Lacticigenium naphtae gen. nov., sp. nov., a halotolerant and motile lactic acid bacterium isolated from crude oil

Takao Iino, Ken-ichiro Suzuki and Shigeaki Harayama

A novel lactic acid bacterium, strain MIC1-18T, was isolated from crude oil collected at an oil–water well in Akita, Japan. Cells of strain MIC1-18T were found to be facultatively anaerobic, mesophilic, neutrophilic, Gram-negative, non-sporulating, motile by means of peritrichous flagella and oval rods, 1.8–2.5 µm long. Optimum growth was observed at 30 °C, pH 7.0 and 3 % (w/v) NaCl. Strain MIC1-18T produced acid from L-arabinose, ribose, glucose, fructose, mannose, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, gentiobiose and 5-ketogluconate. L-Lactic acid was the major end product from glucose. The major cellular fatty acid was C16 : 1\text{v7c}. The cell-wall murein type was A4\text{a} containing Lys–Glu.

The G+C content of the genomic DNA was 37.8 mol%. Phylogenetic analysis based on the 16S rRNA gene revealed that strain MIC1-18T was accommodated as a member of the lactic acid bacteria of the low-G+C content Gram-positive bacteria; the closest neighbour of this organism was Atopococcus tabaci CCUG 48253\text{T}, with only 90.0 % sequence similarity. On the basis of the phenotypic features and phylogenetic position, a novel genus and species, Lacticigenium naphtae gen. nov., sp. nov., are proposed for strain MIC1-18T (=NBRC 101988\text{T}=DSM 19658\text{T}).

Lactic acid bacteria that produce lactic acid as a major end product are important in dairy and fermented food products (Hammes et al., 1990; Lücke, 1996; McKay & Baldwin, 1990; Stiles & Holzapfel, 1997). The lactic acid bacteria are mainly accommodated in the low-G+C content Gram-positive bacteria (Collins et al., 1991; Schleifer & Ludwig, 1996; Vandamme et al., 1996), and consist of more than 30 genera with validly published names, comprising more than 350 named species in the List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.cict.fr/). These lactic acid bacteria have frequently been isolated from milk, meat, plants, animal intestines, dairy foods and fermented products (Stiles & Holzapfel, 1997; Vaughan et al., 2002, 2005). On the other hand, lactic acid bacteria have also been isolated from sponges, raw shells, tobacco and deep sub-sea-floor sediment, which are less significant sources of the bacteria (Collins et al., 2005; Ishikawa et al., 2003; Liu et al., 2002; Toffin et al., 2005). These findings have shown that lactic acid bacteria inhabit a variety of natural environments.

Thus, we attempted to isolate novel lactic acid bacteria from an unusual source, namely crude oil from an oil–water-extracting well. In this paper, we describe the isolation of a halotolerant and motile lactic acid bacterium. On the basis of morphological, biochemical, physiological and phylogenetic properties, a novel genus and species are proposed for this bacterium.

Crude oil was collected from the oil–water separation tank of an oil–water-extracting well in Akita prefecture, Japan. The crude oil was kept in a sealed nylon bag with an O2-absorbing and CO2-generating agent (AnaeroPack; Mitsubishi Gas Chemical) until it was used to inoculate fresh medium.

For enrichment, 0.5 ml crude oil was used to inoculate 20 ml HSm medium in a vial sealed with a tight-fitting butyl rubber stopper. HSm medium was composed of (l1): 0.355 g KCl, 0.14 g KH2PO4, 0.14 g CaCl2·2H2O, 0.25 g NH4Cl, 4.0 g MgCl2·6H2O, 3.45 g MgSO4·7H2O, 18.0 g NaCl, 2.0 mg Fe(NH4)2(SO4)2·6H2O, 1.0 g sodium acetate, 2.0 g yeast extract (Becton Dickinson), 2.0 g trypticase peptone (BBL), 5.0 g NaHCO3, 10.0 ml trace elements solution (Balch et al., 1979) containing 25.0 mg NiCl2·6H2O, 2.0 g (NH4)6Ni(SO4)2·6H2O, 0.3 g Na2SeO3·5H2O and 10.0 mg Na2WO4·2H2O (1 distilled water)–1. Prior to inoculation, the pH of the medium was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of Lacticigenium naphtae MIC1-18T is AB430339.

