A thermophilic, hydrogenogenic and carboxydrotrophic bacterium, *Calderihabitans maritimus* gen. nov., sp. nov., from a marine sediment core of an undersea caldera

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A hydrogenogenic, carboxydrotrophic marine bacterium, strain KKC1T, was isolated from a sediment core sample taken from a submerged marine caldera. Cells were non-motile, Gram-stain-negative, 1.0–3.0 μm straight rods, often observed with round endospores. Strain KKC1T grew at 55–68 °C, pH 5.2–9.2 and 0.8–14 % (w/v) salinity. Optimum growth occurred at 65 °C, pH 7.0–7.5 and 2.46 % salinity with a doubling time of 3.7 h. The isolate grew chemolithotrophically, producing H2 from carbon monoxide (CO) oxidation with reduction of various electron acceptors, e.g. sulfite, thiosulfate, fumarate, ferric iron and AQDS (9,10-anthraquinone 2,6-disulfonate). KKC1T grew heterotrophically on pyruvate, lactate, fumarate, glucose, fructose and mannose with thiosulfate as an electron acceptor. When grown mixotrophically on CO and pyruvate, C16:0 constituted almost half of the total cellular fatty acids. The DNA G+C content was 50.6 mol%. The 16S rRNA gene sequence of KKC1T was most closely related to those of members of the genus *Moorella* with similarity ranging from 91 to 89 %. Based on physiological and phylogenetic novelty, we propose the isolate as a representative of a new genus and novel species with the name *Calderihabitans maritimus* gen. nov., sp. nov.; the type strain of the type species is KKC1T (=DSM 26464T=NBRC 109353T).

Anaerobic, thermophilic, carbon monoxide (CO)-oxidizers growing on CO (carboxydrotrophs) have been isolated from hot water environments, such as deep-sea hydrothermal vents, terrestrial hot springs and bioreactors. Most thermophilic carboxydrotrophs oxidize CO for energy and produce molecular hydrogen or acetate (Sokolova et al., 2009). As many microbes are sensitive to CO exposure, CO-oxidation in the environment may have an important role; that is, ‘scavenging’ toxic CO and converting it to more commonly used substrates (Techtmann et al., 2009). Presently, whereas many isolates have been found in terrestrial hot springs, only one bacterial and three archaeal isolates of H2-producing (hydrogenogenic) carboxydrotrophs have been found in marine environments. These organisms live chemolithotrophically on CO generating H2 using the following reaction: CO + H2O → CO2 + H2, ΔG° = −20 kJ mol−1 (Svetlitchnyi et al., 2001). This reaction is catalysed by carbon monoxide dehydrogenase (CODH) (Ragsdale, 2004). *Caldanaerobacter subterraneus* subsp. *pacificus* (basonym *Carboxydibrachium pacificum*) (Sokolova et al., 2001; Fardeau et al., 2004) was the first isolate from a deep-sea hydrothermal vent. *Thermococcus* strain AM4 was the first hydrogenogenic, carboxydrotrophic archaeon isolated from a deep-sea hydrothermal vent (Sokolova et al., 2004). Culture-based study has shown the inability of many other strains representing the genus *Thermococcus* to consume CO, although genomic studies revealed that *Thermococcus onnurineus* (Lee et al., 2008), *Thermococcus barophilus* (Vannier et al., 2011) and AM4 (Oger et al., 2011) have conserved gene sets, specialized gene clusters encoding CODH and ECH (energy conserving hydrogenase) that enable hydrogenogenic carboxydrotrophy. Sporadic distribution suggests acquisition of the CODH and...
ECH gene sets by lateral gene transfer among strains of the genus *Thermococcus*. Thus, CO oxidation in the thermococci seems neither a major component of metabolism nor common trait in the genus. Here, we report the isolation and characterization of a novel hydrogenogenic, carboxydrotrophic thermophile isolated from the sediment of a marine caldera.

