

Methanobacterium alkalithermotolerans sp. nov., a novel alkaliphilic and hydrogen-utilizing methanogen isolated from an alkaline geothermal spring (La Crouen, New Caledonia)

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1	Methanobacterium alkalithermotolerans sp. nov., a novel alkaliphilic and hydrogen-utilizing		
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-			

27 Abstract

28

An anaerobic, hydrogenotrophic methane-producing archaeon was isolated from an alkaline 29 thermal spring (42°C, pH 9.0) in New Caledonia. This methanogen, designated strain CAN^T, is 30 alkaliphilic, thermotolerant, with Gram-positive staining non-motile cells. Strain CAN^T grows 31 32 autotrophically using hydrogen exclusively as an energy source and carbon dioxide as the sole 33 carbon source (without the requirement of yeast extract or other organic compounds). It grows at 20–45°C (optimum 45°C) and pH 7.3–9.7 (optimum 9.0). NaCl is not required for growth 34 (optimum 0%) but is tolerated up to 1.5%. It resists to novobiocin, streptomycin, and 35 36 vancomycin but is inhibited by ampicillin and penicillin, among other antibiotics. The genome consists of a circular chromosome (2.2 Mb) containing 2,126 predicted protein-encoding genes 37 38 with a G+C content of 36.4 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain CAN^T is a member of the genus *Methanobacterium*, most closely related 39 to the alkaliphilic Methanobacterium alcaliphilum WeN4^T with 98.5% 16S rRNA gene 40 sequence identity. The genomes of strain CAN^T and *M. alcaliphilum* DSM 3459, sequenced in 41 42 this study, share 71.6% average nucleotide identity (ANI) and 14.0% digital DNA-DNA hybridization (dDDH). Therefore, phylogenetic analysis and physiological features determined 43 strain CAN^T as a novel species, for which the name *Methanobacterium alkalithermotolerans* 44 45 sp. nov. is proposed, and strain CAN^T (= DSM 102889^{T} = JCM 31304^{T}) is assigned as the type 46 strain.

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- 48

49 Methanogens are a functional group of Archaea that produce methane as a by-product 50 of their energy metabolism (1, 2). Methanobacterium belongs to the Methanobacteriaceae 51 family of the "Methanobacteriota" phylum (3, 4). Methanobacterium species are mesophilic 52 or thermophilic and occur in various anoxic environments, such as anaerobic digesters (5) and 53 marine sediments (6). More than 41 species of the genus Methanobacterium have been 54 described to date. Some of them can use organic compounds, such as formate, to produce 55 methane (5, 7), while other *Methanobacterium* species grow exclusively through the reduction 56 of carbon dioxide (CO₂) by hydrogen (H₂); examples of such include Methanobacterium aarhusense H2-LR^T (6), Methanobacterium alcaliphilum WeN4^T (8), and Methanobacterium 57 *movens* $TS-2^{T}(7)$. 58

59 In this study, we describe the isolation and characterization of an autotrophic, hydrogenotrophic, alkaliphilic methanogen, which has phenotypic, phylogenetic, and 60 61 phylogenomic characteristics that allow its assignment to a novel species of the genus 62 Methanobacterium. This novel archaeal species was isolated from 'La Crouen' geothermal 63 spring, located north of the southern peridotite nappe of New Caledonia (9). This spring discharges alkaline (pH 9.0), anoxic (O₂(aq)<1 mg/L) and warm (42°C) waters (10, 11), 64 65 enriched in dinitrogen (N₂) with traces of methane (CH₄ <3%) (12). This archaeon is the first 66 Methanobacterium member isolated and characterized from terrestrial thermal sources (or hot 67 springs) and the first example of methanogens isolated and characterized from the alkaline 68 springs of New Caledonia.

