Clostridium algifaecis sp. nov., an anaerobic bacterial species from decomposing algal scum

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Two anaerobic bacterial strains, MB9-7T and MB9-9, were isolated from decomposing algal scum and were characterized using a polyphasic approach. Phylogenetic analysis of 16S rRNA gene sequences showed that strains MB9-7T and MB9-9 are closely related to each other (99.7 % similarity) and they are also closely related to Clostridium tyrobutyricum (96.5 %). The two strains were Gram-stain positive and rod-shaped. Growth occurred at 20–45 °C, at pH 4.0–8.0 and at NaCl concentrations of up to 2 % (w/v). Acid was produced from glucose, xylose and mannose. Products of fermentation in PYG medium were mainly butyrate, acetate, carbon dioxide and hydrogen. The predominant cellular fatty acids were C14 : 0 and C16 : 0. The cellular polar lipids comprised phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, two glycolipids, one phospholipid, one aminophospholipid and two aminolipids. The DNA G+C contents of strain MB9-7T and MB9-9 were 27.9 and 28.7 mol%, respectively. These results support the assignment of the new isolates to the genus Clostridium and also distinguish them from other species of the genus Clostridium. Hence, it is proposed that strains MB9-7T and MB9-9 represent a novel species of the genus Clostridium, with the suggested name Clostridium algifaecis sp. nov. The type strain is MB9-7T (=CGMCC 1.5188T=DSM 28783T).

At the time of writing, the genus Clostridium comprises more than 200 phylogenetically heterogeneous species (http://www.bacterio.net). Based on a comparative study by Collins et al. (1994), half of the species of the genus Clostridium with validly published names fall within the Clostridium butyricum branch (the type species of the genus Clostridium), which is regarded to be the genus Clostridium sensu stricto (cluster I). Other members of the genus Clostridium fall into different clusters, which have only a loose relationship to C. butyricum (Collins et al., 1994; Wiegel et al., 2006). Species of the genus Clostridium have common characteristics, such as being strictly anaerobic, spore-forming, Gram-stain positive, having low G+C contents and producing both acid and alcohol during fermentation. Since members of the genus Clostridium are metabolically versatile and are able to form endospores, they have been detected in diverse environments, and play important ecological roles. For example, Clostridium chromiireducens was reported to help metal reduction in anaerobic soils (Inglett et al., 2011), Clostridium nitrophenolicum degrades p-nitrophenol (Suresh et al., 2007), and Clostridium clariflavum and Clostridium caenicola degrade cellulose (Shiratori et al., 2009). In a study of the diversity and functions of heterotrophic bacteria during algal biomass decomposition in an aquatic ecosystem (Wu et al., 2012), bacterial strains were isolated from decomposing algal slurry collected during an algal bloom season from Lake Taihu (Wuxi City, Jiangsu Province, PR China). Here, we report on the taxonomic description of two novel, strictly anaerobic bacterial strains, MB9-7T and MB9-9.

Strains MB9-7T and MB9-9 were enriched under anaerobic conditions and isolated using the Hungate technique (Hungate, 1969). Both enrichment and pure cultures were grown in PYG

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MB9-7T is KJ001788, and of strain MB9-9 is KJ001789.

Two supplementary figures are available with the online version of this paper.
medium (http://www.dsmz.de/microorganisms/medium); the pH was adjusted to 7.2 before autoclaving, and incubating in an atmosphere of 100 % N₂. Colonies appeared after incubation at 30 °C for 3 days. The isolates were routinely maintained on PYG medium and preserved at −80 °C as a suspension in PYG broth supplemented with 20 % (v/v) glycerol, 1 % (w/v) sodium sulfide and 0.04 % (w/v) L-cysteine hydrochloride.

