Zoogloea caeni sp. nov., a floc-forming bacterium isolated from activated sludge

Yongqi Shao,¹ Bok Sil Chung,^{1,2} Seung Sik Lee,² Woojun Park,³ Sang-Suk Lee⁴ and Che Ok Jeon¹

Correspondence
Che Ok Jeon
cojeon@cau.ac.kr

¹Department of Life Science, Chung-Ang University, Seoul 156-756, Republic of Korea

²Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea

³Division of Environmental Science and Ecological Engineering, Korea University, Seoul 136-701, Republic of Korea

⁴Department of Animal Science and Technology, Sunchon National University, Sunchon 540-742, Republic of Korea

Two floc-forming, nitrogen-fixing bacteria, strains EMB43^T and EMB61, obtained from activated sludge of a domestic wastewater treatment plant in Korea, were characterized. The two strains were very closely related, sharing 99.7 % 16S rRNA gene sequence similarity and showing a level of DNA–DNA relatedness of 93 %, which suggests that they represent members of a single species. Phylogenetic analysis based on 16S rRNA gene sequences showed that the two novel isolates formed a distinct phyletic lineage within the genus *Zoogloea* and were related most closely to *Zoogloea resiniphila* DhA-35^T and *Zoogloea oryzae* A-7^T, with sequence similarities of 97.2 %. Levels of DNA–DNA relatedness between strain EMB43^T and *Z. resiniphila* DhA-35^T and *Z. oryzae* A-7^T were 12.8 and 7.4 %, respectively. Cells of strains EMB43^T and EMB61 were facultatively aerobic, rod-shaped, Gram-negative and motile by means of a polar flagellum. The strains grew at temperatures of 15–40 °C (optimum: 25–30 °C) and at pH 6.0–9.0 (optimum: pH 6.5–7.5). The predominant fatty acids were C_{16:0}, C_{10:0} 3-OH and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), and the predominant polar lipid was phosphatidylethanolamine. The genomic DNA G + C content was 64.9–65.0 mol% and the major isoprenoid quinone was ubiquinone-8 (Q-8). On the basis of phenotypic, chemotaxonomic and molecular data, the isolates are considered to represent a novel species of the genus *Zoogloea*, for which the name *Zoogloea caeni* sp. nov. is proposed. The type strain is EMB43^T (=KCTC 22084^T=DSM 19389^T).

Bacteria of the genus *Zoogloea*, a member of the family *Rhodocyclaceae*, are known to form characteristic cell aggregates embedded in gelatinous matrices, often called zoogloal matrices (Dugan *et al.*, 1992). Historically, the criteria used for taxonomic classification in this genus were based on phenotypic characteristics, which has resulted in confusion in identifying members of the genus and led to *Zoogloea ramigera* ATCC 25935 (=IAM 12670) being transferred to *Duganella zoogloeoidea* based on molecular approaches (Hiraishi *et al.*, 1997). At the time of writing,

the genus *Zoogloea* comprises three recognized species, namely *Z. ramigera* (Crabtree & McCoy, 1967), *Zoogloea resiniphila* (Mohn *et al.*, 1999) and *Zoogloea oryzae* (Xie & Yokota, 2006).

Activated-sludge processes have been used to remove organic compounds as well as nutrients from wastewater, and insight into the bacterial communities is a prerequisite for understanding activated-sludge processes. Therefore, efforts have been made in our laboratory to isolate and characterize members of the bacterial community in activated sludge (Jeon *et al.*, 2003; Lu *et al.*, 2006; Park *et al.*, 2007; Ryu *et al.*, 2007). In the present study, we describe a novel species of the genus *Zoogloea* isolated from an activated-sludge process treating domestic sewage.

Strains EMB43^T and EMB61 were isolated from activated sludge of a domestic wastewater treatment plant in Pohang,

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains EMB43^T and EMB61 are DQ413148 and DQ413150, respectively.

A transmission electron micrograph showing the general morphology of negatively stained cells of strain EMB43^T, and a table detailing the cellular fatty acid compositions of strains EMB43^T and EMB61 and related species are available with the online version of this paper.