For enrichment experiments, a sediment slurry sample was collected in November 2014
using both sterilized spatula and glass bottle from the geothermal spring of La Crouen (165°
53' 20.6" E 21° 32' 06.6" S), located next to the city of Canala (New Caledonia) (13). For
preserving anoxic conditions, the bottle was immediately hermetically sealed and stored at 4°C.
In the laboratory, anaerobic enrichments were carried out using a culture medium containing

74 (per liter of distilled water): 0.1 g KH₂PO₄, 0.1 g K₂HPO₄, 0.5 g NH₄Cl, 0.5 g NaCl, 0.002 g 75 FeSO₄·7H₂O, 0.1 g CaCl₂·2H₂O, 0.1 g MgSO₄·7H₂O, 2 g sodium formate, 1g sodium acetate, 76 0.1 g cysteine hydrochloride, and 1 mL trace element solution SL-10 (14), supplemented with 77 25 mM Tris-Base. The initial pH was adjusted to 9.0 with NaOH. This medium was boiled for 78 5 min and then cooled to room temperature under a flow of O₂-free N₂ gas. The medium (5 mL) 79 was dispensed into Hungate tubes, degassed under a flow of N₂, and subsequently autoclaved 80 (20 min, 120°C). The following sterile solutions were injected in each tube: 0.1 mL of 2% 81 Na₂S·9H₂O, 0.1 mL of 8% Na₂CO₃, 0.1 mL of 10 % tryptone and 0.1 mL of 10% yeast extract. 82 Inoculation at 10% vol. was done with 0.5 mL of the sediment slurry sample. The suspensions were serially diluted in decimal steps using the same medium (up to 10^{-4}), and each tube was 83 84 supplemented by H₂/CO₂ gas mixture (80/20 v/v at 2 bars). The suspensions were incubated for 85 one month at 37°C under shaking (100 rpm). The Hungate technique for anaerobic cultivation 86 was used throughout this study (15).

87 Isolation and growth experiments were performed using the medium described above 88 (without sodium acetate, sodium formate, yeast extract, and tryptone) with the addition of Balch 89 vitamin solution (16), and H_2/CO_2 (80/20 v/v, 2 bars) as the only sources of energy and carbon, 90 and supplemented with 25 mM Tris-Base (except for testing the optimal pH for growth). For 91 the optimum pH tests, the initial pH of the culture media (ranging between 6.9 and 10.1) was 92 adjusted with an anaerobic sterile stock solution of 1M NaOH. The pH was always checked 93 after H_2/CO_2 injection (following inoculation) and at the end of incubation. Water baths were 94 used for incubation at temperatures ranging from 10 to 60°C. For studies of NaCl requirements 95 (tested between 0 and 3%), NaCl was weighted and added directly into the tubes before medium 96 addition. Each test culture was sub-cultured at least once under the same experimental 97 conditions before determining growth rates. The following compounds were tested as potential 98 substrates: (i) acetate, ethanol, formate, iso-butanol, methanol, trimethylamine, or 2-propanol

99 (at a final concentration of 20 mM) with N_2/CO_2 (80/20, v/v; 1 bar), or (ii) only H_2/CO_2 (80/20, 100 v/v) replacing N₂/CO₂ as the gas phase, or (iii) H₂/CO₂ (80/20, v/v) in the presence of acetate 101 (2 mM) as carbon source. Sensitivity to antibiotics (ampicillin, bacitracin, chloramphenicol, 102 cycloserine, kanamycin, metronidazole, novobiocin, penicillin, rifampicin, streptomycin, 103 tetracycline, vancomycin) was tested by adding each antibiotic separately to the basal medium 104 at a final concentration of 25 μ g/mL or 50 μ g/mL. The effect of sulfide concentration (0–10 105 g/L) on the growth rate of the isolate was also tested by adding an increasing concentration of 106 a Na₂S solution. Each growth test and control, carried out in parallel, was run in duplicate 107 Hungate tubes. Controls were (i) inoculated tubes without tested compounds and (ii) 108 uninoculated tubes with tested compounds. Growth was monitored by measuring the turbidity 109 increase at 600 nm by inserting Hungate tubes into the tube holder of a spectrophotometer (Cary 110 50, Varian). Gaseous and liquid end-products of metabolism were periodically measured by gas 111 chromatography (Shimadzu GC 8A) and high-performance liquid chromatography (Thermo Separation Products), as described by Mei et al. (17). 112

113 The presence of putative methanogens from CH₄-producing enrichment cultures was 114 checked by observing the autofluorescence of the cofactor F420 characteristic of methanogens. 115 For this purpose, cell observations were performed under UV light using a Nikon Eclipse E600 116 equipped for epifluorescence and an F420 emission filter. Cellular morphology and purity 117 check of the strain were examined using a phase-contrast microscope (Optiphot; Nikon). For 118 transmission electron microscopy (using a MET FEI Tecnai G2), exponentially grown cells 119 were fixed with glutaraldehyde and osmium tetraoxide, epon-embedded, and stained with 120 uranyl acetate and lead citrate (to watch the cell wall), as described by Quéméneur et al. (18).

