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 BucT, was isolated from a petroleum hydrocarbon-contaminated site in Hungary. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain BucT formed a distinct phyletic lineage within the genus Zoogloea. Its closest relative was found to be Zoogloea caeni EMB43T (97.2 % 16S rRNA gene sequence similarity) followed by Zoogloea oryzae A-7T (95.9 %), Zoogloea ramigera ATCC 19544T (95.5 %) and Zoogloea resiniphila DhA-35T (95.4 %). The level of DNA–DNA relatedness between strain BucT and Z. caeni EMB43T was 31.6 %. Cells of strain BucT 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 C16 : 0, C10 : 03-OH, C12 : 0 and summed feature 3 (C16 : 1v7c and/or iso-C15 : 02-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 BucT 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 BucT (= DSM 28387T = NCAIM B 02570T).

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 zoogloenal 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 BucT 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.
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 (Szoboszlay 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 according to the methods of Smibert & Krieg (1994). Acid production from different carbon sources and the enzymatic activities of strain BucT 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 (Glaserätbebau 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 BucT 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 BucT. 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 BucT 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 among species of the genus Zoogloea are presented for strain BucT and related members of the genus Zoogloea in Table 1. Although strain BucT and Z. oryzae A-7T 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 BucT to fix nitrogen was investigated by PCR amplification of the nifH gene with the forward primer PolF, 5′-TGGGAYCCSARCGCBGACTC-3′, and the reverse primer PolR, 5′-ATSGCCATCATY-TCRCCGGA-3′ (Poly et al., 2001). PCR amplifications were carried out on DNA extracted from strains BucT and Z. caeni EMB43T. As a result nifH PCR products of appropriate size (about 340 bp) were detected for both strains.

Given that strain BucT 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 catalase 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 BucT 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 Dürer 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 unocculated controls to calculate abiotic loss. As a result, it was observed that strain BucT degraded 18.6 ± 1.6% of the petrol/crude oil mixture during the 120 h test period, while Z. caeni EMB43T 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′-TGTYGGGAYGARRTGGAYAA-3′, and the reverse primer XYLE3R, 5′-TCASGTRTASACITCGTAA-3′ (Táncsics et al., 2013), were carried out on DNA extracted from strains BucT and Z. caeni EMB43T.
Table 1. Characteristics of strain BucT and type strains of related species of the genus Zoogloea

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell diameter (µm)</td>
<td>1.2–1.4</td>
<td>0.6–0.9*</td>
<td>0.5–0.7</td>
<td>1.0</td>
<td>1.0–1.2</td>
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<tr>
<td>Colony colour</td>
<td>Greyish white</td>
<td>Yellowish white</td>
<td>White</td>
<td>Colourless</td>
<td>Greyish white</td>
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<tr>
<td>Growth at 37 °C</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<td>Growth at 45 °C</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Denitrification to N₂</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>+</td>
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<tr>
<td>Urease</td>
<td>w</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Denitrification</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<td>Hydrolysis of:</td>
<td></td>
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<tr>
<td>Gelatin</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Casein</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Utilizes as sole carbon source:</td>
<td></td>
<td></td>
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<tr>
<td>Acetate</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Citrate</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Glucose</td>
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<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Mannitol</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>63.2</td>
<td>64.9*</td>
<td>NA</td>
<td>65.1</td>
<td>65.3</td>
</tr>
</tbody>
</table>

*Data from Shao et al. (2009).

Z. caeni EMB43T. The expected PCR product size (about 800 bp) of the targeted C23O gene was detected for strain BucT but not for Z. caeni EMB43T. The XYLE3 PCR product of strain BucT 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 BucT 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 BucT 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 Tamagawa & Komagata (1984). The G+C content of the DNA was calculated from the ratio of deoxyguanosine to thymidine. The cellular membrane of strain BucT contained C16:0, C18:0 3-OH, C12:0 and summed feature 3 (C16:1ω7c and/or iso-C15:0 2-OH) as the major fatty acids (Table S1), which is similar to other species of the genus Zoogloea. The major respiratory lipoquinone 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 BucT 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 BucT was amplified and sequenced using the universal bacterial primers 27F and 1492R (Lane, 1991). The 1405 bp sequence of strain BucT 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 BucT 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 BucT and Z. caeni EMB43T 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).
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 BucT 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 BucT was most closely related to Z. caeni EMB43T with a similarity of 97.2 %; this was followed by Z. oryzae A-7T, Z. ramigera ATCC 19544T and Z. resiniphila DhA-35T 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 BucT (Fig. 1). The level of DNA–DNA relatedness between strains BucT and Z. caeni EMB43T 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 BucT 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. 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, x-chymotrypsin, x-galactosidase, β-galactosidase, β-glucuronidase, x-glucosidase, β-glucosidase, β-glucosaminidase, x-mannosidase or x-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 ($C_{16:1} \omega 7c$ and/or iso-$C_{15:0}$ 2-OH).

The type strain, BucT (=DSM 28387T=NCAIM B 02570T), 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).

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References