Korea. The sludge sample was diluted serially with 1% (w/v) saline solution, spread on R2A agar (Difco) and incubated at 20 °C for 7 days for isolation of bacteria. Subcultivation was performed on R2A agar at 30 °C for 5 days.

Gram staining was performed by using a bioMérieux Gram stain kit according to the manufacturer's instructions. Cell morphology and motility were studied by using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) as described previously (Jeon *et al.*, 2005). Physiological characteristics of strains EMB43^T and EMB61 were examined by growing the isolates on R2A agar at different temperatures (5–50 °C at 5 °C intervals) and in R2A broth adjusted to different pH values (5.0–10.0 at 0.5 pH unit intervals). R2A media with different pH values were prepared as described by Gomori (1955). The formation of flocs was determined by test-tube growth in R2A broth after 3 days incubation and was confirmed by examination with a phase-contrast microscope. Oxidase activity was tested based on oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated based on production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution. Hydrolysis of casein, gelatin, Tweens 80 and 20, aesculin, urea, tyrosine, and starch was investigated on R2A agar according to the methods described by Lányi (1987) and Smibert & Krieg (1994), and the results were read after 7 days at 30 °C. Nitrate reduction was determined in R2A broth supplemented with 0.1% (w/v, 11.7 mM) NaNO₃, which was incubated anaerobically in a GasPak jar at 30 °C for a maximum of 15 days. The concentrations of residual nitrate and any nitrite produced in broth media were determined by using an ICS-1000 ion chromatograph (DIONEX) according to the manufacturer's instructions. Growth under anaerobic conditions was determined on minimal medium agar (Stanier *et al.*, 1966) containing 0.2% (w/v) sodium acetate, and on R2A agar or R2A agar supplemented with 0.1% (w/v) NaNO₃ at 30 °C in a GasPak jar. For the minimal medium agar without NaNO₃, ammonium chloride was used as a nitrogen source. Acid production from carbohydrates was tested as described by Leifson (1963) and additional enzyme activities and biochemical features were determined by using API ZYM and API 20NE kits at 30 °C as recommended by the manufacturer (bioMérieux).

Growth of strains EMB43^T and EMB61 was observed at temperatures of 15–40 °C, with optimum growth at 25–30 °C. The strains grew at pH 6.0–9.0, with optimum growth at pH 6.5–7.5. Fragmented portions of flocs and pellicles could be observed with the naked eye in test tubes when strains EMB43^T and EMB61 were incubated in R2A broth for 3 days. Formation of zoogloeal matrices was also identified by using phase-contrast microscopy. Flocs were formed at late growth stages and cells became embedded in gelatinous matrices to form zoogloae, which were distinguished by a 'tree-like' morphology. All cells that were observed were rods (width, 0.6–0.9 µm; length, 1.1–

2.0 µm), motile by means of a polar flagellum (see Supplementary Fig. S1 in IJSEM Online). Strain EMB61 reduced nitrate to nitrogen gas rapidly without nitrite accumulation. However, because strain EMB43^T reduced nitrate to nitrite quickly, but reduced nitrite to nitrogen gas slowly, nitrite accumulation occurred in R2A broth media. Strains EMB43^T and EMB61 produced acid from several carbohydrates such as D-lactose, *myo*-inositol, D-galactose, D-mannitol and arbutin, results that could be used to differentiate these strains from recognized *Zoogloea* species. Anaerobic growth was not observed for 7 days at 30 °C either on minimal medium agar containing 0.2% (w/v) sodium acetate or on R2A agar without the addition of nitrate, but small colonies were observed on R2A agar after 15 days incubation. However, in the presence of 0.1% (w/v) nitrate, small colonies were obtained after 7 days incubation both on R2A agar and on minimal medium agar containing 0.2% (w/v) sodium acetate, indicating that denitrification contributed more to growth than to fermentation. Several phenotypic features of strains EMB43^T and EMB61 are presented and compared with those of related *Zoogloea* species in Table 1. Some phenotypic features of strains EMB43^T and EMB61, such as urease activity and utilization of glucose and mannitol, were different, which shows that the species represented by these two strains (see below) is metabolically diverse.

