

Zoogloea oleivorans sp. nov., a floc-forming, petroleum hydrocarbon-degrading bacterium isolated from biofilm

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A floc-forming, Gram-stain-negative, petroleum hydrocarbon-degrading bacterial strain, designated Buc^T, was isolated from a petroleum hydrocarbon-contaminated site in Hungary. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain Buc^T formed a distinct phyletic lineage within the genus *Zoogloea*. Its closest relative was found to be *Zoogloea caeni* EMB43^T (97.2% 16S rRNA gene sequence similarity) followed by *Zoogloea oryzae* A-7^T (95.9%), *Zoogloea ramigera* ATCC 19544^T (95.5%) and *Zoogloea resiniphila* DhA-35^T (95.4%). The level of DNA–DNA relatedness between strain Buc^T and *Z. caeni* EMB43^T was 31.6%. Cells of strain Buc^T are facultatively aerobic, rod-shaped, and motile by means of a polar flagellum. The strain grew at temperatures of 5–35 °C (optimum 25–28 °C), and at pH 6.0–9.0 (optimum 6.5–7.5). The predominant fatty acids were C_{16:0}, C_{10:0} 3-OH, C_{12:0} and summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH). The major respiratory quinone was ubiquinone-8 (Q-8) and the predominant polar lipid was phosphatidylethanolamine. The genomic DNA G + C content was 63.2 mol%. On the basis of the chemotaxonomic, molecular and phenotypic data, isolate Buc^T is considered to represent a novel species of the genus *Zoogloea*, for which the name *Zoogloea oleivorans* sp. nov. is proposed. The type strain is Buc^T (=DSM 28387^T=NCAIM B 02570^T).

Members of the genus *Zoogloea* have mostly been isolated from wastewater sludge. They usually form aerobic granular sludge during wastewater treatment processes due to their floc-forming ability (Shao *et al.*, 2009; Weissbrodt *et al.*, 2013; Zhao *et al.*, 2013). Cell aggregates are embedded in gelatinous matrices, the so-called zoogloal matrices (Dugan *et al.*, 1992), which serve as a good basis for microbial biofilm formation. At the time of writing the genus *Zoogloea* comprises four species with validly published names: *Zoogloea caeni* (Shao *et al.*, 2009), *Zoogloea ramigera* (Crabtree & McCoy, 1967), *Zoogloea resiniphila* (Mohn *et al.*, 1999) and *Zoogloea oryzae* (Xie & Yokota, 2006).

Apart from in wastewater treatment processes, microbial biofilms can also play key roles in bioremediation of contaminated ecosystems (Pastorella *et al.*, 2012). Moreover,

relatives of species of the genus *Zoogloea* have recently been identified as potential benzene-degrading bacteria (Jechalke *et al.*, 2013). As petroleum hydrocarbons are frequent environmental contaminants, efforts have been made in our laboratory to isolate and characterize members of bacterial communities from biofilter clean-up facilities set up on hydrocarbon-contaminated sites (Szabó *et al.*, 2011). The present study describes a novel species of the genus *Zoogloea* isolated from a biofilm, which developed on the surface of a biofilter made for petroleum hydrocarbon removal.

Strain Buc^T was isolated from a biofilm sample originating from a Hungarian petroleum hydrocarbon-contaminated site where active bioremediation treatment was in progress. The biofilm sample was homogenized and serially diluted with 0.9% (w/v) saline solution and subsequently spread on R2A agar (DSM medium No. 830) and incubated at 28 °C for 5 days for the isolation of bacteria. Short-term maintenance of isolates was performed on R2A agar at 28 °C for 5 days.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Buc^T is KF667502.

One supplementary figure and one supplementary table are available with the online Supplementary Material.