Marine sediment core sampling was organized by the Geo Biotechnology Development Organization during a cruise of the research vessel 'Umitaka-maru' of Tokyo University of Marine Science and Technology, in August 2011. The sampling site was in a submerged marine caldera, Kikai Caldera, located on the southern coast of Kyushu Island, Japan. Around 6500 years ago, a devastating eruption occurred at Kikai Caldera (known as the Akahoya eruption) which is said to have had critical impact on ancient western Japanese culture and vegetation. An active volcanic islet, Satsuma Iwo-Jima forms a north-west part of the caldera ridge and geothermal activity continues around the coast of the islet, for example, thermal water rich in iron coming up from the sea floor in Nagahama bay causing brown seawater (Kiyokawa et al., 2012). A 2.48 m-long sediment core sample designated TKR04 was taken at 30° 64.42’ N 130° 26.72’ E, 460 m depth, by a piston core sediment sampler. Temperature and pH in the bottom of TKR04 core site were 15.3 °C and 9.14, respectively. The middle of the core was subsampled into a polycarbonate tube and refrigerated until used.

Medium ASW was used for enrichment and isolation, containing 26.7 g NaCl, 6.5 g MgSO4·7H2O, 6.5 g MgCl2·6H2O, 1.25 g CaCl2·2H2O, 0.65 g KCl, 0.5 g NaHCO3, 0.1 g NH4Cl, 0.1 g KH2PO4, 0.1 g NaBr, 10 mg Na2SiO3, 30 mg H3BO3, 15 mg SrCl2·6H2O, 10 mg ferric citrate, 0.05 mg KI, 0.05 mg NaNO3, 10 ml trace mineral solution SL 4 (Pfenning & Lippert, 1966), 1.0 ml vitamin solution (Wolin et al., 1963), 0.5 mg resazurin, 0.1 g Na2S·9H2O and 1000 ml distilled water. The medium was dispensed into 180 mm x 18 mm glass test tubes (5 ml liquid phase) with butyl rubber stoppers, boiled and cooled down under CO gas (atmospheric pressure). After autoclaving, a filter-sterilized solution of NaHCO3, vitamins, yeast extract (if mentioned) and Na2S was added. Sample sediment was cut into 0.5 cm cubes and inoculated into 5 ml ASW medium supplemented with 50 mg yeast extract l⁻¹ and 5 mg glycerol l⁻¹. Cell counts and analysis of gas components in the head spaces were performed as previously described (Yoneda et al., 2012). Hydrogenogenic, carboxydrotrophic growth of cells occurred at 65 °C, pH 7.5. The culture was transferred to ASW medium supplemented with 1.0 g thiosulfate l⁻¹, 50 mg yeast extract l⁻¹ in an anoxic glove box (Coy Laboratory Products). Using this medium, the new strain, designated KKCIᵀ, was isolated using the extinction dilution method. Purity of the isolate was confirmed by routine sequencing of the partial 16S rRNA gene and microscopy. Gram staining was performed using the method of Bartholomew & Mittwer (1952). Transmission electron microscopy was performed as described previously (Yoneda et al., 2012). Spore formation was observed using malachite green staining (Hamouda et al., 2002) with cells of one-week-old culture. Bacterial cells were Gram-stain-negative, round-edged rods ranging from 0.5 to 1.0 μm by 1.0 to 3.0 μm (Fig. S1, available in IJSEM Online). Formation of endospores at the edge of the rods was observed using electron micrography and spore staining (Fig. S2). The isolate was non-motile under light microscopy.

