Thermomicrobium carboxidum sp. nov., and Thermorudis peleae gen. nov., sp. nov., carbon monoxide-oxidizing bacteria isolated from geothermally heated biofilms

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Two thermophilic, Gram-stain-positive, rod-shaped, non-spore-forming bacteria (strains KI3T and KI4T) were isolated from geothermally heated biofilms growing on a tumulus in the Kilauea Iki pit crater on the flank of Kilauea Volcano (Hawai‘i, USA). Strain KI3T grew over an examined temperature range of 50–70 °C (no growth at 80 °C) and a pH range of 6.0–9.0, with optimum growth at 70 °C and pH 7.0. Strain KI4T grew at temperatures of 55–70 °C and a pH range of 5.8–8.0, with optimum growth at 65 °C and pH 6.7–7.1. The DNA G+C contents of strains KI3T and KI4T were 66.0 and 60.7 mol%, respectively. The major fatty acid for both strains was 12-methyl C18:0. Polar lipids in strain KI3T were dominated by glycolipids and phosphatidylinositol, while phosphatidylinositol and phosphoglycolipids dominated in strain KI4T. Strain KI3T oxidized carbon monoxide [6.7 ± 0.8 nmol CO h^{-1} (mg protein)^{-1}], but strain KI4T did not.

16S rRNA gene sequence analyses determined that the strains belong to the class Thermomicrobia, and that strains KI3T and KI4T are related most closely to Thermomicrobium roseum DSM 5159T (96.5 and 91.1 % similarity, respectively). 16S rRNA gene sequence similarity between strain KI3T and strain KI4T was 91.4 %. Phenotypic features and phylogenetic analyses supported the affiliation of strain KI3T to the genus Thermomicrobium, while results of chemotaxonomic, physiological and biochemical assays differentiated strains KI3T and KI4T from Thermomicrobium roseum. Strain KI3T (=DSM 27067T=ATCC BAA-2535T) is thus considered to be the type strain of a novel species, for which the name Thermomicrobium carboxidum sp. nov. is proposed. Additionally, the characterization and phylogenetic position of strain KI4T showed that it represents a novel species of a new genus, for which the name Thermorudis peleae gen. nov., sp. nov. is proposed. The type strain of Thermorudis peleae is KI4T (=DSM 27169T=ATCC BAA-2536T).

The class Thermomicrobia (phylum Chloroflexi) comprises, at the time of writing, four genera (Gupta et al., 2013), each represented by a single species: Thermomicrobium roseum (Jackson et al., 1973), Sphaerobacter thermophilus (Hugenholtz & Stackebrandt, 2004), ‘Nitrolancetus hollan-dicus’ (Sorokin et al., 2012) and ‘Thermobaculum terrenum’ (Botero et al., 2004; Kunisawa, 2011). Genome sequences for strains of these species (Wu et al., 2009; Kiss et al., 2010; Pati et al., 2011; Sorokin et al., 2012) have revealed cox operons that encode carbon monoxide (CO) dehydrogenase in Thermomicrobium roseum DSM 5159T and S. ther-mophilus DSM 20745T. Although Thermomicrobium was first described by Jackson et al. (1973), its CO-oxidizing capacity was only recently documented (Wu et al., 2009).

Strains KI3T and KI4T were recovered from a layered geothermally heated biofilm growing on a large steaming tumulus in Kilauea Iki (19° 24’ 51.1” N 155° 14’ 57.4” W). Biofilm samples were collected aseptically and transferred to Whirlpak sample bags. Samples were shipped overnight at ambient temperatures to a laboratory at Louisiana State University and processed immediately upon arrival.

Biofilm subsamples (0.5 g fresh weight) were added to 5 ml of Thermomicrobium Cellulose (TC) medium in sealed serum bottles. Medium TC had the same composition as the Thermomicrobium medium (TM) described by Jackson et al.
(1973) with tryptone omitted and 0.5 % (w/v) microcrystalline cellulose added (pH 6.5). The resulting slurries were incubated at 70 °C for 30 min and then diluted serially. CO (100 p.p.m.) was added to the headspace of each dilution tube, and CO uptake was monitored over time as described previously (King, 1999). All enrichments were incubated with shaking at 100 r.p.m. The greatest dilution with evidence of CO oxidation was plated onto medium TC solidified with gellan gum (PhytaGel; Sigma Aldrich). Plates were wrapped with DuraSeal (Diversified Biotech) and incubated at 70 °C.

Individual colonies with distinct morphotypes were selected, inoculated into liquid medium TC and screened for CO uptake. Enrichments were replated as necessary to obtain pure cultures.