Colony morphology was studied on R2A agar medium using direct and stereomicroscopic observations of single colonies. Gram staining was performed according to Claus (1992). Cell morphology and motility were studied by using phase-contrast microscopy and transmission electron microscopy (Morgagni 268). For transmission electron microscopy analysis cells were negatively stained with 1% (w/v) uranyl acetate (Szoboszlai *et al.*, 2008). Oxidase activity was studied by the method of Tarrand & Gröschel (1982). Catalase production and the Voges–Proskauer reaction were demonstrated by the methods of Cowan & Steel (1974). Acid production from D-glucose was checked by the classical test according to Hugh & Leifson (1953). Growth at different temperatures (from 5 to 45 °C) was determined by using both R2A broth and R2A agar medium, while pH tolerance (pH 3–11, using increments of 0.5 pH units) was determined using R2A broth. Urease activity, reduction of nitrate, hydrolysis of starch and Tween 80, and gelatinase and phosphatase activities were studied according to Smibert & Krieg (1994). Acid production from different carbon sources and the enzymatic activities of strain Buc^T were investigated with API 50 CH, API 20 NE and API ZYM kits (bioMérieux), according to the manufacturer's instructions. Growth under anaerobic conditions was determined in R2A broth media with and without the addition of 0.15% (w/v) KNO₃ at 28 °C. To ensure anaerobic conditions 100 ml serum bottles (Glasgerätebau Ochs) with 75 ml R2A broth were crimp sealed and sparged with nitrogen under sterile conditions. The dissolved oxygen concentration inside the bottles was measured non-invasively by using a Fibox 3 trace v3 fibre optic oxygen meter with PSt3 sensor spots (PreSens).

Growth of strain Buc^T was observed at temperatures of 5–35 °C, with optimum growth observed at 25–30 °C. Growth was observed at pH 6–9; optimal growth was observed between pH 6.5 and 7.5. Colonies on R2A agar exhibited a wax-like consistency due to the large amount of zoogloal matrices produced by the cells of strain Buc^T. Accordingly, typical zoogloal flocs were formed by the strain in R2A broth, and flocs were easily observable with the naked eye after 1–2 days of incubation. All cells observed were rods (about 1.2–1.4 µm wide and 2.5–3 µm long) with thick capsules. Cells were Gram-stain-negative and motile by means of a polar flagellum (Fig. S1, available in the online Supplementary Material). Strain Buc^T reduced nitrate to nitrite readily, but formation of gaseous nitrogen was not observed. Anaerobic growth was not observed for 7 days at 28 °C in R2A broth without the addition of nitrate. However, in the presence of nitrate rapid growth was observed, indicating that nitrate reduction contributed to anaerobic growth while fermentation did not. Acetate and most carbohydrates (API 20 NE, API 50 CH) could not be used as sole sources of carbon. Some phenotypic features that are often compared between species of the genus *Zoogloea* are presented for strain Buc^T and related members of the genus *Zoogloea* in Table 1. Although strain Buc^T and *Z. oryzae* A-7^T showed similar

metabolic capabilities, they differed substantially in their optimum growth temperatures, colony morphologies and in their ability to reduce nitrate to nitrogen gas (Table 1).

The genetic ability of strain Buc^T to fix nitrogen was investigated by PCR amplification of the *nifH* gene with the forward primer PolF, 5'-TGCGAYCCSAARGCBGACTC-3', and the reverse primer PolR, 5'-ATSGCCATCATY-TCRCCGGA-3' (Poly *et al.*, 2001). PCR amplifications were carried out on DNA extracted from strains Buc^T and *Z. caeni* EMB43^T. As a result *nifH* PCR products of appropriate size (about 340 bp) were detected for both strains.

