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

Correspondence
Takao Iino
ino-takao@nite.go.jp
NITE Biological Resource Center, National Institute of Technology and Evaluation, 2-5-8, Kazusakamatari, Kisarazu, Chiba 292-0818, Japan

A novel lactic acid bacterium, strain MIC1-18\textsuperscript{T}, was isolated from crude oil collected at an oil–water well in Akita, Japan. Cells of strain MIC1-18\textsuperscript{T} 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 \( \mu \text{m} \) long. Optimum growth was observed at 30 °C, pH 7.0 and 3 % (w/v) NaCl. Strain MIC1-18\textsuperscript{T} produced acid from L-arabinose, ribose, glucose, fructose, mannose, N-acetylgalactosamine, 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 C\textsubscript{16} : 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 MIC1-18\textsuperscript{T} 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\textsuperscript{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 MIC1-18\textsuperscript{T} (=NBRC 101988\textsuperscript{T} =DSM 19658\textsuperscript{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 O\textsubscript{2}-absorbing and CO\textsubscript{2}-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 (l\textsuperscript{–1}): 0.355 g KCl, 0.14 g KH\textsubscript{2}PO\textsubscript{4}, 0.14 g CaCl\textsubscript{2}.2H\textsubscript{2}O, 0.25 g NH\textsubscript{4}Cl, 4.0 g MgCl\textsubscript{2}.6H\textsubscript{2}O, 3.45 g MgSO\textsubscript{4}.7H\textsubscript{2}O, 18.0 g NaCl, 2.0 mg Fe(NH\textsubscript{4})\textsubscript{2}(SO\textsubscript{4})\textsubscript{2}.6H\textsubscript{2}O, 1.0 g sodium acetate, 2.0 g yeast extract (Becton Dickinson), 2.0 g trypticase peptone (BBL), 5.0 g NaHCO\textsubscript{3}, 10.0 ml trace elements solution (Balch et al., 1979) containing 25.0 mg NiCl\textsubscript{2}.6H\textsubscript{2}O, 2.0 g (NH\textsubscript{4})\textsubscript{2}Ni(SO\textsubscript{4})\textsubscript{2}.6H\textsubscript{2}O, 0.3 g Na\textsubscript{2}SeO\textsubscript{3}, 5H\textsubscript{2}O and 10.0 mg Na\textsubscript{3}WO\textsubscript{4}.2H\textsubscript{2}O (1 distilled water) \textsuperscript{–1}. Prior to inoculation, the pH of the medium was...
adjusted to 7.0 with 6 M HCl, dissolved air was removed by flushing with H2/CO2 (4:1, v/v; approx. 150 kPa), and 10 ml vitamin solution l−1 (Wolin et al., 1963) and 10 ml sterile stock solution l−1 containing 0.5 g Na2S/cysteine–HCl were added. The enrichment culture was cultivated at 25 °C for 3 weeks and transferred several times to fresh HSm medium. Subsequently, serial decimal dilutions (10−1 to 10−10) of the enrichment culture were made with 20 % (w/v) saline, and 0.1 ml of the diluted samples was spread on HSm agar (1.5 %, w/v) plates and cultivated aerobically at 25 °C for 3 weeks. Visible colonies grown on HSm agar medium were picked up and transferred to fresh HSm agar plates. The purification procedure was repeated several times until the cultures were deemed pure and a uniformly shaped single culture, designated MIC1-18T, was obtained. The following study for MIC1-18T basically used HSm medium flushed with N2/CO2 (4:1, v/v) instead of H2/CO2 (4:1, v/v; approx. 150 kPa).

Cells of strain MIC1-18T 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 MIC1-18T had a Gram-positive cell-wall structure. Spore formation was not observed under a phase-contrast microscope.

Strain MIC1-18T was a facultatively anaerobic bacterium and catalase-negative. The growth temperature for strain MIC1-18T 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 MIC1-18T produced acid from L-arabinose, ribose, glucose, fructose, mannose, N-acetylglucosamine, amygdalin, arbutin, salicin, clofibiose, maltose, sucrose, trehalose, gentiobiose 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 determined by HPLC with an organic acid column from glucose under aerobic and anaerobic conditions, as ketogluconate. Lactic acid was the major end product