For analysis of fatty acid methyl esters, cells of strains EMB43^T and EMB61 were harvested from R2A agar plates after incubation for 3 days. Analysis of fatty acid methyl esters was performed according to the instructions of the Microbial Identification system (MIDI; Microbial ID, Inc.). Analyses of polar lipids and isoprenoid quinones were carried out by using the methods described by Komagata & Suzuki (1987). The DNA G + C content of strains EMB43^T and EMB61 was determined by using a high-performance liquid chromatograph fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE; GROM) according to the method of Tamaoka & Komagata (1984). The major respiratory lipoquinone of strains EMB43^T and EMB61 was ubiquinone-8 (Q-8). The cellular membrane of strains EMB43^T and EMB61 contained C_{16:0}, C_{10:0} 3-OH and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) as major fatty acids (see Supplementary Table S1, available in IJSEM Online). Polar lipids were dominated by a large amount of phosphatidylethanolamine, but a small amount of an amino group-containing lipid was also present. The DNA G + C contents of strains EMB43^T and EMB61 were 64.9 and 65.0 mol%, respectively. Strains EMB43^T and EMB61 were able to grow in nitrogen-free minimal broth medium (Stanier *et al.*, 1966) containing 0.2% (w/v) sodium acetate. PCR amplifications of the *nifH* gene by using the forward primer PolF, 5'-TGCGAYCCSAARGCBGACTC-3', and the reverse primer PolR, 5'-ATSGCCATCATYTCRCCGGA-3' (Poly *et al.*, 2001), on DNA extracted from strains EMB43^T and EMB61, *Z. oryzae* A-7^T, *Z. resiniphila* DhA-35^T and *Z. ramigera* ATCC 19544^T were carried out to confirm their

Table 1. Characteristics of strains EMB43^T and EMB61 and related *Zoogloea* species

Strains: 1, EMB43^T (data from the present study); 2, EMB61 (present study); 3, *Z. resiniphila* DhA-35^T (present study and Mohn *et al.*, 1999); 4, *Z. oryzae* A-7^T (Xie & Yokota, 2006); 5, *Z. ramigera* ATCC 19544^T (Unz, 1984; Xie & Yokota, 2006). Symbols: +, positive; –, negative; NA, data not available. All are positive for nitrate reduction.

Characteristic	1*	2*	3	4	5
Cell diameter (µm)	0.6–0.9	0.6–0.9	0.5–0.7	1.0	1.0–1.2
Growth at 45 °C	–	–	+	–	–
Catalase	+	+	–*	+	+
Urease	–	+	–*	+	+
Denitrification	+	+	–*	+	+
Hydrolysis of:					
Gelatin	+	+	+	–	+
Casein	–	–	+	–	+
Utilizes as sole carbon source:					
Acetate	+	+	+	–	+
Citrate	–	–	–*	–	+
Glucose	+	–	+	–	–
Mannitol	–	+	+	–	+
DNA G + C content (mol%)	64.9	65.0	NA	65.1	65.3

*Data from the present study.

nitrogen-fixing capabilities. Expected PCR product sizes (about 340 bp) of the *nifH* gene were detected for strains EMB43^T, EMB61 and *Z. oryzae* A-7^T, but not for *Z. resiniphila* DhA-35^T or *Z. ramigera* ATCC 19544^T (data not shown). The predominant fatty acids, major lipoquinone and polar lipids, and DNA G+C contents of strains EMB43^T and EMB61 were in accordance with those of members of the genus *Zoogloea* (Unz, 1984; Mohn *et al.*, 1999; Xie & Yokota, 2006).