Given that strain Buc^T was isolated from a petroleum hydrocarbon-rich environment, its ability to degrade a petrol/crude oil mixture was tested by means of a simple volumetric method. Moreover, as several *Betaproteobacteria* are known to degrade aromatic hydrocarbons and to possess the subfamily I.2.C catechol 2,3-dioxygenase gene (C23O) (Táncsics *et al.*, 2012, 2013), the presence of this functional gene was investigated via PCR. To test its petroleum hydrocarbon-degrading ability strain Buc^T was grown in R2A broth for 3 days at 28 °C. Subsequently 5 ml of the culture was transferred into 100 ml OIR III broth medium [5 g (NH₄)₂SO₄, 0.5 g KH₂PO₄, 1 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.2 g CaCl₂·6H₂O, 0.01 g FeSO₄·7H₂O, 0.5 g peptone, 0.5 g yeast extract, 1000 ml dH₂O] containing 2 ml petrol/crude oil mixture (3:2, v/v) and incubated in a rotary shaker for 120 h at 20 °C and at 150 r.p.m. After incubation the volume of unmetabolized petroleum hydrocarbons was measured by extracting them from the OIR III broth using 3 × 50 ml n-hexane. The resultant 150 ml n-hexane solution containing the unmetabolized petroleum hydrocarbons was filtered through a Düren 619 G ¼ filter paper containing Na₂SO₄ to remove the residual aqueous phase. To maximize recovery of petroleum hydrocarbons the OIR III broth was also treated with 50 ml chloroform and the chloroform phase was then added to the 150 ml n-hexane/petroleum hydrocarbon solution. Subsequently, solvents were separated from the petrol/crude oil mixture by means of fractional distillation using a Heidolph Rotary Evaporator. After distillation the pure petrol/crude oil mixture was incubated at 65 °C for 45 min to remove solvent residues. The amount of the petrol/crude oil mixture metabolized was calculated as the mass difference between the initial and the residual amount of the applied mixture. Measurements were carried out in triplicate with uninoculated controls to calculate abiotic loss. As a result, it was observed that strain Buc^T degraded 18.6 ± 1.6% of the petrol/crude oil mixture during the 120 h test period, while *Z. caeni* EMB43^T was unable to utilize petroleum hydrocarbons at all.

PCR amplifications of the subfamily I.2.C C23O gene by using the forward primer XYLE3F, 5'-TGYTGGGAYGAR-TGGGAYAA-3', and the reverse primer XYLE3R, 5'-TCASGTRTASACITCSGTRAA-3' (Táncsics *et al.*, 2013), were carried out on DNA extracted from strains Buc^T and

Table 1. Characteristics of strain Buc^T and type strains of related species of the genus *Zoogloea*

Strains: 1, Buc^T (data from the present study); 2, *Z. caeni* EMB43^T (data from the present study except where indicated); 3, *Z. resiniphila* DhA-35^T (Mohn *et al.*, 1999; Shao *et al.*, 2009); 4, *Z. oryzae* A-7^T (Xie & Yokota, 2006); 5, *Z. ramigera* ATCC 19544^T (Unz, 1984; Xie & Yokota, 2006). +, Positive; –, negative; w, weakly positive; NA, data not available. All strains are positive for nitrate reduction.

Characteristic	1	2	3	4	5
Cell diameter (µm)	1.2–1.4	0.6–0.9*	0.5–0.7	1.0	1.0–1.2
Colony colour	Greyish white	Yellowish white	White	Colourless	Greyish white
Growth at 37 °C	–	+	+	+	+
Growth at 45 °C	–	–	+	–	–
Denitrification to N ₂	–	+	–	+	+
Catalase	+	+	–	+	+
Urease	w	–	–	+	+
Denitrification	+	+	–	+	+
Hydrolysis of:					
Gelatin	–	–	+	–	+
Casein	–	–	+	–	+
Utilizes as sole carbon source:					
Acetate	–	+	+	–	+
Citrate	–	–	–	–	+
Glucose	–	–	+	–	–
Mannitol	–	–	+	–	+
DNA G + C content (mol%)	63.2	64.9*	NA	65.1	65.3

*Data from Shao *et al.* (2009).

Z. caeni EMB43^T. The expected PCR product size (about 800 bp) of the targeted C23O gene was detected for strain Buc^T but not for *Z. caeni* EMB43^T. The XYLE3 PCR product of strain Buc^T was sequenced and the putative subfamily I.2.C C23O gene sequence was deposited in GenBank under accession number KJ433487.

