Haloanaerobium fermentans sp. nov., a strictly anaerobic, fermentative halophile isolated from fermented puffer fish ovaries

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A strain of strictly anaerobic and moderately halophilic bacteria isolated from salted puffer fish ovaries was studied phenotypically, genotypically and phylogenetically. On the basis of its physiological and morphological characteristics, the new isolate is considered to be a member of the genus Haloanaerobium. It is a motile, rod-shaped, non-spore-forming, Gram-negative, obligate anaerobe that grows in the presence of 25% (w/v) NaCl. The optimum salt concentration for growth is 10% (w/v). It grows well at 15 and 45 °C, but not at 10 or 50 °C. The optimum temperature for growth is 35 °C. It grows at pH 6–9.0 and the optimum pH for growth is 7.5. It ferments N-acetylglucosamine, cellubiose, fructose, galactose, D-glucose, lactose, maltose, D-mannose, raffinose, D-ribose, sucrose and D-xylose. It ferments D-glucose with the production of hydrogen, carbon dioxide, ethanol and organic acids such as acetate, formate and lactate. 16S rRNA gene sequence information confirmed the phylogenetic position of the new isolate, strain R-9T, as a member of the genus Haloanaerobium. DNA–DNA hybridization data revealed that isolate R-9T exhibited low levels of reassociation (less than 30%) with previously described Haloanaerobium species. Based on these results, the new isolate appears to represent a new Haloanaerobium species, for which the name Haloanaerobium fermentans sp. nov. is proposed. The type strain is R-9T (= JCM 10494T).

Keywords: fermented seafood, halophilic fermentative bacteria, Haloanaerobium fermentans

INTRODUCTION

Members of the genus Haloanaerobium are widely distributed in surface saline ecosystems such as hyper-saline lakes and subsurface ecosystems such as oil-fields. Eight species of this genus have been described previously. They have been isolated from various hypersaline environments: the Great Salt Lake (Zeikus et al., 1983; Tsai et al., 1995), Sivash Lake (Zhilina et al., 1992), Retba Lake (Cayol et al., 1994, 1995), gas-bearing sandstones and brine waters (Rengpipat et al., 1988), petroleum reservoir fluid (Bhupathiraju et al., 1994, 1999) and oil-injection water (Ravot et al., 1997).

In a previous study, we described halophilic bacteria that grew only anaerobically in the manufacturing process of a Japanese seafood, puffer fish ovaries fermented with rice-bran or fugunoko nukaduke (Kobayashi et al., 1995), but the detailed characteristics of these anaerobes were not reported. In this study, we undertook a phenotypic, genotypic and phylogenetic characterization of R-9T, an isolate of the new halophilic anaerobe that is representative of one of the dominant species in these fermented ovaries. The precise identification and phylogenetic position of the new isolate was determined by phenotypic characterization, 16S rRNA gene sequencing and DNA–DNA hybridization. On the basis of our results, we conclude that our isolate should be placed in a new species of Haloanaerobium.

METHODS

Bacterial strains and media. A new isolate (R-9T) was obtained previously from salted puffer fish ovaries in the manufacturing process of a Japanese traditional food
To isolate and purify the new isolate, we used GYP agar consisting of (l−1 distilled water): 10 g glucose, 10 g yeast extract, 5 g Bactopeptone, 2 g meat extract (Kyokuto), 2 g CH₃COONa, 3 H₂O, 5 g CaCO₃, 5 ml salt solution (l−1: 40 g MgSO₄·7H₂O, 2 g MnSO₄·4H₂O, 2 g FeSO₄·7H₂O, 2 g NaCl). 0.25 g Tween 80, 200 g NaCl and 12 g agar. A GasPak anaerobic system (BBL) was used for cultivation.