DNA was extracted using the NaOH method described by Mesbah et al. (1989). To determine the 16S rRNA gene sequence, primers B27F (5’-AGAGTTTGATCCTGGCGTACG-3’) and U1525R (5’-AAGAGGTTGWTCCARCCT-3’) (Lane, 1991) were used for PCR amplification. PCR products were sequenced using the BigDye Terminator v3.1/1.1 Cycle Sequencing kit (Applied Biosystems) and a 3130 Genetic analyser (Applied Biosystems) following the protocol provided by the manufacturer. A close to full-length 16S rRNA gene sequence (1503 bp) was determined. BLAST analysis showed this strain was closest to the genus *Moorella* which belongs to the phylum ‘Firmicutes’, class Clostridia, order Thermoanaerobacterales and family Thermoanaerobacteraceae, using NCBI taxonomy. The similarity of the KKCIᵀ sequence was 91 % to *Moorella humifera* 64-FGQᵀ (Nepomnyashchaya et al., 2012), 90 % to *Clostridium thermoacetica* DSM 521ᵀ (basonym Clostridium thermoaceticum) (Fontaine et al., 1942; Collins et al., 1994), *Moorella glycerini* JW/AS-Y6ᵀ (Slobodkin et al., 1997b) and ‘Moorella perchloratireducens’ An10d (Balk et al., 2008), and 89 % to *Moorella thermoautotrophica* JW 701/3ᵀ (basonym Clostridium thermoautotrophicum) (Wiegel et al., 1981; Collins et al., 1994). KKCIᵀ also showed sequence similarity of 89 % to *Syntrophothermus lipocalidus* TGB-C1ᵀ (Sekiguchi et al., 2000), a member of the order Clostridiales and family Syntrophomonadaceae. 16S rRNA gene sequences of species of the genus *Moorella* and some selected members of order Thermoanaerobacterales and family Syntrophomonadaceae were obtained from the DDBJ/EMBL/GenBank databases and aligned using MEGA 5.0 software (Tamura et al., 2011). A phylogenetic tree was reconstructed by using the online phylogeny program PhyML 3.0 (Dereeper et al., 2008). The tree showed that KKCIᵀ was related to species of the genus *Moorella* although branching distinctly and deeply from them (Fig. S3). The genus *Syntrophothermus* appeared to be the second closest genus to KKCIᵀ compared with the other genera from the order Thermoanaerobacterales. A phylogenetic tree using the sequences related to members of the genus *Moorella* and *S. lipocalidus* was reconstructed (Fig. 1). The tree demonstrated a similar result to Fig. S3 supporting data that KKCIᵀ is phylogenetically distinct based on 16S rRNA gene sequence comparisons.

The G+C content of KKCIᵀ genomic DNA was determined as described previously (Yoneda et al., 2012).
The DNA G+C content was 50.6 ± 0.4 mol% (mean ± SD; determined in duplicate).

Media supplemented with yeast extract (50 mg l⁻¹) and thiosulfate (1.0 g l⁻¹) was used to determine the salinity, pH and temperature ranges for growth, and antibiotic sensitivity. The salinity range for growth was tested by decreasing or increasing the concentrations of NaCl, MgSO₄ .7H₂O, MgCl₂ .6H₂O and CaCl₂ .2H₂O at constant ratios in the ASW medium. Salinity was scored as the total weight percentage (w/v) of these salts. In salinity tests over 8 %, only NaCl and MgCl₂ .6H₂O were increased to avoid formation of a precipitate. KKC₁T grew within a salinity range of 0.82–14.1 %, but not lower than 0.61 % or over 14.4 %. The optimum salinity for KKC₁T growth was 2.46 %. Therefore medium containing a reduced amount of salt (hASW) was used for further physiological tests. The components of hASW medium were identical to ASW medium except for the following salts per litre: 16.0 g NaCl, 3.9 g MgSO₄ .7H₂O, 3.9 g MgCl₂ .6H₂O and 0.27 g CaCl₂ .2H₂O. The pH for growth of KKC₁T was determined with the addition of the following buffers at 10 mM; citric acid and MES (pH 4.4–5.9), MES (pH 6.1–7.5), HEPES (pH 6.9–8.2), TAPS (pH 7.9–8.5) and CAPS (pH 8.0–9.5). Growth was observed between pH 5.2 and 9.2 and was optimal between pH 7.0 and 7.5. No growth was observed at pH 4.8 or 9.5. The temperature range for growth of KKC₁T was 55–68 °C with an optimum at 65 °C. Strain KKC₁T did not grow at 50 or 70 °C. When cells were grown under optimal conditions in hASW medium, the doubling time of KKC₁T was 3.7 h. A maximum cell density of 3.3 × 10⁸ cells ml⁻¹ was achieved after 64 h of cultivation. Antibiotic sensitivity was tested in medium supplemented with 100 µg ml⁻¹ of chloramphenicol, erythromycin, ampicillin, streptomycin, tetracycline, penicillin or rifampicin using optimum growth conditions.