For the analysis of fatty acid methyl esters, strain Buc^T was cultivated on R2A agar at 28 °C. Sufficient cells of comparable physiological age could be harvested from the third quadrant of the plates. Analysis of fatty acid methyl esters was performed according to the instructions of the Microbial Identification system (MIDI; Microbial ID). Fatty acid analyses together with analyses of respiratory quinones and polar lipids were carried out by the Identification Service, Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The DNA G + C content of strain Buc^T was determined from bacterial cells disrupted using a Constant Systems TS 0.75 kW disrupter (IUL Instruments). After purification of the DNA on hydroxyapatite according to the procedure of Cashion *et al.* (1977), it was degraded to nucleosides using P1 nuclease and bovine alkaline phosphatase, as described by Mesbah *et al.* (1989). The nucleosides were separated by reversed-phase HPLC by the methods described by Tamaoka & Komagata (1984). The G + C content of the DNA was calculated from the ratio of deoxyguanosine to thymidine. The cellular membrane of strain Buc^T contained C_{16:0}, C_{10:0} 3-OH, C_{12:0} and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) as the major fatty acids (Table S1), which is similar to

other species of the genus *Zoogloea*. The major respiratory lipoinone detected was ubiquinone-8 (Q-8), while ubiquinone-7 (Q-7) was detected as a minor component. Investigation of the polar lipid profile showed that the main component was phosphatidylethanolamine. The DNA G + C content of strain Buc^T was 63.2 mol%. Overall, the chemotaxonomic data were in accordance with those of members of the genus *Zoogloea* (Unz, 1984; Mohn *et al.*, 1999; Xie & Yokota, 2006; Shao *et al.*, 2009).

The 16S rRNA gene of strain Buc^T was amplified and sequenced using the universal bacterial primers 27F and 1492R (Lane, 1991). The 1405 bp sequence of strain Buc^T was compared with 16S rRNA gene sequences using the EzTaxon server (<http://www.ezbiocloud.net/eztaxon>; Kim *et al.*, 2012) to determine an approximate phylogenetic affiliation. Sequence similarity values between strain Buc^T and related strains were calculated by using the FASTA3 program at EBI (<http://www.ebi.ac.uk/fasta33/nucleotide.html>). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods with Kimura's two-parameter calculation model and the maximum-parsimony algorithm (Kimura, 1980) using MEGA version 5.0 (Tamura *et al.*, 2011). Tree topologies and distances were evaluated by bootstrap analysis based on 1000 replicates. For DNA–DNA hybridization experiments between strains Buc^T and *Z. caeni* EMB43^T cells were disrupted using a Constant Systems TS 0.75 kW disrupter (IUL Instruments) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977).