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out as previously described (Zhang et al., 2011). The almost complete 16S rRNA gene sequences (1476 nt) of the new isolates were used for calculating similarities to their phylogenetic neighbours using the EzTaxon server version 2.1 (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). Phylogenetic analysis based on 16S rRNA gene sequences of the two strains and the type strains of all species of the genus Clostridium with validly published names was performed using the software package MEGA version 4.1 (Tamura et al., 2007). Multiple alignments with sequences of the genus Clostridium were performed using the CLUSTAL X program (version 1.64b; Thompson et al., 1997). Phylogenetic trees were then reconstructed using the maximum-likelihood and neighbour-joining methods (Fitch, 1971; Saitou & Nei, 1987) with Kimura’s two-parameter calculation model (Kimura, 1980). The robustness of the topology in the phylogenetic trees was evaluated by bootstrap analyses based on 1000 resamplings (Felsenstein, 1985). Genetic distances between strain MB9-7ᵀ, MB9-9 and strains of species of the genus Clostridium were calculated on the basis of pairwise alignments according to the p-distance algorithm. A large tree was firstly reconstructed based on 16S rRNA gene sequences of the whole of the genus Clostridium. Based on the tree topology and branch stability, 13 sequences were then selected for the reconstruction of a small tree to show the phylogenetic relationship of strains MB9-7ᵀ and MB9-9 to other members of the genus Clostridium. Analysis of 16S rRNA gene sequences confirmed that strains MB9-7ᵀ and MB9-9 are closely related to each other (99.7 % similarity) and fall into the radius of Clostridium sensu stricto (Wiegel et al., 2006), with the highest sequence similarity (96.6 %) being to Clostridium tyrobutyricum. The phylogenetic trees (Fig. 1) reconstructed with the neighbour-joining and maximum-likelihood methods also indicated that both strains clustered within Clostridium sensu stricto.

The morphological and physiological characteristics of strains MB9-7ᵀ and MB9-9 were investigated using cells cultivated on PYG medium at 30 °C. Cellular morphology was examined with transmission (JEM 1400; JEOL) and scanning (Quanta 200; FEI) electron microscopy. Gram staining was carried out according to the methods of Johnson et al. (1995). Spore formation was determined using a staining method (Schaeffer & Fulton, 1933). The temperature range for growth was tested in RCM broth (http://www.dsmz.de/microorganisms/medium), incubated for 48 h at 4, 8, 15, 20, 25, 30, 37, 40, 45, 50 and 55 °C. The pH range for growth was tested at pH 3, 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 and 11 by adjusting the pH of RCM broth with 5 M NaOH or HCl. Growth in the presence of 0–8 % (w/v, at intervals of 1 %) NaCl was examined in RCM broth. For phenotypic characterization, all tests were carried out in triplicate in anaerobic PYG medium (pH 7.2). Catalase activity was determined with bubble production using 3 % (v/v) H₂O₂. Hydrolysis of gelatin, aesculin and starch, and hydrogen sulfide production were determined following methods described by Holdeman et al. (1977). Substrate utilization was determined in PY broth (http://www.dsmz.de/microorganisms/medium) supplemented with 1 % (w/v) Casamino acids, peptone, tryptone, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, sucrose, arabinose, ribose, xylose, starch, trehalose, fucose, pyruvate and malate. Growth was judged by the increase in optical density after 2 days’ cultivation. The fermentation products in PYG broth were analysed after 3 days of cultivation. Acetate, butyrate, formate, lactate, propionate and pyruvate were analysed by ion-exchange chromatography (ICS2500; Dionex), as described by Lin et al. (2007). Ethanol and butanol production were determined by HPLC equipped with an electric conductivity monitor (Agilent). Gas production was measured by gas chromatography with a packed TDX-01 column (Techcomp). Biochemical characteristics were tested using API 20A and API ZYM kits (bioMérieux) according to the manufacturer’s instructions. Strains MB9-7ᵀ, MB9-9 and C. tyrobutyricum JCM 11008ᵀ were tested in parallel in the experiments described above.