Growth of KKC₁T occurred slowly with ampicillin with a low cell yield (3.7 × 10⁷ cells ml⁻¹). All other antibiotics inhibited growth.

To determine if the isolate was capable of carboxydotrophic growth (growth under 100 % CO gas), the following electron acceptors (10 mM) were tested; sulfate, sulfite, thiosulfate, colloidal elemental sulfur (3.0 g l⁻¹), fumarate, perchlorate, ferric citrate, amorphous Fe(III) oxide, Fe₂O₃, MnO₂ (7.0 g l⁻¹), nitrate, AQDS (9,10-anthraquinone 2,6-disulfonate) and humate (1.0 g l⁻¹). hASW medium omitting MgSO₄ .7H₂O and with the addition of 9.0 g of MgCl₂ .6H₂O was used as a basal medium. Amorphous Fe(III)-oxide was prepared as described by Slobodkin et al. (1997a). Growth with metal-containing compounds, nitrate, AQDS and humate was tested in media without Na₂S. Concentration of acetate, succinate and fumarate was analysed using HPLC (Prominence, Shimadzu) equipped with a 4.6 × 150 mm Cosmosil SC₁₈-PAQ column (Nacalai Tesque). Column temperature was set at 30 °C and 20 mM phosphoric acid (1.0 ml min⁻¹ flow rate) was used for the mobile phase. The spectrophotometric detector was set at 210 nm. Sulfide production, Fe(III) reduction and Mn(IV) reduction were detected spectrophotometrically as described by Cord-Ruwisch (1985), Yoneda et al. (2012) and Boogerd & de Vrind (1987), respectively. Growth was observed in the presence of sulfate, sulfite, thiosulfate, fumarate, perchlorate, ferric citrate, amorphous Fe(III) oxide, Fe₂O₃ and MnO₂, and weakly with AQDS (approximately 1.0 × 10⁵ cells ml⁻¹). Sulfide production was observed with sulfite and thiosulfate. With sulfate, sulfide production was not detected in either the gas or liquid phase. Fe²⁺ production was detected with ferric citrate, amorphous Fe(III) oxide, and at a low level with Fe₂O₃. With AQDS, the culture exhibited a vivid orange colour indicating AQDS reduction. MnO₂ particles changed slightly to a dim blackish colour compared with unused MnO₂ particles; however, reduction of MnO₂ was not detected spectrophotometrically in the liquid phase by the leuco-berbelin blue-I (LBB) method. Fumarate was reduced to succinate. Weak growth, approximately 4.3 × 10⁷ cells ml⁻¹, occurred with nitrate; however, no growth occurred when transferred to fresh medium. No growth occurred with humate. Elemental sulfur inhibited growth. KKC₁T grew without any addition of extra electron acceptors when the medium was reduced using Na₂S. In all growth-positive conditions, H₂ and CO₂
production along with CO consumption occurred. When cell density reached a maximum of around $1.0 \times 10^8$ cells ml$^{-1}$, CO (initial concentration of 43.9 $\mu$mol ml$^{-1}$ in head space) concentration was decreased to 1.8, 33.1 and 2.8 $\mu$mol ml$^{-1}$ and hydrogen was produced up to 42.0, 9.4 and 43.2 $\mu$mol ml$^{-1}$ in the gas phase with amorphous Fe(III) oxide, thiosulfate, and without an additional electron acceptor, respectively. Small amounts of acetate (1.0–2.0 mM) were also detected in cultures grown under CO atmosphere with ferric citrate, amorphous Fe(III) oxide, MnO$_2$ and without an electron acceptor. Acetate was below detection level when cells were grown with sulfate, sulfite, thiosulfate, fumarate, perchlorate, Fe$_2$O$_3$ or AQDS.