The type strains of Haloanaerobium praevalens (DSM 2228T), Haloanaerobium acetoethylicum (DSM 3532T), Haloanaerobium saccharolyticum subsp. saccharolyticum (DSM 66437T), Haloanaerobium saccharolyticum subsp. senegalense (DSM 7379T), Haloanaerobium alcaliphilum (DSM 8275T), Haloanaerobium lacusrosei (DSM 10165T) and Haloanaerobium congolense (DSM 11287T) were obtained from the DSMZ (Braunschweig, Germany). Haloanaerobium salsuginis ATCC 51327T was obtained from the ATCC (Manassas, VA, USA). Isolate R-9T was stabbed into anaerobic bacteria culture medium (ABCM; Eiken) consisting of (l−1 distilled water): 3 g glucose, 5 g yeast extract, 10 g peptone, 3 g soypeptone, 10 g trypto, 2 g plant extract, 5 g soluble starch, 3 g meat extract, 2 g NaCl, 2.5 g K₂HPO₄, 0.005 g haemin, 0.3 g L-cysteine.HCl and 0.3 g sodium thioglycolate, supplemented with 10% (w/v) NaCl and 0.5% CaCO₃. The culture was maintained at 5 °C and subcultured every 6 months. The inocula used were prepared from 48- to 72-h-old cultures in ABCM medium supplemented with 10% (w/v) NaCl incubated at 30 °C under anaerobic conditions.

Cultural, physiological and biochemical characteristics. Gram staining was performed as described previously (Dussault, 1955). Cell morphology and motility were observed by light microscope. Cells for electron microscopy were stained with uranyl acetate and lead citrate. Staining was performed as described previously (Dussault, 1961). DNA–DNA hybridization studies were carried out by the microplate hybridization method with photoelution labelling and fluorometric detection as described previously (Ezaki et al., 1989). The DNA base composition was determined by the HPLC method as reported previously (Katayama-Fujimura et al., 1984).

16S rRNA gene sequencing and phylogenetic analysis. The 16S rRNA gene was amplified by PCR. Sequencing of the 16S rRNA gene was carried out by using an Applied Biosystems PCR kit (Dye Terminator cycle sequencing kit). For amplification of the 16S rRNA gene, a universal primer set was used that corresponded to positions 8–27 (forward primer) and 1492–1510 (reverse primer) of the Escherichia coli numbering system (Weisburg et al., 1991). The reaction mixture was analysed with the Applied Biosystems 373A DNA sequencer as described previously (Satomi et al., 1997). The final sequence was determined from overlapping sequence data using the GENETYX computer program.

Nucleotide substitution rates (K_{sub} values) (Kimura, 1980) were determined and a distance matrix tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using the CLUSTAL w program (Thompson et al., 1994). The sequence for positions 99–1359 in the E. coli numbering was used in the analyses.

Reference sequences of other members of the genus Haloanaerobium and related genera were obtained from the DDBJ database. The following sequences were used for analysis (with accession numbers): Haloanaerobium praevalens DSM 2228T (AB022034), Haloanaerobium acetoethylicum DSM 3532T (X89071), Haloanaerobium saccharolyticum subsp. saccharolyticum DSM 66437T (X89069), Haloanaerobium saccharolyticum subsp. senegalense DSM 7379T (X89070), Haloanaerobium alcaliphilum DSM 8275T (X81850), Haloanaerobium lacusrosei DSM 10165T (L39787), Haloanaerobium congolense DSM 11287T (U76632), Haloanaerobium salsuginis ATCC 51327T (L22890), Haloanaerobium kushneri ATCC 700103T (U86446), Halobacteroides halobius DSM 5150T (L37423), Sporohalobacter lortetii ATCC 35059T (MS9122), Halothermothrix orenii OMC 548T (L22016) and Megashaera elsdenii ATCC 17752 (M26493).

