**Megasphaera hexanoica** sp. nov., a medium-chain carboxylic acid-producing bacterium isolated from a cow rumen

Byoung Seung Jeon,¹ Seil Kim² and Byoung-In Sang¹,*

**Abstract**

Strain MH₁, a strictly anaerobic, Gram-stain-negative, non-spore-forming, spherical coccus or coccoid-shaped microorganism, was isolated from a cow rumen during a screen for hexanoic acid-producing bacteria. The microorganism grew at 30–40°C and pH 5.5–7.5 and exhibited production of various short- and medium-chain carboxylic acids (acetic acid, butyric acid, propionic acid, isobutyric acid, isovaleric acid, hexanoic acid, heptanoic acid and octanoic acid), as well as H₂ and CO₂ as biogas. Phylogenetic analysis based on 16S rRNA gene sequencing demonstrated that MH¹ represents a member of the genus *Megasphaera*, with the closest relatives being *Megasphaera indica* NMBH1-10¹ (94.1 % 16S rRNA sequence similarity), *Megasphaera elsdenii* DSM 20460¹ (93.8 %) and *Megasphaera paucivorans* DSM 16981¹ (93.8 %). The major cellular fatty acids produced by MH¹ included C₁₂:₀, C₁₄:₀, C₁₆:₀, C₁₈:₁cis 9, and C₁₈:₀; and the DNA G+C content of the MH¹ genome is 51.8 mol%. Together, the distinctive phenotypic and phylogenetic characteristics of MH¹ indicate that this microorganism represents a novel species of the genus *Megasphaera*, for which the name *Megasphaera hexanoica* sp. nov. is herein proposed. The type strain of this species is MH¹ (=KCCM 43214¹=JCM 31403¹).

Hexanoic acid is a valuable linear six-carbon carboxylic acid that can be used in the production of multiple biofuels and biochemicals [1, 2]. Various anaerobic bacteria have been reported as hexanoic-acid-producers [1, 3, 4], including *Megasphaera elsdenii* and *Megasphaera indica* [5, 6].

Species of the genus *Megasphaera* have been isolated from various sources, including the rumen and faeces of cattle and other animals, hay, breweries, human samples, etc. [5, 7–13]. These strains are of particular interest for their high levels of hexanoic acid production [6, 14, 15]. In a previous study, Roddick and Britz [6] showed that in situ removal of hexanoic acid resulted in increased production of hexanoic acid (up to 19 g l⁻¹) when using glucose as a carbon source. Meanwhile, Choi et al. [15] reported that extractive fermentation increased hexanoic acid production up to 28.4 g l⁻¹ when using sucrose as a carbon source. Notably, enriched species such as *Megasphaera elsdenii* were also found to affect the production of C₄–C₆ carboxylic acids during thin stillage fermentation when equipped with an electrolytic extraction module [14, 16].

Taxonomically, the genus *Megasphaera* is a member of the family Veillonellaceae, order Veillonellales, class Negativicutes, and phylum Firmicutes, and is characterized by Gram-stain-negative, spherical, obligate anaerobic bacteria [5].

Seven strains of members of the genus *Megasphaera* have been identified as type strains of species with validly published names. As a representative strain, *M. elsdenii* DSM 20460¹ was isolated from rumen of sheep. The closest taxonomic relative of *M. elsdenii* DSM 20460¹, *M. indica* NMBHI-10¹ was isolated from the faeces and intestine of humans [5]. *Megasphaera massiliensis* NP5¹ was isolated from the faeces of a HIV-infected patient [17]. Conversely, *Megasphaera cerevisiae* DSM 20462¹, *Megasphaera paucivorans* DSM 16981¹ and *Megasphaera sueciensis* DSM 17042¹ were isolated from brewery-derived samples [10] and *Megasphaera micronuciformis* DSM 17226¹ was isolated from a human liver abscess and whitlow [13]. In this study a strictly anaerobic, hexanoic-acid-producing bacterial strain, MH¹, was isolated from a cow rumen as described previously [18]. On the basis of the results of a polyphasic taxonomic investigation the novel strain represents a novel species of the genus *Megasphaera*.