Cells of strains MB9-7ᵀ and MB9-9 were Gram-stain positive, approximately 2.0–4.0 μm × 0.2–0.3 μm; subterminal endospores were formed in swollen sporangia (Fig. S1, available in the online Supplementary Material). After 3 days’ incubation at 30 °C on PYG agar, colonies were creamy-white, flat, circular and usually 1.0–1.5 mm in diameter. Strains MB9-7ᵀ and MB9-9 were able to grow at temperatures of 20–45 °C (optimal, 30–37 °C), at pH 4.0–8.0 (optimum, pH 5.5–6.5), and at NaCl concentrations lower than 2 % [(w/v) optimal, 0 %]. Strain MB9-7ᵀ and MB9-9 used Casamino acids, peptone, tryptone, cellobiose, sucrose, fructose, galactose, glucose, lactose, maltose, mannose, ribose, xylose, pyruvate and malate as carbon and energy sources. Starch, fucose, arabinose and trehalose did not support growth. The fermentation products from glucose were mainly butyrate, acetate, and trace amounts of malate and lactate. CO₂ and H₂ were detected in the headspace during fermentation. Ethanol was not produced. Additional physiological and biochemical characteristics of strain MB9-7ᵀ and MB9-9 are provided in the species description, and properties differentiating the isolate from type strains of closely related species are listed in Table 1.

For chemotaxonomic analyses, biomass of strains MB9-7ᵀ, MB9-9 and C. tyrobutyricum JCM 11008ᵀ was harvested from PYG broth incubated at 30 °C for 3 days. Cellular fatty acids were extracted and methylated according to the standard protocol of Sherlock Microbial Identification System version 6.0 (MIDI), analysed by GC (model 6890;
The predominant cellular fatty acids of strain MB9-7\textsuperscript{T} were iso-C\textsubscript{16:0} (35.3\%), iso-C\textsubscript{14:0} (21.9\%) and summed feature 3 (iso-C\textsubscript{16:0}30\%) to distinguish strains MB9-7\textsuperscript{T} and MB9-9 from other species of the genus Clostridium. Therefore, we propose that strains MB9-7\textsuperscript{T} and MB9-9 represent a novel species of the genus Clostridium, with the proposed name Clostridium algifaecis sp. nov.

**Description of Clostridium algifaecis sp. nov.**

Clostridium algifaecis sp. nov. (algi.fae.cs. L. fem. n. alga an alga; L. n. faex faecis dregs; N.L. gen. n. algifaecis of algal dregs).

Cells are Gram-stain positive, rod-shaped (0.2–0.3 × 2.0–4.0 μm), strictly anaerobic. Colonies on PYG agar are creamy-white, flat, circular and usually 1.0–1.5 mm in diameter after 3 days’ incubation at 30 °C. Growth occurs at 20–45 °C (optimum, 30–37 °C), at pH 4.0–8.0 (optimum, pH 5.5–6.5) and at NaCl concentrations lower than 2\%.

Oxidase and catalase activities are negative. Hydrogen sulfide is produced. Uses Casamino acids, peptone, tryptone, cellobiose, sucrose, fructose, galactose, glucose, lactose, maltose, mannose, ribose, xylose, pyruvate and malate as carbon and energy sources. The fermentation products from glucose are mainly butyrate, acetate, and trace amounts of malate and lactate. CO\textsubscript{2} and H\textsubscript{2} are generated during fermentation. Ethanol is not produced. In API 20A strips, indole is not produced. Urea, gelatin and aesculin are not hydrolysed. Acids are produced from glucose, xylose and mannose, but not from 13 other tested substrates. In API ZYM strips, leucine arylamidase, acid phosphatase, valine arylamidase and naphthol-AS-BI-phosphohydrolase activities