Sequencing of the 16S rRNA gene was carried out as described by Lane (1991). The resulting 16S rRNA gene sequences (1460 nt) of strains EMB43^T and EMB61 were compared with available 16S rRNA gene sequences from GenBank by using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine an approximate phylogenetic affiliation, and gene sequences were aligned with those of closely related species by using the CLUSTAL W program (Thompson *et al.*, 1994). Phylogenetic trees were constructed by using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms available in PHYLIP version 3.6 (Felsenstein, 2002). Sequence similarity values between the novel isolates and related strains were computed by using the FASTA3 program at EBI (<http://www.ebi.ac.uk/fasta33/nucleotide.html>). Bootstrap analysis was performed according to the algorithm of the Kimura two-parameter model (Kimura, 1980) of the neighbour-joining method in the PHYLIP package. DNA–DNA hybridization was carried out to evaluate the level of genomic DNA relatedness among the two novel isolates and closely related *Zoogloea* species by using the fluorometric microplate method (Ezaki *et al.*, 1989). Fluorometric data recorded after 30 min reaction of the substrates were used for calculation of DNA–DNA hybridization values. The

signals produced by self-hybridization were inferred as 100% and the levels of DNA–DNA relatedness were calculated from the mean values of five replications.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strains EMB43^T and EMB61 formed a distinct phyletic lineage within the genus *Zoogloea* (Fig. 1). The overall topology of the neighbour-joining tree was similar to that of the maximum-likelihood and maximum-parsimony trees (data not shown). Comparative 16S rRNA gene sequence analysis revealed that strains EMB43^T and EMB61 were very closely related (99.7%), and they showed a level of DNA–DNA relatedness of 93%, which suggests that they represent members of a single species. Strains EMB43^T and EMB61 were related most closely to *Z. oryzae* A-7^T, *Z. resiniphila* DhA-35^T and *Z. ramigera* ATCC 19544^T, with 16S rRNA gene sequence similarities of 97.2, 97.2 and 96.6%, respectively. However, levels of DNA–DNA relatedness between strain EMB43^T and *Z. resiniphila* DhA-35^T and *Z. oryzae* A-7^T were 12.8 and 7.4%, respectively, which are clearly below the 70% threshold generally accepted for species delineation (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002). The physiological, biochemical and phylogenetic data presented suggest that strains EMB43^T and EMB61 represent a single novel species of the genus *Zoogloea*, for which the name *Zoogloea caeni* sp. nov. is proposed.

Description of *Zoogloea caeni* sp. nov.

Zoogloea caeni (cae'ni. L. gen. n. *caeni* of mud, referring to the isolation of the type strain from activated sludge).

Colonies on R2A agar are white, glistening, translucent, raised and circular with entire margins. Cells are facultatively

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strains EMB43^T and EMB61 and related taxa. Bootstrap values are shown as percentages of 1000 replicates; only values >50 % are shown. *Comamonas denitrificans* 123^T was used as an outgroup. Bar, 0.01 changes per nucleotide position.

aerobic, Gram-negative, short rods (about 0.6–0.9 µm wide and 1.1–2.0 µm long) that are motile by means of a polar flagellum. Positive for nitrate reduction to nitrogen gas and for nitrogen fixation. Growth occurs optimally at 25–30 °C and pH 6.5–7.5. Catalase- and oxidase-positive. Hydrolyses Tween 20 and gelatin, but not tyrosine, aesculin, casein, Tween 80 or starch. Produces acid from D-lactose, myo-inositol, D-galactose, D-mannitol and arbutin, but not from raffinose, L-arabinose, melibiose, D-glucose, D-fructose, D-mannose or salicin. Negative for indole production and arginine dihydrolase activity. Negative for assimilation of L-arabinose, N-acetylglucosamine, maltose, D-mannose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid (API 20NE). Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase, but not lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Weak enzymic activities are observed for valine arylamidase and acid phosphatase. Contains a large amount of phosphatidylethanolamine as the major polar lipid. The major isoprenoid quinone is ubiquinone-8 (Q-8). The major fatty acids are C_{16:0}, C_{10:0} 3-OH and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH). The DNA G+C content is 64.9–65.0 mol% (HPLC).

The type strain, EMB43^T (=KCTC 22084^T=DSM 19389^T), was isolated from activated sludge of a domestic waste-

water treatment plant. EMB61 is a second strain of the species.

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