MH¹ was isolated from cow rumen. Briefly, samples were suspended in saline solution and cultivated on Reinforced Clostridia Medium (RCM) agar containing 5 g hexanoic acid.
1\textsuperscript{–1} at 37 °C in an anaerobic chamber (Coy) with 90 % N\textsubscript{2}, 5 % H\textsubscript{2}, and 5 % CO\textsubscript{2}. For solid medium, 15 g agar 1\textsuperscript{–1} was added and the medium was purged with argon gas before autoclaving at 121 °C for 20 min.

The isolated strain was subsequently maintained in modified peptone–yeast extract–fructose medium (mPYF, Table S1, available in the online Supplementary Material) under anaerobic conditions, and stored as a glycerol stock (10 %; v/v) at −70 °C.

For all analyses, M. paucivorans DSM 16981\textsuperscript{T}, M. succinicipiens DSM 17042\textsuperscript{T}, M. micronuciformis DSM 17226\textsuperscript{T}, M. cerevisiae DSM 20462\textsuperscript{T}, M. elsdenii KCTC 5187\textsuperscript{T} (=DSM 20460\textsuperscript{T} =ATCC 25940\textsuperscript{T}), and Anaeroglobus geminatus CCUG 44773\textsuperscript{T} were used as reference strains [9, 10, 13, 29]. Each strain was obtained from the corresponding culture collection and maintained at 30–37 °C in TFY medium containing 20 g yeast extract 1\textsuperscript{–1}, 30 g peptone 1\textsuperscript{–1}, 0.5 g cysteine–HCl hydrate 1\textsuperscript{–1} and 5 g fructose 1\textsuperscript{–1}.

The growth temperature range and optimum growth conditions for MH\textsuperscript{T} were determined on mPYF medium. Growth was assessed by measuring the OD\textsubscript{600} at 30–45 °C and pH 5.0–8.0. The aerobicity of the strain was tested by cultivation under aerobic or anaerobic atmospheric conditions for 10 days. MH\textsuperscript{T} exhibited growth at between 30 and 40 °C (optimal temperature: 37 °C) and between a pH of 5.5 and 7.5 (optimum pH: 6.5), which was consistent with the growth range of species of the genus Megasphaera. Likewise, MH\textsuperscript{T} did not show any growth under aerobic conditions.

For evaluation of colony and cell morphology, MH\textsuperscript{T} was cultivated on mPYF plates (pH 7.0) at 37 °C for 2–3 days. Colony morphology was evaluated after 3 days of incubation, while cell morphology was evaluated by Gram stain analysis using a Gram staining set (Difco), according to the manufacturer’s instructions, as well as by scanning electron microscopy (SEM; FEI-Nova SEM; FEI) analysis. Meanwhile, cell motility and catalase production were evaluated by microscopic observation of cultures during exponential growth, using a FV-1000 confocal microscope (Olympus) and by addition of 3 % (v/v) H\textsubscript{2}O\textsubscript{2} to cells smeared on standard microscope slides, respectively. MH\textsuperscript{T} exhibited coccoid cells that were 0.5–0.8 µm long by 0.8–1.2 µm wide (Fig. 1 and Table 1), which were non-motile and catalase-negative.

To compare the cellular fatty acid composition of MH\textsuperscript{T} with those of the reference strains listed above, each strain was cultivated on RCM agar at 30 °C for 3 days to achieve late exponential phase. Cellular fatty acids were then extracted and prepared as fatty acid methyl esters, as previously described [19], and analyzed using a Microbial Identification System (MIDI, Sherlock MIS Software version 6.2; MIDI). The cellular fatty acid profile of each strain is summarized in Table 2. As in other strains of members of the genus Megasphaera, primary cellular fatty acids (CFA) of MH\textsuperscript{T} included C\textsubscript{12}:0 (16.6 %), C\textsubscript{14}:0 (8.1 %), C\textsubscript{15}:0 (6.3 %), summed feature 5 [C\textsubscript{15}:0 DMA(dimethyl acetal) and/or C\textsubscript{14}:0 3-OH(hydroxyl)]; 6.56 %], C\textsubscript{16}:0 (12.7 %), C\textsubscript{18:1} cis 9 (8.2 %), and C\textsubscript{18:0} (9.4 %). However, ratios of the CFA showed considerable differences as well, CFAs such as C\textsubscript{9}:0 (1.6 %), C\textsubscript{13}:0 (3.2 %), C\textsubscript{17}:0 (5.4 %), and C\textsubscript{17}:0 DMA (2.3 %), which showed relatively lower contents, was also detected. Therefore, these CFAs of MH\textsuperscript{T} distinguished it from other members of the genus Megasphaera.