**Fig. 1.** Phylogenetic tree reconstructed with the neighbour-joining method, showing the relationships of strains MB9-7\textsuperscript{T}, MB9-9 and representatives of the genus Clostridium sensu stricto (cluster I of the genus Clostridium) within the family Clostridiaceae based on almost complete 16S rRNA gene sequences (1476 bp). Numbers represent confidence levels (percentages higher than 70\% are shown) from 1000-replicate bootstrap sampling. Nodes with filled circles indicate branches that are also found in the maximum-likelihood tree. Bar, evolutionary distance (K\textsubscript{muc}) of 0.02.
Table 1. Phenotypic characteristics that differentiate strains MB9–7T and MB9–9 from closely related members of the genus Clostridium

Strains: 1, MB9–7T; 2, MB9–9; 3, C. tyrobutyricum JCM 11008T. All data are from this study unless indicated otherwise. +, Positive; −, negative; w, weakly positive. All strains tested were positive for the hydrolysis of casein, H2S production, activities of acid phosphatase and naphthol-AS-BI-phosphohydrolase. All strains produced acids from glucose and xylose, but not from sucrose, maltose, salicin, arabinose, glycerol, cellobiose, melilose, sorbitol, raffinose, rhamnose or trehalose. All strains were negative for indole production, as well as glucose and xylose, but not from sucrose, maltose, salicin, arabinose, glycerol, cellobiose, melilose, sorbitol, raffinose, rhamnose or trehalose. All strains tested were positive for the hydrolysis of starch, aesculin and gelatin. All strains were negative for the following enzymic activities: urease, esterase lipase (C8), x-glucosidase, N-acetyl-β-glucosaminidase, x-galactosidase, β-galactosidase, x-glucosidase, esterase (C4), lipase (C14), β-glucoronidase, N-acetyl-β-glucosaminidase, x-mannosidase and β-fucosidase activities are absent. The predominant cellular fatty acids (>10%) are straight chain C16:0 and C14:0, and summed feature 3 (containing C16:1ω7c/C16:1ω6c). The polar lipid profile contains phosphatidylglycerol, diposphatidylglycerol, phosphatidyethanolamine, two glycolipids, one phospholipid, one aminophospholipid and two aminolipids. The DNA G+C content is 27.9–28.7 mol%.

The type strain is MB9–7T (=CGMCC 1.5188T=DSM 28783T), which was isolated from decomposing algal scum.

Acknowledgements

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References


Table 2. Cellular fatty acid composition of strains MB9–7T, MB9–9 and related type strains of the genus Clostridium

Strains: 1, MB9–7T; 2, MB9–9; 3, C. tyrobutyricum JCM 11008T. All data are from this study; ND, not detected.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
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<tr>
<td>Saturated straight-chain</td>
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<tr>
<td>C14:0</td>
<td>21.9</td>
<td>29.5</td>
<td>2.5</td>
</tr>
<tr>
<td>C16:0</td>
<td>35.3</td>
<td>22.5</td>
<td>70.3</td>
</tr>
<tr>
<td>Unsaturated</td>
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<tr>
<td>C12:0 at 12–13</td>
<td>1.8</td>
<td>1.6</td>
<td>ND</td>
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<tr>
<td>C13:0:10c</td>
<td>ND</td>
<td>6.0</td>
<td>ND</td>
</tr>
<tr>
<td>C14:0:9c</td>
<td>4.1</td>
<td>5.7</td>
<td>4.7</td>
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<tr>
<td>C16:0:9c</td>
<td>2.2</td>
<td>2.5</td>
<td>ND</td>
</tr>
<tr>
<td>C17:0 cyclo</td>
<td>5.1</td>
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<tr>
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</tr>
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<td>C18:1:9c</td>
<td>ND</td>
<td>ND</td>
<td>1.8</td>
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<tr>
<td>Summed features</td>
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<tr>
<td>1 (C13:0:0OH/iso-C15:1)</td>
<td>6.7</td>
<td>6.4</td>
<td>ND</td>
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<tr>
<td>2 (C16:1:07c/C16:1:06c)</td>
<td>18.3</td>
<td>18.7</td>
<td>11.6</td>
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