RESULTS

Morphology and cultural characteristics

Cells of isolate R-9T were Gram-negative rods (1–0.5 µm wide by 2–7–3.3 µm long). Cells grew only anaerobically on plate medium. Colonies on ABCM plate medium supplemented with 10% (w/v) NaCl were smooth, convex, opaque and yellowish. Spores were not observed microscopically and no growth was obtained after pasteurization. Strain R-9T was motile by means of peritrichous flagella (Fig. 1).
Physiological and biochemical characteristics

Isolate R-9\textsuperscript{T} was not able to grow in the presence of less than 5\% (w/v) NaCl and growth was not inhibited in the presence of 25\% (w/v) NaCl. The optimal NaCl concentration for growth was 10\% (w/v). Isolate R-9\textsuperscript{T} grew well at 15 and 45 °C but not at 10 or 50 °C. The optimum temperature for growth was 35 °C. It grew well in the pH range of 6.0 to 9.0. The optimum pH for growth was 7.5. It grew in an anaerobic box with a GasPak anaerobic system (BBL) but not outside the anaerobic box. Isolate R-9\textsuperscript{T} fermented D-glucose to hydrogen, carbon dioxide and other acidic end-products. It produced acetate, formate, lactate and ethanol, but not butyrate or propionate. After 6 d incubation, isolate R-9\textsuperscript{T} had produced 258 mg acetic acid l\textsuperscript{-1}, 20 mg formic acid l\textsuperscript{-1}, 41 mg lactic acid l\textsuperscript{-1} and 603 mg ethanol l\textsuperscript{-1} in the broth culture. Isolate R-9\textsuperscript{T} produced hydrogen sulfide on SIM medium. Fer-

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Table 1. Differentiation of isolate R-9\textsuperscript{T} from other *Haloanaerobium* species on the basis of biochemical and physiological characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>R-9\textsuperscript{T}</th>
<th>H. <em>praevalens</em> DSM 2228\textsuperscript{a}</th>
<th>H. <em>acetoethylicum</em> DSM 3532\textsuperscript{a}</th>
<th>H. <em>saccharolyticum</em> subsp. <em>saccharolyticum</em> DSM 6643\textsuperscript{a}</th>
<th>H. <em>saccharolyticum</em> subsp. <em>senegalense</em> DSM 7379\textsuperscript{a}</th>
<th>H. <em>alcaliphilum</em> DSM 8275\textsuperscript{a}</th>
<th>H. <em>lucusrosae</em> DSM 10165\textsuperscript{a}</th>
<th>H. <em>congenolese</em> DSM 11287\textsuperscript{a}</th>
<th>H. <em>salsuginis</em> DSM 700103\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
<td>1.1-2 x 2.7-3.3</td>
<td>0.9-1.1 x 2.2-2.6</td>
<td>0.4-0.7 x 1.1-1.6</td>
<td>0.5-0.7 x 1.1-1.5</td>
<td>0.4-0.6 x 2.5</td>
<td>0.8 x 3.3-5</td>
<td>0.5 x 2.3</td>
<td>0.5-1 x 2.4</td>
<td>0.3-0.4 x 2.6-4.6</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>33</td>
<td>27</td>
<td>32</td>
<td>31</td>
<td>32</td>
<td>31</td>
<td>32</td>
<td>34</td>
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<td>NaCl concentration (°C):</td>
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<td></td>
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</tr>
<tr>
<td>Range</td>
<td>7.25</td>
<td>20-30</td>
<td>6-20</td>
<td>3-30</td>
<td>5-25</td>
<td>25-25</td>
<td>7.5-34</td>
<td>4-24</td>
<td>6-24</td>
</tr>
<tr>
<td>Optimum</td>
<td>10</td>
<td>12.5</td>
<td>10</td>
<td>10</td>
<td>7.5-12.5</td>
<td>10</td>
<td>18-20</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Temperature (°C):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimum</td>
<td>35</td>
<td>37</td>
<td>34</td>
<td>37-40</td>
<td>40</td>
<td>37-40</td>
<td>40</td>
<td>42</td>
<td>40</td>
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<td>pH:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>6-9.0</td>
<td>6-9.0</td>
<td>54.8-0</td>
<td>6-8.0</td>
<td>6.3-8.7</td>
<td>5.8-10</td>
<td>NT</td>
<td>6-3.8-5</td>
<td>5-6.8-0</td>
</tr>
<tr>
<td>Optimum</td>
<td>7.5</td>
<td>7.0-7.4</td>
<td>6.3-7.4</td>
<td>7.5</td>
<td>6.7-7.0</td>
<td>7.0</td>
<td>6.1</td>
<td>6-5.7-5</td>
<td></td>
</tr>
</tbody>
</table>