A supplementary figure showing phase-contrast and transmission electron micrographs of cells of strain MIC1-18T is available with the online version of this paper.
Cells of strain MIC1-18\textsuperscript{T} were oval rods and were approximately 0.6–0.7 μm in width and 1.8–2.5 μm in length (see Supplementary Fig. S1 in IJSEM Online). The cells usually occurred singly or in pairs, but sometimes as short chains. Motility was observed under a phase-contrast microscope. Electron micrography demonstrated the presence of peritrichous flagella. Conventional Gram staining of the cells was negative. However, the Gram test with 3% (w/v) KOH as described by Powers (1995) indicated that strain MIC1-18\textsuperscript{T} had a Gram-positive cell-wall structure. Spore formation was not observed under a phase-contrast microscope.

Strain MIC1-18\textsuperscript{T} was a facultatively anaerobic bacterium and catalase-negative. The growth temperature for strain MIC1-18\textsuperscript{T} ranged from 4 to 30 °C, with an optimum at 30 °C. No growth was observed at 35 °C. The pH range for growth was 6.5–8.5, the optimum being pH 7.0. No growth was observed at pH 6.0 or 9.0. Growth occurred at <17% (w/v) NaCl, with an optimum of 3% (w/v) NaCl; no growth was observed at 18% (w/v) NaCl. By using the API 50 CHL system (bioMérieux), strain MIC1-18\textsuperscript{T} produced acid from L-arabinose, ribose, glucose, fructose, mannose, N-acetylg glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, gentisiose and 5-ketogluconate. Lactic acid was the major end product from glucose under aerobic and anaerobic conditions, as determined by HPLC with an organic acid column (Waters). The stereoisomer of the lactic acid produced was mostly the L form; the ratio of L- to D-lactic acid was 97.3 : 2.7 by using the UV method for determination of D- and l-lactic acid (product code 11 112 821 035; R-Biopharm). Elemental sulfur (1%, w/v), sulfate (10 mM), thiosulfate (10 mM), sulfite (1 mM), nitrate (10 mM), nitrite (1 mM) and fumarate (10 mM) were not utilized as electron acceptors in the presence of 0.2% (w/v) each of yeast extract and Polypepton (Nihon Pharmaceutical) as carbon and energy sources. Strain MIC1-18\textsuperscript{T} was susceptible to 100 μl gentamicin ml\textsuperscript{-1} and resistant to 100 μg ml\textsuperscript{-1} each of kanamycin and streptomycin. The generation time was calculated to be 8.1 h in HSM medium containing 20 mM glucose at 30 °C, pH 7.0 and 3% (w/v) NaCl, based on the increase in turbidity.

The major cellular fatty acid was C\textsubscript{16:1}ω7c (57.4%), based on the MIDI microbial identification system (Microbial ID; Agilent Technologies) described by Sasser (1990). C\textsubscript{14:0} (9.9%), C\textsubscript{16:0} (16.6%) and C\textsubscript{18:1}ω9c (14.0%) were also contained as minor components. The cell-wall amino acids were lysine, alanine and glutamic acid in an approximately molar ratio of 1 : 1.5 : 1.6, determined based on the method described by Tamura et al. (1994). It was inferred that strain MIC1-18\textsuperscript{T} possessed murine type A4\textsubscript{4} containing 1-Lys–L-Glu as described by Schleifer & Kandler (1972), based on the method described by Nozawa et al. (2007). The genomic DNA G + C content of strain MIC1-18\textsuperscript{T} was 37.8 mol%, determined by HPLC as described by Tamaoka & Komagata (1984).