121 The purity of the strain was further checked after DNA extraction (19) by the amplification 122 of partial 16S rRNA gene using the bacteria-specific primers 341F/518R (20) to verify the 123 absence of any bacterial contaminant. Near-complete 16S rRNA genes were amplified using the primer pairs A109F/1492R for *Archaea* domain (21, 22), then sequenced by GATC-Biotech
(Konstanz, Germany). The 16S rRNA gene sequence was deposited in Genbank and aligned
with available sequences from the NCBI database using the MUSCLE program (23),
implemented in MEGA7 software (24). Evolutionary analyses were conducted from 16
nucleotide sequences (including the isolate, fourteen *Methanobacterium* type species, and *Methanopyrus kandleri*) using three different methods: maximum-likelihood (ML) (25),
maximum-parsimony (MP) (26), and neighbor-joining (NJ) (27).

131 High molecular weight DNA of the isolate and Methanobacterium alcaliphilum DSM 3459 132 was extracted using a phenol-chloroform method (28) modified by adding a step including 133 several cycles of grinding the frozen cell pellet in liquid nitrogen to optimize cell wall lysis. 134 Whole genomes were then sequenced at the GeT-PlaGe platform (INRAe Toulouse, France) 135 by combining long reads (Oxford Nanopore) and short reads (Illumina MiSeq) technologies, as 136 described by Quéméneur et al. (13). The genomes of the isolate and Methanobacterium 137 alcaliphilum DSM 3459 were assembled with Unicycler v0.4.6 (29) and SPAdes v3.7.1 (30). 138 Their sequences were deposited in the NCBI database and annotated using the MaGe system 139 (Microscope Genome Annotation) (31). The average nucleotide identity value (ANI) was 140 calculated using OrthoANI with Usearch (http://www.ezbiocloud.net/tools/ani). Digital (or in 141 silico) DNA-DNA hybridization (dDDH) was calculated using the Genome-to-Genome 142 Distance Calculator (GGDC) 2.1 (http://ggdc.dsmz.de/) (32). Whole-genome- and proteome-143 based taxonomic analyses were performed using the bioinformatics platform Type (Strain) 144 Genome Server (TYGS) (https://tygs.dsmz.de) (33).

Methane production and a simultaneous decrease in both H_2 and CO_2 were detected in all enrichment cultures of the 'La Crouen' slurry sample after two weeks of incubation at 37°C. The highest dilution culture (10⁻⁴) was transferred into a minimum liquid medium with H_2/CO_2 as sole energy and carbon sources. The Roll-tube method described originally by Hungate (15) 149 was used for strain isolation in a solid medium. The culture was ten-fold serially diluted in roll 150 tubes containing a molten 1.6% agar medium. Single white and circular colonies (1 mm in 151 diameter after 2-week incubation) were resuspended in basal medium with the addition of Balch 152 vitamins, which serves to inoculate a second round of ten-fold serially diluted in roll tubes. 153 After the third round of roll tube isolation, one single colony was finally grown in liquid culture, 154 resulting in the strain designated CAN^T, further characterized physiologically and 155 metabolically.

156 Cells of strain CAN^T are straight rods $1.3-4.0 \ \mu m$ long and $0.5-0.7 \ \mu m$ wide, occurring 157 singly, in pairs, or chains (Fig. 1A and 1B). They are non-motile and Gram-positive staining 158 (Fig. 1B).

Strain CAN^T grows only under strictly anaerobic conditions. It is mesophilic but thermotolerant and grows at temperatures ranging from 20°C to 45°C (Table 1). The optimal temperature for growth is 45°C. The pH range for growth is 7.3–9.7, with an optimum growth at pH 9.0 (checked 15 min after H₂/CO₂ addition and at the end of the experiments). NaCl is not required for growth, and the NaCl range for growth is 0–1.5% (optimum at 0%), indicating that strain CAN^T is adapted to the low-salinity, warm, and high-pH fluid emitted by the alkaline spring of La Crouen (New Caledonia).