The following substrates (10 mM) were tested under N$_2$ atmosphere: yeast extract (1.0 g l$^{-1}$), peptone (1.0 g l$^{-1}$), pyruvate, lactate, formate, acetate, propionate, butyrate, fumarate, malate, succinate, citrate, methanol, ethanol, 1-propanol, 1-butanol, glycerol, glucose, sucrose, lactose, maltose, xylose, arabinose, fructose, mannose and soluble starch (1.0 g l$^{-1}$). Basal medium supplemented with 10 mM sulfate or thiosulfate, or without addition of an extra electron acceptor was used. Chemolithotrophic growth on hydrogen and carbon dioxide (80 % H$_2$/20 % CO$_2$ in the headspace) was tested in basal medium supplemented with various electron acceptors used in the carboxydrotroph test described above. With sulfate, strain KKC1$^T$ grew only on fumarate, producing 0.2 mM acetate, but sulfide was not detected. Strain KKC1$^T$ grew on pyruvate, lactate, fumarate, glucose, fructose and mannose with thiosulfate and produced sulfide. With pyruvate, lactate, glucose, fructose and mannose, 5.7, 4.3, 6.9, 1.6 and 1.0 mM acetate was produced, respectively, when cultures reached a maximum cell density of around $1.0–2.0 \times 10^8$ cells ml$^{-1}$. Quite a small amount of H$_2$, 2.3 $\mu$mol ml$^{-1}$ (in head space) on glucose and 2.2 $\mu$mol ml$^{-1}$ on fructose, was detected. No growth was observed on yeast extract, peptone, malate, succinate, citrate, formate, acetate, propionate, butyrate, methanol, ethanol, 1-propanol, 1-butanol, glycerol, sucrose, lactose, maltose, xylose, arabinose or starch with thiosulfate. KKC1$^T$ did not grow on organic compounds without electron acceptors or on hydrogen or carbon dioxide under all conditions tested.

For cellular fatty acids (CFAs) and polar lipid analysis, KKC1$^T$ cells were grown in hASW medium under 100 % CO atmosphere with the addition of thiosulfate (2.0 g l$^{-1}$), pyruvate (1.0 g l$^{-1}$), NaHCO$_3$ (reduced to 0.2 g l$^{-1}$) and yeast extract (50 mg l$^{-1}$). CFAs were analysed at TechnoSuruga Laboratory (Shizuoka, Japan) using the Microbial Identification System version 6 (MIDI) and GC (Sasser, 1990). The CFA pattern was simple as C$_{16:0}$ was the most dominant CFA representing up to 49.45 % of the total CFAs. This is more than four times higher than the second highest CFA, C$_{15:0}$ (11.57 %) (Table 1). Other major CFAs present at more than 5 % were iso-C$_{16:0}$ (6.95 %), C$_{17:0}$ (6.04 %) and C$_{18:0}$ (5.19 %). The complete profile with all other CFAs is shown in Table S1. Polar lipids were detected using TLC as described by Minnikin et al. (1984). One major lipid and some minor lipids were detected on TLC plates (Fig. S4). All of these lipids were positive using the Dittmer-Lester reagent (as blue spots) and periodate-Schiff reagent (as reddish-violet spots), but negative with ninhydrin and 1-naphthol reagents, indicating that they were phosphatidylylycerol.