An almost-complete 16S rRNA gene sequence (1487 bp) was determined for strain MIC1-18\textsuperscript{T}. The 16S rRNA gene was amplified by PCR with the universal primers 27F (positions 8–27 in the Escherichia coli numbering system) and 1492R (positions 1510–1492) as described previously (Iino et al., 2007). After alignment of the obtained sequence with related sequences in public DNA databases by using ARB software (http://www.arb-home.de/), phylogenetic trees were constructed by the neighbour-joining (NJ) method with the CLUSTAL_X program (Felsenstein, 1985; Kimura, 1980; Saitou & Nei, 1987; Thompson et al., 1997) and by the maximum-likelihood (ML) method with the MOLPHY software (Adachi & Hasegawa, 1995). Phylogenetic analysis showed that strain MIC1-18\textsuperscript{T} was included in the low-G + C content Gram-positive bacteria (Collins et al., 1991; Schleifer & Ludwig, 1996). The topologies of the trees generated by the NJ and ML methods were almost identical, and strain MIC1-18\textsuperscript{T} formed a distinct subline branching at the periphery of the 16S rRNA gene sequence cluster that embraced the genera Alkalibacterium (Ntougias & Russell, 2001), Alloiooccus (Aguirre & Collins, 1992), Allolustis (Collins et al., 2003), Ato pococcus (Collins et al., 2005), Atopostipes (Cotta et al., 2004), Carnobacterium (Collins et al., 1987), Dolosigranulum (Aguirre et al., 1993), Desemzia (Stackebrandt et al., 1999), Isobaculum (Collins et al., 2002), Marinilactibacillus (Ishikawa et al., 2003) and Trichococcus (Scheff et al., 1984) (Fig. 1). The branching of strain MIC1-18\textsuperscript{T} at the base of this group was supported by bootstrap values of 95 and 92% in the NJ and ML analyses, respectively. The 16S rRNA gene sequence of strain MIC1-18\textsuperscript{T} had similarities of 88.2–93.3% to those of bacteria belonging to the 11 above-mentioned genera.

Morphological, biochemical and physiological properties of strain MIC1-18\textsuperscript{T}, along with those of members of phylogenetically related genera, are summarized in Table 1. Strain MIC1-18\textsuperscript{T} showed a negative reaction to conven-
tional Gram staining. The Gram-negative property was only observed in *Alkalibacterium olivapovliticus* and occasionally in *Isobaculum melis* (Collins et al., 2002; Ntougias & Russell, 2001). Furthermore, strain MIC1-18T could be distinguished from members of phylogenetically related genera by its cell-wall amino acid composition. Strain MIC1-18T possessed murein type A4a (L-Lys–L-Glu) constituting lysine, alanine and glutamic acid, which differs from those of all phylogenetically related genera except *Atopococcus* (Collins et al., 2005).

In addition to the differences described above, cell shape, motility, genomic DNA G+C content and growth under anaerobic conditions are markedly different between strain MIC1-18T and the genus *Atopococcus*, which was phylogenetically related most closely to strain MIC1-18T (Collins et al., 2005). Cells of strain MIC1-18T are oval rods that are motile by means of peritrichous flagella, whereas cells of *Atopococcus tabaci*, the sole representative of the genus *Atopococcus*, are coccoid and non-motile; also, the genomic DNA G+C content of strain MIC1-18T is lower than that of *Atopococcus tabaci*. Furthermore, strain MIC1-18T grows under anaerobic conditions, whereas *Atopococcus tabaci* does not. Strain MIC1-18T was also readily distinguished from members of the genera *Alkalibacterium* and *Marinilactibacillus* by the following properties in addition to the differences in Gram staining and cell-wall amino acid composition. Strain MIC1-18T was neutrophilic and produced L-lactic acid as the end product from glucose, whereas *Alkalibacterium olivapovliticus* (Ntougias & Russell, 2001), *Alkalibacterium ibuiriense* (Nakahjima et al., 2005) and *Alkalibacterium psychrotolerans* (Yumoto et al., 2004) are alkaliphilic and produce DL-lactic acid. Similarly, strain MIC1-18T did not grow at 35 °C and produced L-lactic acid as the end product from glucose, whereas *Marinilactibacillus psychrotolerans* (Ishikawa et al., 2003) and *Marinilactibacillus piezotolerans* (Toffin et al., 2005) grew at 37 °C and weakly produced formic and acetic acids and ethanol in addition to L-lactic acid. Strain MIC1-18T was distinguished from *Atloiooccus otitis* (Aguirre & Collins, 1992; Faden & Dryja, 1989), *Atopostipes suicioalcalis* (Cotta et al., 2004) and *Dolosigranulum piezotolerans* (Toffin et al., 2005) grew at 37 °C and weakly produced formic and acetic acids and ethanol in addition to L-lactic acid. Strain MIC1-18T was distinguished from members of the genera *Alloiococcus*, *Atopostipes* and *Dolosigranulum* by the following properties in addition to the differences in Gram staining and cell-wall amino acid composition.