166 H_2 and CO_2 were the only substrates used for growth and methanogenesis (Table 1, Fig. 2). 167 Acetate, ethanol, formate, isobutanol, methanol, trimethylamine, and 2-propanol were not used 168 as substrates for growth or methane production. Yeast extract and tryptone were not required 169 for growth. The maximal growth rate was found to be $0.02 h^{-1}$ using H_2/CO_2 as sole energy and 170 carbon sources for growth in optimal conditions (45°C, pH 9.0, and 0% NaCl).

171 Strain CAN^T was resistant to antibiotics such as novobiocin, streptomycin, and 172 vancomycin added at 25 μ g/mL or 50 μ g/mL in the medium. It also grew in the presence of 25 173 μ g/mL of tetracycline, but it was sensitive to this antibiotic at 50 μ g/mL. Ampicillin, bacitracin, 174 chloramphenicol, cycloserine, kanamycin, metronidazole, penicillin, and rifampicin at 25 175 μ g/mL inhibited cell growth. Sulfide concentration greater than 1.5 g/L also inhibited cell 176 growth of strain CAN^T.

177 Phylogenetic analysis based on the almost full-length 16S rRNA gene sequence (1227 nt) indicated that strain CAN^T is a member of the genus Methanobacterium, in the order 178 179 Methanobacteriales, class Methanobacteria, phylum "Methanobacteriota" (Fig. 3). The phylogenetic position of strain CAN^T was confirmed by using three different methods: 180 181 maximum-likelihood (ML, Fig 3), maximum-parsimony (MP, Fig. S1A), and neighbor-joining (NJ, Fig. S1B). The closest relatives of strain CAN^T are type strains of the 182 species: *Methanobacterium alcaliphilum* WeN4^T (98.5% identity) isolated from sediments of a 183 184 low-salt alkaline lake of the Wadi el Natrun (Egypt) (8), Methanobacterium movens TS-2^T 185 (98.4% identity) isolated from sediments of the alkaline saline Tuosu Lake (Qaidam basin, China) (7), and *Methanobacterium flexile* GH^T (97.5% identity) isolated from sediments of an 186 187 alkaline saline lake, the Gahai Lake (Qaidam basin, China) (7). It is more distantly related to 188 the other alkaliphilic Methanobacterium type strains of the species: Methanobacterium 189 subterraneum A8p^T (95.1% identity).

190 The 16S rRNA gene sequence of strain CAN^T was closely related (>99% identity) to 191 a few environmental sequences retrieved in March 2022 by NCBI web BLAST search (34) and 192 originated from deep subsurface gas-associated water (DQ841242) (35) as well as H₂-fed 193 bioreactor operated at halo-alkaline conditions (KJ491002) (36). Sequences of the 16S rRNA 194 gene affiliated to the genus Methanobacterium were also detected in the alkaline spring of La 195 Crouen (representing 4.5% of prokaryotes in the sample studied) (11) and in the hyperalkaline 196 springs of the Ligurian Alps ophiolite massif and in subsurface fluids of the Sultanate of Oman 197 ophiolite massif (19, 37-39), where the methanogen could potentially consume H_2 produced by 198 serpentinization reactions.

The complete genome sequence of strain CAN^{T} is a unique circular chromosome 2,2 199 200 Mb long with a 36.4 mol% G+C content (Table 2). Its G+C content is identical to that of M. *flexile* GH^T (36.4 mol%), higher than that of *M. alcaliphilum* WeN4^T (33.5 mol%), and lower 201 than *M. movens* TS-2^T (39.1 mol%). Its size is much smaller than that of its closest phylogenetic 202 neighbor *M. alcaliphilum* WeN3 (2.4 Mb). The ANI and dDDH values between CAN^{T} and *M*. 203 204 alcaliphilum WeN3 were 71.55% and 14.0%, i.e., far below the species boundaries of 95~96% and 70%, respectively, recommended by Chun *et al.* (40). The CAN^T strain genome comprises 205 206 a single chromosome and 2,189 genes, including 2,126 protein-encoding genes and 49 total 207 RNA encoding genes. A complete pathway for hydrogenotrophic methanogenesis was found 208 using MaGe with both genes encoding formylmethanofuran dehydrogenase and methyl 209 coenzyme-M reductase that catalyze the initial and final step of methanogenesis on H₂/CO₂, 210 respectively. The genome of *M. alcaliphilum* WeN3 comprises 2,375 genes, including 2,325 211 protein-encoding genes and 46 total RNA encoding genes. The distribution of protein-encoding 212 genes of both strain CAN^T and *M. alcaliphilum* WeN3 into COGs functional categories is very 213 similar (Supplementary Table S1). In a whole-proteome-based phylogenetic analysis (Fig. S2), 214 strain CAN^T is also closely related to *M. alcaliphilum* (consistently with the 16S rRNA gene-215 based phylogenetic analysis) and clustered with other members of the family 216 Methanobacteriaceae, including the genera Methanobacterium, Methanobrevibacter, and 217 Methanothermobacter (previously placed in the genus Methanobacterium (41)).