Biochemical characterization of MH\textsuperscript{T} and the reference strains was carried out in duplicate using an API 50CH system (bioMérieux) and API 20A kits (bioMérieux), according to the manufacturer’s instructions. Prior to analysis, each strain was cultivated on mPYF medium at 37 °C for 1 day. Notably, the results, particularly the utilization of D-fructose and D-mannose and non-utilization of D-glucose, maltose, and mannitol, clearly distinguished MH\textsuperscript{T} from other related strains of members of the genus Megasphaera (Table 1).

To characterize the metabolic end-products of MH\textsuperscript{T} when cultivated in the presence of 2 % fructose as a carbon source, the fermentation broth was extracted using ether as a solvent and analyzed using a gas chromatography time of flight mass spectrometer (ToF/MS; Leco St. Joseph). Ion spectra of the metabolic end-products were then compared with those in the National Institute of Standards and Technology (NIST) library. These analyses detected the following end-products: acetic acid, butyric acid, isobutyric acid, pentanoic acid, isovaleric acid, hexanoic acid, heptanoic acid, and octanoic acid (Fig. 2). In addition, Jeon et al. have reported also that MH\textsuperscript{T} produced 86 mM of hexanoic acid and 4.5 mM of octanoic acid using butyric acid and fructose. When propionic acid and fructose was utilized, pentanoic acid and heptanoic acid increased to 56.2 and 21.1 mM [18]. For that reason, MH\textsuperscript{T} has been reported to utilize C\textsubscript{2}-C\textsubscript{6} fatty acids as electron acceptors [18].

When cultivated in PYG broth containing glucose as the sole carbon source, MH\textsuperscript{T} showed slight growth and produced...
acetic acid (0.3 mM), isobutyric acid (2.5 mM), butyric acid (0.026 mM), pentanoic acid (0.03 mM), hexanoic acid (3.8 mM), heptanoic acid (1.9 mM) and octanoic acid (1.3 mM). The fatty acids profile of MH T was compared with that of M. elsdenii DSM 20460 (Fig. S1). M. elsdenii DSM 20460 T produced acetic acid (10.8 mM), isobutyric acid (3.5 mM), butyric acid (37.2 mM), pentanoic acid (0.1 mM), and hexanoic acid (13.1 mM). Lanjeker et al., have reported the volatile fatty acids profile of M. indica NMBHI-10 T. M. indica NMBHI-10 T produced acetic acid (12.8 mM), isobutyric acid (1.2 mM), butyric acid (14.2 mM), valeric acid (0.7 mM), hexanoic acid (29.5 mM), succinic acid (4.9 mM) and formic acid (10.4 mM) [5]. On the basis of these characteristics, the fatty acid profile of MH T showed distinctive differences.

The susceptibility of MH T and the reference strains to vancomycin and colistin was subsequently evaluated via the antibiotic disc method (Oxoid), as described previously [20]. For these analyses, each strain was grown in liquid mPYF medium at 37 °C for 1 day and spread on mPYF plates. Antibiotic discs containing vancomycin (5 µg) or colistin (10 µg) were then placed on the plates, and the plates were incubated at 37 °C for 3 days. Zones of clearance surrounding the discs indicated sensitivity to the antibiotic. The antibiotic susceptibility of each strain is summarized in Table 1. MH T and M. elsdenii DSM 20460 T had antibiotic susceptibility only to colistin, while M. indica NMBHI-10 T showed resistance to both vancomycin and colistin.

Table 1. Characteristics of MH T and closely related strains

| Strains: 1, MH T; 2, M. elsdenii DSM 20460 T; 3, M. succiniciens DSM 17042 T; 4, M. paucivorans DSM 16981 T; 5, M. micronuciformis DSM 17226 T; 6, M. cerevisiae DSM 20462 T; 7, M. indica NMBHI-10 T; 8, M. massiliensis NP3 T; 9, Anaeroglobus geminatus CCUG 44773 T. Results for metabolic end products of MH T are from this study with cells that were cultured for 3 days at 37 °C in TGY. +, positive; −, negative; w, weak; z, variable; nd, not determined; r, resistant; s, susceptible. All strains, except for M. massiliensis NP3 T, showed negative reactions for nitrate reduction, formation of indole, oxidase and catalase activities and hydrolysis of aesculin, gelatin and arginine. M. massiliensis NP3 T showed negative reactions for formation of indole, catalase activity and hydrolysis of arginine. All strains, except for M. massiliensis NP3 T, were positive for the utilization of pyruvate.