Utility of:  
- L-Arabinose: –  
- N-Acetylglucosamine: + + + +  
- Cellobiose: – + + + +  
- Fructose: + + + + + +  
- Galactose: – + – + + +  
- D-Glucose: + + + + + +  
- Glycerol: – – + + + –  
- Lactose: + – – – + +  
- Maltose: + NT NT + + + +  
- Mannose: + + + + + +  
- Pyruvate: – – + – + +  
- Raffinose: + NT NT – +  
- D-Ribose: + NT NT + + +  
- Rhamnose: – NT – – –  
- Starch: + + + + – –  
- Sucrose: + + + + + +  
- D-Xylose: + + + + + +  

Fermentation products:  
- Acetate, formate, lactate, H\textsubscript{2}, CO\textsubscript{2}  
- Acetate, butyrate, propionate, H\textsubscript{2}, CO\textsubscript{2}  
- Acetate, ethanol, H\textsubscript{2}, CO\textsubscript{2}  
- Acetate, H\textsubscript{2}, CO\textsubscript{2}  
- Acetate, lactate, butyrate, H\textsubscript{2}, CO\textsubscript{2}  
- Acetate, ethanol, H\textsubscript{2}, CO\textsubscript{2}  
- Acetate, H\textsubscript{2}, CO\textsubscript{2}  
- Acetate, formate, lactate, ethanol, H\textsubscript{2}, CO\textsubscript{2}  

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![Fig. 1. Transmission electron micrograph of negatively stained cells of Haloanaerobium fermentans strain R-9T. Bar, 2 µm.](image-url)
Table 2. Degree of DNA–DNA relatedness between the new isolate and other Haloanaerobium species

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Strain</th>
<th>Relatedness to labelled DNA from R-9(^T) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>New isolate</td>
<td>R-9(^T) (= JCM 10494(^T))</td>
<td>100</td>
</tr>
<tr>
<td><em>H</em>. praevans</td>
<td>DSM 2228(^T)</td>
<td>22</td>
</tr>
<tr>
<td><em>H</em>. acetoethylicum</td>
<td>DSM 3532(^T)</td>
<td>23</td>
</tr>
<tr>
<td><em>H</em>. saccharolyticum subsp. saccharolyticum</td>
<td>DSM 6643(^T)</td>
<td>20</td>
</tr>
<tr>
<td><em>H</em>. saccharolyticum subsp. senegalense</td>
<td>DSM 7379(^T)</td>
<td>11</td>
</tr>
<tr>
<td><em>H</em>. alcaliphilum</td>
<td>DSM 8275(^T)</td>
<td>20</td>
</tr>
<tr>
<td><em>H</em>. lacusrosei</td>
<td>DSM 10165(^T)</td>
<td>19</td>
</tr>
<tr>
<td><em>H</em>. congolense</td>
<td>DSM 11287(^T)</td>
<td>21</td>
</tr>
<tr>
<td><em>H</em>. salsuginis</td>
<td>ATCC 51327(^T)</td>
<td>11</td>
</tr>
</tbody>
</table>

*H*. Haloanaerobium.

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Fig. 2. Phylogenetic tree showing the relationship of Haloanaerobium fermentans strain R-9\(^T\) (= JCM 10494\(^T\)) to other strains of the genus Haloanaerobium and related genera based on a comparison of 16S rRNA gene sequences. The tree, constructed by the neighbour-joining method (Saitou & Nei, 1987), was based on a comparison of positions 99–1359 (*E. coli* numbering; Weisburg *et al.*, 1991). The tree was rooted with Megasphaera elsdonii.
mentative substrates included N-acetylglicosamine, cellobirose, fructose, galactose, d-glucose, lactose, maltose, D-mannose, raffinose, D-ribose, sucrose and D-xylose. L-Arabinose, glycerol, pyruvate, rhamnose and starch were not fermented (Table 1). Sulfate, sulfate, thiosulfate, elemental sulfur and formate did not stimulate the growth of isolate R-9\textsuperscript{T}.