In conclusion, strain MIC1-18T was significantly different from 11 related genera, namely *Alkalibacterium*, *Atloiooccus*, *Allofustis*, *Atopococcus*, *Atopostipes*, *Carnobacterium*, *Dolosigranulum*, *Desemzia*, *Isobaculum* and *Trichococcus*, on the basis of phylogenetic position, morphology and biochemical and physiological properties. Consequently, we propose a novel genus and species for strain MIC1-18T, to be named *Lacticigenium naphtae* gen. nov., sp. nov.

**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequences of strain MIC1-18T and related species. The tree was based on an alignment of 1142 bp of 16S rRNA gene sequence and constructed by using the neighbour-joining method. Numbers at nodes indicate bootstrap percentages derived from 1000 bootstrap replications, determined by neighbour-joining analysis. Bar, 0.01 substitutions per nucleotide position.
Table 1. Morphological, biochemical and physiological properties of strain MIC1-18\(^T\) (*Lacticigenium naphtae* gen. nov, sp. nov.) and phylogenetic relatives

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Lacticigenium naphtae</em> MIC1-18(^T)</td>
<td>(data from present study)</td>
</tr>
<tr>
<td>2</td>
<td><em>Alkalibacterium</em> [three species (n=3); Nakajima <em>et al.</em>, 2005; Ntougias &amp; Russell, 2001; Yumoto <em>et al.</em>, 2004]</td>
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<td>3</td>
<td><em>Allofustis</em> (n=1; Collins <em>et al.</em>, 2003)</td>
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<td>4</td>
<td><em>Alloiooccus</em> (n=1; Aguirre &amp; Collins, 1992; Faden &amp; Dryja, 1989)</td>
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<td>5</td>
<td><em>Atopococcus</em> (n=1; Collins <em>et al.</em>, 2005)</td>
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<td>6</td>
<td><em>Atopostipes</em> (n=1; Cotta <em>et al.</em>, 2004)</td>
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<td>7</td>
<td><em>Carnobacterium</em> (n=9; Collins <em>et al.</em>, 1987)</td>
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<td>8</td>
<td><em>Desemzia</em> (n=1; Stackebrandt <em>et al.</em>, 1999)</td>
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<td>9</td>
<td><em>Dolosigranulum</em> (n=1; Aguirre <em>et al.</em>, 1993)</td>
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<td>10</td>
<td><em>Isobaculum</em> (n=1; Collins <em>et al.</em>, 2002)</td>
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<td>11</td>
<td><em>Marinilactibacillus</em> (n=2; Ishikawa <em>et al.</em>, 2003; Toffin <em>et al.</em>, 2005)</td>
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<td>12</td>
<td><em>Trichococcus</em> (n=5; Liu <em>et al.</em>, 2002; Pikuta <em>et al.</em>, 2006; Scheff <em>et al.</em>, 1984)</td>
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**ND**, No data available.