Table 1 summarizes the physiological and biochemical differences between strain CAN^T and several type species of the genus *Methanobacterium* (including the four closest phylogenetic neighbor type species and other alkaliphilic members). Strain CAN^T shares similar properties with several *Methanobacterium* members, such as the exclusive utilization of H₂/CO₂ for methanogenesis. However, strain CAN^T differs from its closest phylogenetic neighbors, *M. alcaliphilum* WeN4^T, *M. movens* TS-2^T, and *M. flexile* GH^T, by its ability: (i) to

grow at 45°C (its optimum temperature), similarly to *M. aarhusense* H2-LR^T (6), and (ii) to 224 225 grow autotrophically in mineral medium without any organic additions (e.g., yeast extract), similarly to *Methanobacterium subterraneum* A8p^T (5). To our knowledge, strain CAN^T is also 226 singular by its ability to grow optimally at higher pH (i.e., pH 9.0) than other type species of 227 228 the genus Methanobacterium. Indeed, the two other alkaliphilic Methanobacterium members, i.e., *M. alcaliphilum* WeN4^T (8) and *M. subterraneum* A8p^T (5), display optimal growth at pH 229 230 8.4 and pH 7.8-8.8, respectively. Based on morphological, phenotypic, phylogenetic, and 231 genomic analyses, strain CAN^T is proposed to represent a novel species of the genus 232 Methanobacterium, M. alkalithermotolerans sp. nov.

233

234 Description of Methanobacterium alkalithermotolerans

235

Methanobacterium alkalithermotolerans (al.ka.li.ther.mo.to'le.rans. N.L. n. *alkali*, alkali (from
Arabic article *al*, the; Arabic n. *qaliy*, ashes of saltwort); Gr. fem. adj. *therme*, heat; L. pres.
part. *tolerans*, tolerating, enduring; N.L. part. adj. *alkalithermotolerans*, thermophilic alkalitolerating).

240

241 Displays similar traits as described for the genus. Cells are non-motile, straight rods 1.3-4.0 242 µm long and 0.5–0.7 µm wide, occurring singly, in pairs, or in chains. Colonies are white and 243 circular, 1 mm in diameter after two weeks of incubation. Alkaliphilic, mesophilic but 244 thermotolerant. Growth occurs at 20–45°C (optimum 45°C), at pH 7.3–9.7 (optimum pH 9.0) 245 with 0–1.5% NaCl (optimum 0%). Added NaCl and yeast extract were not required for growth. Hydrogen and carbon dioxide were used as substrates for growth and methanogenesis. Acetate, 246 247 ethanol, formate, isobutanol, methanol, trimethylamine, and 2-propanol were not used for 248 growth. Resistant to novobiocin, streptomycin, and vancomycin (at 50 µg/mL) but sensitive to

249	ampicillin, bacitracin, chloramphenicol, cycloserine, kanamycin, metronidazole, penicillin,
250	rifampicin (at 25 μ g/mL), and tetracycline (at 50 μ g/mL). Most closely related to
251	Methanobacterium alcaliphilum WeN4 ^T (98.5% 16S rRNA gene sequence identity). The G+C
252	content of the genomic DNA is 36.4%. The GenBank accession number for the 16S rRNA gene
253	sequence of strain CAN ^T is KR349725, and that for the whole genome sequence is CP058560.
254	The type strain CAN ^T (= DSM 102889^{T} = JCM 31304^{T}) was isolated from a sediment slurry of
255	La Crouen geothermal spring near Canala, New Caledonia.