N-carbon and energy sources. Strain MIC1-18T was susceptible to ampicillin, bacitracin, chloramphenicol, rifampicin, tetracycline and vancomycin (100 μg ml−1), weakly susceptible to 100 μl gentamicin ml−1 and resistant to 100 μg ml−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 C16:1ω7c (57.4 %), based on the MIDI microbial identification system (Microbial ID; Agilent Technologies) described by Sasser (1990). C14:0 (9.9 %), C16:0 (16.6 %) and C18:1ω9c (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 MIC1-18T possessed murein type A4γ containing l-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 MIC1-18T 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 MIC1-18T. 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 MIC1-18T 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 MIC1-18T formed a distinct subline branching at the periphery of the 16S rRNA gene sequence cluster that embraced the genera Alkalibacterium (Ntougias & Russell, 2001), Alloiovoccus (Aguirre & Collins, 1992), Allofustis (Collins et al., 2003), Atopococcus (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 & Komagata, 1984) (Fig. 1). The branching of strain MIC1-18T 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 MIC1-18T 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 MIC1-18T, along with those of members of phylogenetically related genera, are summarized in Table 1. Strain MIC1-18T 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., 1992; Faden & Dryja, 1989), *Atopostipes suiciacalis* (Cotta et al., 2004) and *Dolosigranulum pigrum* (Aguirre et al., 1993) by its lower genomic DNA G+C content. Furthermore, the cell shape, motility, end products from glucose and growth under anaerobic conditions of strain MIC1-18T were different from *Atopococcus otitis*, *Atopostipes suiciacalis* and *Dolosigranulum pigrum*. Strain MIC1-18T was distinguished from members of the genera *Carnobacterium* (Collins et al., 1987), *Isobaculum* (Collins et al., 2002) and *Trichococcus* (Scheff et al., 1984) by growth at 8% (w/v) NaCl. It is interesting that growth at 8% (w/v) NaCl distinguished between one phylogenetic cluster containing strain MIC1-18T and genera such as *Atopococcus*, *Alkalibacterium* and *Marinilactibacillus*, and the other cluster containing genera such as *Carnobacterium*, *Isobaculum* and *Trichococcus*.

In conclusion, 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 *Alloiococcus otitis* (Aguirre & Collins, 1992; Faden & Dryja, 1989), *Atopostipes suiciacalis* (Cotta et al., 2004) and *Dolosigranulum pigrum* (Ishikawa et al., 2003) by 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.

### 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 MIC1-18T (*Lacticigenium naphtae* gen. nov, sp. nov.) and phylogenetic relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell form</td>
<td>Oval rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Ovoid</td>
<td>Coccoid</td>
<td>Short rods</td>
<td>Straight, slender rods</td>
<td>Short rods</td>
<td>Ovoid</td>
<td>Rods</td>
<td>Straight rods</td>
<td>Spherical to ovoid</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
<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>
<td>Motile/non-motile</td>
<td>ND</td>
</tr>
</tbody>
</table>
| Gram staining | Facultatively anaerobic | L (L) | Facultatively anaerobic | L (L) | Facultatively anaerobic | Aerobic | Facultatively anaerobic | L (L) | Facultatively anaerobic | L (L) | ND | +/
| Metabolism    | - | +/− | + | + | Facultatively anaerobic | Aerobic | Facultatively anaerobic | L (L) | Facultatively anaerobic | L (L) | ND | ND |
| End products from glucose* | L (L) | L (L) | ND | No product | ND | F, A, L | A, L | F, A, L |
| Optimum growth at: | Temperature (°C) | 30 | 27–37 | ND | ND | 30 | 28–30 | ND | ND | ND | ND | 37–40 | 23–30 |
|                | pH | 7.0 | 9.0–10.5 | ND | ND | ND | ND | ND | ND | ND | ND | 7.0–9.0 | 6.0–7.5 |
|                | Growth at 8% (w/v) NaCl | + | + | ND | ND | + | ND | ND | ND | ND | ND | − | + |
| Cell-wall murein | A4\(\alpha\), L-Lys–L-Glu | A4\(\beta\), Orn–D-Asp or Orn–D-Glu | A1\(\alpha\), L-Lys direct | ND | A4\(\alpha\), L-Lys–L-Glu | A4\(\alpha\), L-Lys–D-Asp | m-Dpm† | A4\(\alpha\), Lys–D-Glu | A4\(\alpha\), Lys–D-Asp | A3\(\alpha\), Lys–L-Thr–Glu | A4\(\alpha\), Orn–D-Glu | A4\(\alpha\), L-Lys–D-Asp |
| DNA G+C content (mol%) | 38 | 39–43 | 39 | 44–45 | 46 | 44 | 33–37 | 40 | 42 | 39 | 34–42 | 45–49 |

*F, Formic acid; A, acetic acid; L, lactic acid; E, ethanol. Stereoisomers of organic acids are indicated in parentheses.
†m-Dpm, meso-diaminopimelic acid.

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

*Lacticigenium* [Lact.i.ci.ge’ni.um. N.L. n. *acidum lacticum* lactic acid; N.L. neut. suff. -genium (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-sporulating 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 C<sub>16:1ω7c</sub>. Cell-wall murin is type A4<sub>v</sub> 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 30 °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). Acid is produced from l-arabinose, ribose, glucose, fructose, mannone, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, gentiobiose 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 genomic DNA is 37.8 mol% (as determined by HPLC).

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

**Acknowledgements**

The authors thank Mr Takahiro Iwami and Ms Yayoi Sakiyama for technical support. This study was partly supported by the New Energy and Industrial Technology Development Organization (NEDO).

**References**