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Table 2. Comparison of cellular fatty acids of strain MH\textsuperscript{T} and some closely related strains

Strains: 1, MH\textsuperscript{T}; 2, M. eladenii DSM 20460\textsuperscript{T}; 3, M. sueciensis DSM 17042\textsuperscript{T}; 4, M. paucivorans DSM 16981\textsuperscript{T}; and 5, M. micronuciformis DSM 17226\textsuperscript{T}; 6, M. cerevisiae DSM 20462\textsuperscript{T}; 7, M. indica NMBHI-10\textsuperscript{7}; 8, Anaeroglobus geminatus CCUG 44773\textsuperscript{T}. All data are from this study. Strains were cultured without shaking for 2 days on RCM. Values are percentages of total fatty acids that were determined in this study. Fatty acids comprising less than 1% of the total in all strains were not listed.

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*The values were from Lanjekar et al. [5].†Summed features consist of one or more fatty acids that could not be separated. Summed feature 2 consists of C\textsubscript{13}:1\textit{cis} 12, C\textsubscript{14}:0 ALDE, and/or C\textsubscript{11}:1 2-OH; Summed feature 2 consists of C\textsubscript{12}:0 3-OH and/or C\textsubscript{13}:0 DMA; Summed feature 4 consists of C\textsubscript{15}:1\textit{cis} 7 and/or C\textsubscript{15}:2; Summed feature 5 consists of C\textsubscript{15}:0 DMA and/or C\textsubscript{14}:0 3-OH; Summed feature 6 consists of C\textsubscript{15}:0 Anteiso 3-OH and C\textsubscript{16}:1\textit{cis} 7 DMA; Summed feature 7 consists of C\textsubscript{17}:2 and C\textsubscript{17}:1\textit{cis} 8; Summed feature 10 consists of C\textsubscript{18}:1\textit{cis} 11/\textit{trans} 9/\textit{trans} 6.
modifications: 27f (forward) 5'-AGA GTT TGA TCM TGG
CTC AG-3' and 1492r (reverse) 5'-TAC GGY TAC CTT
GTT ACG ACT T-3'. The amplification product (1490 bp)
was sequenced using a Sanger DNA sequencer (ABI
3730XL; Applied Biosystems) by Macrogen (Seoul, Korea).

The 16S rRNA sequence of MH\textsuperscript{T} was then compared with
those of closely related strains obtained from the GenBank
(www.ncbi.nlm.nih.gov/genbank/) and Eztaxon databases
(www.ezbiocloud.net/eztaxon) [22]. Alignment and phylo-
genetic analysis were conducted using MEGA7 software [23], and
multiple alignments were generated using the CLUSTAL-W algo-
rithm. Reconstruction of phylogenetic trees was carried out
using the maximum-likelihood (ML) [24], maximum-parsi-
mony (MP) [25], and neighbour-joining (NJ) [26] methods,
and distance matrices were generated as described previously
[27]. The branch stability of the phylogenetic tree was assessed
with 1000 neighbour-joining bootstrap replications. On the
basis of the results of 16S rRNA sequencing, the closest rela-
tives of MH\textsuperscript{T} were \textit{M. indica} NMBHI-10\textsuperscript{T} (94.1 % similarity)
and \textit{M. elsdenii} DSM 20460\textsuperscript{T} (93.8 %), followed by \textit{M. pauci-
vorans} DSM 16981\textsuperscript{T} (93.7 %), \textit{Anaeroglobus gemen-
nitus} CCUG 44773\textsuperscript{T} (93.4 %) and \textit{M. micronuciformis} DSM 17226\textsuperscript{T} (93.0 %). In addition, the
results of analysis of 16S rRNA using BLAST indicated that
MH\textsuperscript{T} has high similarity with some uncultivated clones or
unreleased strains, and these clones have been found in
human caecum (GenBank accession number LT576402), por-
cine intestine (GenBank accession number EU728714) and
sheep rumen (GenBank accession number AB507598). Mean-
while, the results of phylogenetic tree analysis clearly indicated
that MH\textsuperscript{T} represents a novel member of the genus \textit{Megasphaera},
as reported by Marchandin \textit{et al.} [13], this DNA G+C content
was lower than those of \textit{M. elsdenii} DSM 20460\textsuperscript{T} (54.8 mol%)
[20] and \textit{M. indica} NMBHI-10\textsuperscript{T} (57.7 mol%), and higher than
those of \textit{M. paucivorans} DSM 16981\textsuperscript{T} (37.7 mol%), \textit{M. sue-
ciensis} DSM 17042\textsuperscript{T} (43.3 mol%), \textit{M. micronuciformis} DSM 17226\textsuperscript{T} (45.7 mol%), and \textit{M. cerevisiae} DSM 20462\textsuperscript{T} (46.5 mol
%)(Table 1).