256

257 **Conflicts of interest**

259 The authors declare that there are no conflicts of interest.

260

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270

272 Figures legends

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Fig 1: Phase-contrast micrographs of cells of strain CAN^T (A; bar 10 μ m) and transmission electron micrograph showing cells of strain CAN^T (B; bar 1 μ m), with Gram-positive type cell wall (C; bar 0.5 μ m).

277

Fig. 2: Kinetics of hydrogen (H₂) consumption, methane (CH₄) production, and growth (optical density OD 600 nm \times 10) of strain CAN. This monitoring was determined at pH 9.0 and 37°C in the minimal medium with the addition of H₂/CO₂ (80/20 v/v, 2 bars) as the only source of energy and carbon. Values are means ± standard deviations of triplicated cultures.

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Fig. 3: Maximum-likelihood (ML) phylogenetic tree based on 1227 aligned bp of 16S rRNA
gene sequences showing the position of strain CAN^T among its closest neighbors belonging to
the genus *Methanobacterium. Methanopyrus kandleri* AV19^T (NR_074539) was used as an
outgroup (not shown). Bootstrap values (based on 1000 replicates) are shown at branch nodes.
Accession numbers are indicated in brackets. Bar: 0.02 substitutions per 100 nucleotides.

Table 1. Differential characteristics between strain CAN^T (obtained in this study) and data obtained from previous studies for several type species 290

of the genus Methanobacterium. Strains: 1, CAN^T; 2, M. alcaliphilum WeN4^T (8); 3, M. movens TS-2^T (7); 4, M. flexile GH^T (7); 5, M. aarhusense 291

Characteristics	1	2	3	4
Origin	Sediment of thermal spring, New Caledonia	Alkaline lake sediment, Egypt	Sediment of Tuoso Lake, China	Sedim Lak, C
Cell size (µm)	0.5–0.7 x 1.3–4.0	0.5–0.6 x 2–25	0.4–0.5 x 2–5	0.3–0.
Gram staining	-	-	+	+

H2-LR^T (6); 6, M. subterraneum A8p^T (5). 292

Characteristics	1	2	3	4	5	6
Origin	Sediment of thermal	Alkaline lake	Sediment of Tuoso Lake China	Sediment of Gahai Lak China	Marine sediment, Denmark	Deep groundwater, Sweden
Cell size (µm)	0.5–0.7 x 1.3–4.0	0.5–0.6 x 2–25	0.4–0.5 x 2–5	0.3–0.5 x 2–5	0.7 x 5–18	0.1-0.15 x 0.6-1.2
Gram staining	-	-	+	+		+
Motility	-	-	+	-	-	-
Growth substrates						
$H_2 + CO_2$	+	+	+	+	+	+
Formate	-	-	-	+	-	+
Alcohols	-	-	-		-	-
Acetate requirement	-	-	-		-	-
Yeast extract requirement	-	+	+	+	-	-
Optimum temperature (°C)	45	37	35–38	35–38	45	30–40
Optimum pH for growth	9.0	8.4	7.2–7.5	7.0–.5	7.5-8.0	7.8-8.8
Optimum NaCl (%)	0	0–0.6	0-0.3	0-0.1	0.05–.9	0.1–0.2
DNA G+C content $(mol\%)^1$	36.41*	57 ²	39.5 ²	37.3 ²	34.9 ²	$39.5^{1}(54.5^{2})$

293 294 295

+, Positive; -, negative. ¹ G+C content determined from genomic data. ² G+C content determined by using HPLC.

- Table 2. Genome statistics of strain CAN^T, and *Methanobacterium alcaliphilum* DSM3459
- (both genomes obtained in this study).

Genome / Sample Name	Strain CAN ^T	Methanobacterium alcaliphilum DSM 3459
GenBank accession number	CP058560	JALKIF00000000
MaGe Genome ID	MAGE_000013558.1	MAGE_000016954.1
Genome Size (bp)	2,187,077	2,377,238
Contig count	NA	65
Scaffold count	1	65
GC (%)	36.4	33.4
Gene Count	2,189	2,375
Protein count	2,126	2,325
rRNA Count	7	4
tRNA Count	40	40
Other RNA	2	2

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