**G + C content of DNA and DNA–DNA hybridization**

The DNA base composition of isolate R-9\textsuperscript{T} was found to be 33.3 mol% G + C. The levels of DNA hybridization between the new isolate and seven *Haloanaerobium* species used in this study were less than 30\% (Table 2).

**16S rRNA gene sequence comparison**

In order to investigate the phylogenetic relationship of isolate R-9\textsuperscript{T} to other bacteria, the sequence of a 16S rRNA gene PCR product was determined and then analysed. A comparative analysis of the 16S rRNA gene sequences revealed that isolate R-9\textsuperscript{T} belonged to the low-G + C-content, Gram-positive phylum. Further sequence alignments and phylogenetic analyses show the taxonomic and phylogenetic position of the new isolate to be among the members of the genus *Haloanaerobium* (Fig. 2). The sequence similarities of isolate R-9\textsuperscript{T} to other *Haloanaerobium* species ranged from 95-1\% (*Haloanaerobium salsuginis*) to 97-7\% (*Haloanaerobium saccharolyticum* subsp. *saccharolyticum*).

**DISCUSSION**

The strain that we investigated is a strictly anaerobic, rod-shaped, fermentative bacterium that is Gram-negative, non-spore-forming and motile by means of peritrichous flagella. It is catalase-negative, moderately halophilic and has a genomic DNA G + C content of 33\% mol\. On the basis of these characteristics, the isolate should be considered a member of the order *Haloanaerobiales* (Rainey et al., 1995).

The phylogenetic analyses of 16S rRNA gene sequence information clarifies the taxonomic and phylogenetic position of isolate R-9\textsuperscript{T} in the genus *Haloanaerobium*. Isolate R-9\textsuperscript{T} could not be accommodated in the same species as any of the seven *Haloanaerobium* species described previously due to the low DNA–DNA hybridization values (Table 2).

Phenotypic studies also revealed that isolate R-9\textsuperscript{T} could not be assigned to any of the previously described species of the genus *Haloanaerobium* (Table 1). Isolate R-9\textsuperscript{T} differs from *Haloanaerobium praevalens* and *Haloanaerobium alcaliphilum* in its ability to ferment a wide range of carbohydrates and in its fermentation products (volatile organic acids and ethanol). In addition, isolate R-9\textsuperscript{T} and *Haloanaerobium praevalens* can be differentiated by motility. *Haloanaerobium lacusrosei* (Cayol et al., 1995) is an extremely halophilic bacterium that grows optimally in 18–20\% (w/v) NaCl. Therefore, isolate R-9\textsuperscript{T} could be differentiated from *Haloanaerobium lacusrosei* on the basis of optimal salt concentration, since optimal growth of isolate R-9\textsuperscript{T} was observed in 10\% (w/v) NaCl, as well as some differences of substrate profiles, such as the ability to ferment glycerol and starch. Isolate R-9\textsuperscript{T} can be differentiated from *Haloanaerobium saccharolyticum* (both subspecies) (Zhilina et al., 1992; Cayol et al., 1994) on the basis of its ability to produce ethanol. In addition, the ability to ferment D-arabinose, glycerol, pyruvate and raffinose can be used to differentiate isolate R-9\textsuperscript{T} from *Haloanaerobium saccharolyticum* subsp. *saccharolyticum*. Likewise, the ability to ferment galactose, glycerol and D-xylose can be used to differentiate it from *Haloanaerobium saccharolyticum* subsp. *senegalense*. Other combinations of properties distinguish isolate R-9\textsuperscript{T} from the three other *Haloanaerobium* species: from *Haloanaerobium acetoethylicum* (Rengpipat et al., 1988) by fermentation of galactose and pyruvate; from *Haloanaerobium salsuginis* (Bhupathiraju et al., 1994) by fermentation of L-arabinose, cellobirose, pyruvate and rhamnose and by motility; and from *Haloanaerobium congolense* (Ravot et al., 1997) by fermentation of lactose and D-xylose and by motility.

