C_{17:0}$ (10.2 %), $C_{18:1}\omega7c$ (12.7 %), $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (15.1 %). The general pattern of predominant fatty acids of strain BL06$^T$ was similar to those of described *Alishewanella* species, and the differences in the fatty acids characteristic of the genus *Alishewanella* compared with the genus *Shewanella*, as described by Vogel *et al.* (2000), were also present in the fatty acids of strain BL06$^T$; for instance, the lack of iso-branched 15-carbon and iso-branched 13-carbon in the genus *Alishewanella*. The fatty acid profiles are presented in Supplementary Table S2 (available in IJSEM Online).

The *Alishewanella* species described thus far, including the species proposed (strain BL06$^T$) in the present study, are Gram-negative rods. They have oxidase and catalase activities and are able to hydrolyse gelatin. They do not produce indole, urease, β-galactosidase or arginine dihydrolase (Kim *et al.*, 2009; Roh *et al.*, 2009; Vogel *et al.*, 2000). Strain BL06$^T$ has similar phenotypic characteristics to other *Alishewanella* species, but the phenotypic data in Table 1 reveal the enzymological and metabolic variability among members of the genus *Alishewanella*. In conclusion, the morphological, physiological and biochemical characteristics, the phylogenetic consensus tree and the results of DNA–DNA hybridization suggest strongly that the strain is genotypically, phenotypically and phylogenetically different from *A. fetalis*, *A. aestuarii* and *A. jeotgali*. Therefore, strain BL06$^T$ is presented here as representing a novel species of the genus *Alishewanella*, with the name *Alishewanella agri* sp. nov.

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

*Alishewanella agri* (a′gri. L. gen. n. *agri* of a field).

Cells are Gram-negative, non-motile, aerobic, rod-shaped, 1–2 μm long and 0.5 μm wide and exist as single cells after incubation on LA for 2 days at 30 °C. Colonies are 1–3 mm in diameter, circular, raised and a light ivory colour. Growth occurs at 10–44 °C in 0–6 % NaCl, with optimal growth at 30 °C in 2 % NaCl. The pH range for growth is 5.5–12.0, with an optimum pH of 6.0–8.0. Catalase and oxidase reactions are positive. Positive for reduction of nitrates to nitrites, aesculin hydrolysis and gelatin hydrolysis, but negative for β-galactosidase activity. Cannot produce indole and does not ferment glucose. No arginine dihydrolase or urease activity. Enzyme activities are present for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Enzyme activities are not present for lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. In API 20NE, D-glucose and maltose are assimilated, but L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid are not. According to the API 50CH test, D-glucose, aesculin, maltose, sucrose and starch are assimilated, but not glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl D-xylopyranoside, D-galactose, D-fructose, L-sorbose, L-rhamnose, dulcitol, *myo*-inositol, D-mannitol, D-sorbitol, methyl D-mannopyranoside, methyl D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, D-lactose, melibiose, trehalose, insulin, melezitose, raffinose, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. Growth also occurs on TSA, R2A and MA. Major fatty acids are $C_{16:0}$, $C_{17:1}\omega8c$, $C_{17:0}$, $C_{18:1}\omega7c$, $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH. The G+C content of genomic DNA of the type strain is 54.8 mol%.

The type strain, BL06$^T$ (=KCTC 22400$^T$=JCM 15597$^T$), was isolated from soil in Pohang, South Korea.

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