In conclusion, the distinct phenotypic, chemotaxonomic
and phylogenetic properties of MH\textsuperscript{T} presented above dem-
strate that this organism represents a novel species of the genus \textit{Megasphaera},
for which we propose the name \textit{Megasphaera hexanoica} sp. nov.

**DESCRIPTION OF MEGASPHAERA HEXANOICA SP. NOV.**

\textit{M. hexanoica} sp. nov. (he.xa.no'i.ca. N.L. n. acidum hexa-
noicum, hexanoic acid; N.L. fem. adj. hexanoica, referring
to the production of hexanoic acid).

Strictly anaerobic, Gram-strain-negative, non-spor-forming,
non-motile, cocoid-shaped microorganism that grows in dip-
lococci. The average size of each cell is 0.8–1.2\,\mu m and the

**Fig. 2.** Total ion chromatograph of metabolic end products by GC-ToF/MS. 1. Acetic acid; 2. 2-methylpropionic acid (isobutyric acid); 3. butanoic acid; 4. 3-methylbutanoic acid (isovaleric acid); 5. pentanoic acid (valeric acid); 6. hexanoic acid (caproic acid); 7. heptanoic acid; 8. octanoic acid (caprylic acid); 9. 2-phenylmalonic acid
microorganism exhibits optimal growth in mPYF medium at 37°C. The strain produces acidic metabolites using D-arabinose, D-fructose, D-arabitol, inositol, potassium gluconate and potassium 5-ketogluconate but not glycerol, erythritol, L-arabinose, D-ribose, D-xylene, D-adonitol, methyl β-D-xylpyranoside, D-glucose, L-sorbose, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin ferric citrate, salicin, cellobiose, maltose, lactose, melibiose, D-sucrose, trehalose, inulin, melezitose, D-rafinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol or potassium 2-ketogluconate. The strain is variable in utilization of D-Mannose as a carbon source and showed weak utilization for galactose and DL-lactate. Addition of acetate and butyrate to the culture medium markedly enhances the growth rate of the microorganism. Colonies are visible on mPYF plates after 2 days at 37°C, and appear slightly yellowish, convex and opaque, with circular edges and diameter of 0.5–1 mm, after 6–7 days. Growth of the strain occurs at 30–40°C, with optimal growth at 37°C. The major end-products of the microorganism are acetic acid, isovaleric acid, and n-caproic acid. The type strain is resistant to a 5 µg vancomycin disc. The major cellular fatty acids are C12:0, C16:0, and C18:0.

The type strain MH(T) (KCCM43214(T)=JCM31403(T)) was isolated from the rumen of a cow. The DNA G+C content of the type strain is 51.8 mol%.

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Seil Kim and Byoung-In Sang developed the concept of the study, contributed to data analysis and preparation of the manuscript with the

**Fig. 3.** Neighbor-joining tree based on 16S rRNA gene sequences, showing the phylogenetic position of *M. hexanoica KCCM 43214(T)* within closely related taxa in the *Megasphaera* group. GenBank accession numbers of the 16S rRNA gene sequences are given in parentheses. Black circles indicate that the corresponding branches were also recovered both by maximum-likelihood and maximum-parsimony methods. Bootstrap values (based on 1000 replications) greater than or equal to 70% are shown as percentages at each node. Bar, 0.01 substitutions per nucleotide position.
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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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