Recently, *Haloanaerobium kushneri* was isolated from an oil brine (Bhupathiraju et al., 1999). Since *Haloanaerobium kushneri* was not included as a reference in the DNA–DNA hybridization study, we could not compare the genotype of our isolate with that of *Haloanaerobium kushneri*. However, the information available for *Haloanaerobium kushneri* indicates that there are some differences (ability to ferment L-arabinose and some physiological characteristics such as ability to grow at 15 °C or at pH 4 values greater than 8.0) between this species and our isolate. We compared the 16S rRNA gene sequence of isolate R-9\textsuperscript{T} with the sequence of *Haloanaerobium kushneri* and found a level of similarity of 96.6\%. This value is low enough to warrant placement of the two organisms in separate species.

**Ecological importance of isolate R-9\textsuperscript{T}**

(*Haloanaerobium fermentans*)

Because *Haloanaerobium fermentans* was isolated in the manufacturing process of fermented puffer fish ovaries, its true ecological niche is unknown. However, the presence of this bacterium as one of the dominant species in a salt-preserved food (Kobayashi et al., 1995) indicates the significance of strictly anaerobic, halophilic bacteria during fermentation processes in food products. To our knowledge, there have been few reports of halophilic anaerobes present in food products and associated with the microbiological problems of their manufacturing processes. A halophilic anaerobe, *Bacteroides halosmophilus*, was isolated from Mediterranean salted anchovies (Baum-
gartner, 1937), but this isolate has been lost. Similar anaerobes were also isolated from a spoiled sample of sugar salted herring (Knöchel & Huss, 1984a, b), but these isolates were not characterized in detail. Recently, we isolated a strictly anaerobic halophile from canned Swedish fermented herrings (Surströmming) and these isolates were identified as *Haloanaerobium praevalens* (unpublished data). Therefore, this report is the first description of a novel halophilic anaerobe isolated from food products. In the manufacturing process of salted puffer fish ovaries, as well as in broth medium, significant amounts of organic acids were found; 30–1570 µg acetic acid g⁻¹, 30–110 µg formic acid g⁻¹ and 310–2050 µg lactic acid g⁻¹ were detected in the fermented ovaries. These halophilic anaerobes probably contribute to the manufacturing process of this Japanese traditional food product, fermented puffer fish ovaries (*fugunoko nukaduke*).

Description of *Haloanaerobium fermentans* sp. nov.


Cells are Gram-negative rods (1.0–1.2 µm wide by 2.7–3.3 µm long). The bacterium is motile by means of peritrichous flagella. Spores are not observed. Cells grow only anaerobically on solid medium. Colonies on ABCM plate medium supplemented with 10 % (w/v) NaCl are smooth, convex, opaque and yellowish. It is a moderately halophilic bacterium that grows in the presence of 7–25 % (w/v) NaCl, with an optimal NaCl concentration of 10 % (w/v). It grows well at 15 and 45 °C but not at 10 or 50 °C. The optimum temperature for growth is 35 °C. It grows well in the pH range of 6.0 to 9.0 with the optimum pH for growth being 7.5. It ferments d-glucose to hydrogen, carbon dioxide, ethanol and other acidic end-products such as acetate, formate and lactate. Butyrate and propionate are not produced. Hydrogen sulfide is produced. The fermentative substrates include N-acetylglucosamine, cellobiose, fructose, galactose, d-glucose, lactose, maltose, d-mannose, raffinose, d-ribose, sucrose and d-xylene. L-Arabinose, glycerol, pyruvate, rhamnose and starch are not fermented. The DNA base composition is 33.3 mol% G+C (HPLC).

Isolated from salted ovaries in the manufacturing process of a Japanese traditional food product, fermented puffer fish ovaries or *fugunoko nukaduke*. The 16S rRNA gene sequence of the new isolate R-9⁰ has been deposited in the DDBJ database under accession no. AB023308. The type strain is R-9⁰, which has been deposited with the Japan Collection of Microorganisms under accession number JCM 10494⁰.

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Haloanaerobium fermentans sp. nov.