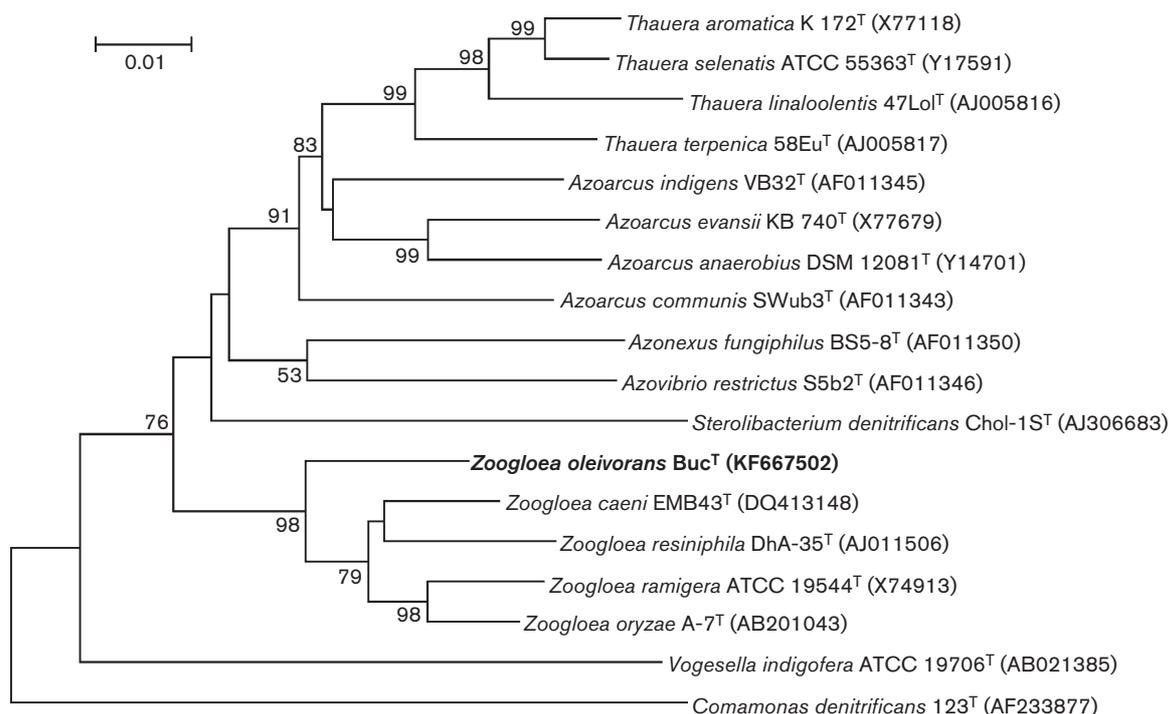


Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain Buc^T and related taxa. Bootstrap values are shown at nodes as percentages of 1000 replicates; only values over 50% are shown. Bar, 0.01 changes per nucleotide position.

DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in-situ* temperature probe (Varian).

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain Buc^T 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 strain Buc^T was most closely related to *Z. caeni* EMB43^T with a similarity of 97.2%; this was followed by *Z. oryzae* A-7^T, *Z. ramigera* ATCC 19544^T and *Z. resiniphila* DhA-35^T with 16S rRNA gene similarities of 95.9, 95.5 and 95.4%, respectively. However, it must be noted that, based on the topologies of the 16S rRNA gene-based phylogenetic trees used during this study, no clear closest relative of the genus *Zoogloea* could be ascertained for strain Buc^T (Fig. 1). The level of DNA–DNA relatedness between strains Buc^T and *Z. caeni* EMB43^T was 31.6%, clearly below the cut-off-point recommended for the delineation of bacterial species (Wayne *et al.*, 1987). The physiological, biochemical and phylogenetic data presented suggest that strain Buc^T represents a novel species of the

genus *Zoogloea*, for which the name *Zoogloea oleivorans* sp. nov. is proposed.

Description of *Zoogloea oleivorans* sp. nov.

Zoogloea oleivorans [o.le.i.vo'rans. L. n. *oleum* oil; L. v. *vorare* to devour; N.L. part. adj. *oleivorans* capable of utilizing oil (hydrocarbons)].

Colonies on R2A are greyish white, raised and circular with entire margins, and have a wax-like consistency. Cells are facultatively aerobic, Gram-stain-negative, motile rods (about 1.2–1.4 µm wide and 2.5–3 µm long) with thick capsules. Growth occurs optimally at pH 6.5–7.5 and 25–30 °C, but not above 35 °C. Catalase- and oxidase-positive. Able to use petroleum hydrocarbons as carbon and energy sources. Positive for nitrate reduction, but negative for indole production and gelatin, starch, aesculin, casein and Tween 80 hydrolysis. Most carbohydrates (API 20 NE, API 50 CH) cannot be used as sole sources of carbon. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and phosphoamidase, but not lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase or α-fucosidase. Weak enzymic activities are observed for urea and L-arginine. 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, C_{12:0} and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH).

The type strain, Buc^T (=DSM 28387^T=NCAIM B 02570^T), was isolated from a biofilm developed on the surface of a biofilter made for petroleum hydrocarbon removal. The genomic DNA G+C content of the type strain is 63.2 mol% (HPLC).

Acknowledgements

This project was supported by the research grants OTKA PD 104307, and Research Centre of Excellence-8526-5/2014/TUDPOL. A. T. was supported by the Bolyai János Research Grant of the Hungarian Academy of Sciences. We thank László Szabó (Institute of Material and Environmental Chemistry, Research Centre for Natural Sciences, Hungarian Academy of Sciences) for preparing the transmission electron micrographs.

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