The phylogeny of the 16S rRNA genes showed that strain KKC1$^T$ is distinct from any other bacterial genus with sequence similarities below 91 %, which is below the generally accepted cut-off value (95%) that separates members of different species and genera (Tindall et al., 2010). In addition, compared with members of the genus *Moorella*, there are two major physiological differences (Table 2). Firstly, the salinity range for growth was different, reflecting the isolation source. At the time of writing, other isolates in the genus *Moorella* have been obtained from soil, animal manure or fresh water environments including terrestrial hot springs and bio-reactors (see DSMZ Catalogue). KKC1$^T$ originated from marine sediments and requires a salinity of over 0.82 % for growth. While some species of the genus *Moorella* can tolerate salinity levels up to 2 %, the salinity tolerance of KKC1$^T$ was much higher at up to 14 %. Secondly, the product of CO oxidation is acetate in other species of the genus *Moorella* with the only exception being *M. thermoacetica* strain AMP (Jiang et al., 2009): KKC1$^T$ grown under CO produced mainly H$_2$ and only small amounts of acetate. KKC1$^T$ showed the ability to couple CO oxidation with sulfite, thiosulfate, fumarate, ferric iron, AQDS or possibly MnO$_2$ as an electron acceptor. This broad usage of electron acceptors is not reported in other marine carboxydrotrophs: e.g. *Caldanaerobacter subterraneus* subsp. *pacificus* (Sokolova et al., 2001) and isolates of the genus *Thermococcus* (Sokolova et al., 2004; Sokolova et al., 2009). Reduction of sulfate, thiosulfate and elemental sulfur are coupled to CO oxidation (Sokolova et al., 2009). *Desulfotomaculum carboxydovorans* CO-1-SRB$^T$ is the only known hydrogenogenic bacteria which can reduce sulfate with 100 % CO in the head space, and sulfite with lactate (Parshina et al., 2005); however, combination of sulfite reduction with CO oxidation has not yet been reported. *D. carboxydovorans* produces H$_2$S from H$_2$ and sulfate, so that sulfide production under CO atmosphere might be occurring indirectly using H$_2$ produced from CO oxidation.

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<tr>
<th>CFA</th>
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<td>C$_{14:0}$</td>
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<td>C$_{18:0}$</td>
<td>5.19</td>
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*Calderihabitans maritimus* gen. nov., sp. nov.
Strain KKC1\textsuperscript{T} has a novel feature producing H\textsubscript{2}S directly using CO as an energy source with sulfite or thiosulfate since this isolate cannot grow on H\textsubscript{2} and CO\textsubscript{2}. Based on this distinct phylogeny and physiology, we propose KKC1\textsuperscript{T} as a representative of a new genus and novel species with the name Calderihabitans maritimus gen. nov., sp. nov.

### Description of Calderihabitans gen. nov.

**Calderihabitans** (Cal.de.ri.ha’bi.tans. N.L. fem. n. *caldera* from the Portuguese *n. caldera* cauldron; -i- connecting vowel; L. masc. n. *habitans* dweller, inhabitant; N.L. masc. n. *Calderihabitans* inhabitant of caldera, referring to the isolation source of the type strain).

Thermophilic, strictly anaerobic eubacteria. Cells are Gram-stain-negative, straight-rod-shaped. Form round endospores. Grow chemolithotrophically on CO producing hydrogen as a main product. Able to reduce sulfite, thiosulfate, fumarate, Fe\textsubscript{2}O\textsubscript{3}, ferric citrate, amorphous Fe(III)-oxide or AQDS, or without addition of an electron acceptor. Growth on CO is not maintained with nitrate. Small amounts of acetate are produced during growth on CO. Does not grow on H\textsubscript{2} or CO\textsubscript{2}. Can utilize pyruvate, lactate, fumarate, glucose, fructose and mannose in the presence of thiosulfate with production of sulfide. Growth is not maintained with yeast extract, butanol, glycerol, sucrose, lactose, maltose, xylose, propionate, butyrate, methanol, ethanol, 1-propanol, 1-butanol, glycerol, sucrose, lactose, maltose, xylose, arabinose or soluble starch with thiosulfate. Does not grow on organic compounds without an electron acceptor. Does not require yeast extract for growth. The major CFA is C\textsubscript{16:0}.

The type strain KKC1\textsuperscript{T} (=DSM 26464\textsuperscript{T}=NBRC 109353\textsuperscript{T}), was isolated from a sediment core sample of the undersea caldera, Kikai Caldera, located on the southern coast of...
Kyushu Island, Kagoshima Prefecture, Japan. The G+C content of the type strain is 50.6 mol% (HPLC).

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