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<th>3</th>
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<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<tbody>
<tr>
<td>Cell form</td>
<td>Oval rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Ovoid</td>
<td>Coccioid</td>
<td>Short rods</td>
<td>Straight, slender rods</td>
<td>Short rods</td>
<td>Ovoid</td>
<td>Rods</td>
<td>Straight rods</td>
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<td>Motile</td>
<td>ND</td>
<td>ND</td>
<td>Non-motile</td>
<td>Non-motile</td>
<td>Motile/non-motile</td>
<td>Motile</td>
<td>Non-motile</td>
<td>Non-motile</td>
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<td>Gram staining</td>
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<td>Facultatively anaerobic</td>
<td>Aerobic</td>
<td>Facultatively anaerobic</td>
<td>Aerobic</td>
<td>Facultatively anaerobic</td>
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<td>Aerobic</td>
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<td>End products from glucose*</td>
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<td>No product</td>
<td>ND</td>
<td>F, A, L</td>
<td>ND</td>
<td>F, A, L</td>
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<td>A, L</td>
<td>L (L)</td>
<td>A, L</td>
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<td>Optimum growth at:</td>
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<td>ND</td>
<td>30</td>
<td>28–30</td>
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<td>ND</td>
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<td>23–30</td>
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<td>pH</td>
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<td>7.0–9.0</td>
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<td>Growth at 8% (w/v) NaCl</td>
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<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<td>ND</td>
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<td>Cell-wall murein</td>
<td>A4(\alpha), L-Lys–L-Lys–</td>
<td>A4(\beta), Orn-D-Asp or Orn–D-Glu</td>
<td>A1(\alpha), L-Lys direct</td>
<td>A4(\alpha), L-Lys–L-Glu</td>
<td>A4(\alpha), L-Lys–L-Glu</td>
<td>m-Dpm(\dagger)</td>
<td>A4(\alpha), Lys–D-Glu</td>
<td>A4(\alpha), Lys–D-Asp</td>
<td>A3(\alpha), Lys–Thr–Gly</td>
<td>A4(\beta), Orn-D-Glu</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>38</td>
<td>39–43</td>
<td>39</td>
<td>44–45</td>
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<td>42</td>
<td>39</td>
<td>34–42</td>
<td>45–49</td>
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*F, Formic acid; A, acetic acid; L, lactic acid; E, ethanol. Stereoisomers of organic acids are indicated in parentheses.
† m-Dpm, meso-diaminopimelic acid.
**Description of Lacticigenium gen. nov.**

*Lacticigenium* [Lact.ic.i genetically. N.L. n. *acidum lacticum* lactic acid; N.L. neut. suff. *-igenium* (from Gr. *v. genmao* to produce) that which produces; N.L. neut. n. *Lacticigenium* a bacterium that produces lactic acid].

Facultatively anaerobic, mesophilic, neutrophilic, non-sporing and motile by peritrichous flagella. Gram reaction is negative with conventional Gram stain, but positive with the KOH test. Cells are oval rods. Catalase is not produced. The major cellular fatty acid is C16:1ω7c. Cell-wall murein is type A4α containing L-Lys–L-Glu. The G+C content of the genomic DNA is 38 mol% (as determined by HPLC). Represents a distinct phylogenetic lineage in the low-G+C content Gram-positive bacteria based on 16S rRNA gene sequence analysis. The type species is *Lacticigenium naphtae*.

**Description of Lacticigenium naphtae* sp. nov.**


Displays the following properties in addition to those given in the genus description. Cells are 0.6–0.7 μm in size. Growth occurs at or below 30 °C, but not at 35 °C, with an optimum at 3 °C. The pH range for growth is 6.5–8.5, with an optimum around pH 7.0. Growth occurs below 17% (w/v) NaCl, with an optimum at 3% (w/v). Acids are produced from L-arabinose, ribose, glucose, fructose, mannose, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, gentio- and 5-ketogluconate. L-Lactic acid is the major end product from glucose. Sulfate, sulfite, thiosulfate, elemental sulfur, nitrate, nitrite and fumarate are not used as electron acceptors. The G+C content of genome DNA is 37.8 mol% (as determined by HPLC).

The type strain is MIC1-18T (=NBRC 101988T = DSM 19658T), which was isolated from a crude-oil sample collected from an oil–water well in Akita, Japan.

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**References**