Colonial characteristics were determined after growth for 4 days at 70 °C on TM (Jackson et al., 1973). Gram staining was performed using standard methods (Smibert and Krieg, 1994). Cell morphology was determined using a Zeiss Axioscope fitted with a Neofluar ×100 objective and an AxioCam MR digital camera. Strain KI3T formed off-white colonies, while strain KI4T developed small semi-translucent pink colonies. Cells of both strains KI3T and KI4T were Gram-stain-positive, non-spore-forming, non-motile and rod-shaped. Cells of strain KI3T (0.7 μm width, 1.0–2.0 μm length) were often found in pairs; cells of strain KI4T (1.0 μm width, 1.5–2.0 μm length) were often found in short chains (Fig. 1).

The 16S rRNA genes from these strains were amplified by PCR (Lane, 1991; Sambrook & Russell, 2001) and sequenced using an ABI 3130XL Genetic Analyzer (Applied Biosystems) at the Louisiana State University Genomics Facility (Baton Rouge, LA, USA). Bidirectional 16S rRNA gene sequence reads were assembled and edited using Sequencher 4.8 (Gene Codes Corporation). The 16S rRNA gene sequences were aligned to the SILVA reference 16S rRNA non-redundant database and classified taxonomically using SINA v1.2.11 (Pruesse et al., 2012). Phylogenetic trees were reconstructed using the neighbour-joining, maximum-parsimony and maximum-likelihood methods in MEGA v5.1 (Tamura, 2011), with bootstrap values based on 1000 replications (Fig. 2). Distances were computed using the Kimura two-parameter model in MEGA v5.1. The closest relative of strains KI3T and KI4T was Thermomicrobium roseum DSM 5159T, at 16S rRNA gene sequence similarities of 96.5 and 91.1 %, respectively. 16S rRNA gene sequence similarity between strains KI3T and KI4T was 91.4 %. Lower sequence similarities (<87.6 %) were found with all other described species of the class Thermomicrobia.

DNA G+C contents (mol%) were analysed by the Identification Service of the DSMZ (Braunschweig, Germany) following standard methods (Cashion et al., 1977; Mesbah et al., 1989). Fatty acid extraction and analyses were also performed by the DSMZ using standard procedures (Miller, 1982; Kuykendall et al., 1988), and the protocol of the Sherlock Microbial Identification System (MIDI), with biomass obtained from isolates grown under the same conditions. Three peaks that were not present in the MIDI peak naming table were assigned to fatty acids based on their equivalent chain-lengths (ECL), including 12-methyl C18 : 0 (ECL = 18.431), 5,12-methyl C18 : 2 (ECL = 18.043) and C18 : 0 n-5,7,9 (ECL = 19.102) (Scholfield and Dutton, 1971; Takagi and Itabashi, 1981; Duque et al., 1993). However, several other peaks (0.35–2.23 % of the total fatty acids) could not be assigned (Table S1, available in the online Supplementary Material). The fatty acid profiles of strains KI3T and KI4T were primarily composed of 12-methyl C18 : 0 (56.21 and 69.48 %, respectively) and were similar to that of Thermomicrobium roseum (Table S1). The presence of 5,12-methyl C18 : 2 (12.21 %) distinguishes strain KI3T from the other strains examined. The DNA G+C contents of strains KI3T and KI4T were 66.0 and 60.7 mol%, respectively.

Polar lipids were analysed by the Identification Service of the DSMZ following standard methods (Tindall et al., 2007), with biomass obtained from isolates grown under the same conditions. Polar lipids in strain KI3T were dominated by one major glycolipid and phosphatidylinositol, with six minor glycolipids and three minor phosphoglycolipids (Fig. S1); phosphatidylinositol and five phosphoglycolipids dominated the polar lipids in strain

Fig. 1. Photomicrographs of cells of strain KI3T (a) and strain KI4T (b). Bars, 10 μm.
KI4\textsuperscript{T}, which was also characterized by six relatively abundant glycolipids (Fig. S1). The polar lipid profiles of strains KI3\textsuperscript{T} and KI4\textsuperscript{T} differed distinctly from that of Thermomicrobium roseum DSM 5159\textsuperscript{T}.

Temperature optima of strains KI3\textsuperscript{T} and KI4\textsuperscript{T} were assessed by cultivating strains in TM at 30, 40, 50, 55, 60, 65, 70 and 80 °C (all at pH 6.5). Optimal growth pH was determined by cultivating strains in TM with a pH range of 3–10 (all at 65 °C) prepared using 0.1 M solutions of acetic acid and sodium acetate (for pH 3.1–6.5), Na2HPO4 and HCl (for pH 7.0–9.0), and Na2HPO4 and NaOH (for pH 10.0). Growth was also assessed using TM buffered with 0.1 M solutions of pivalic acid (for pH 4.1–4.5), MES (for pH 5.0–6.6), Tris (for pH 7.1–8.5) and sodium bicarbonate (for pH 9.1–9.5). Strain KI3\textsuperscript{T} grew optimally at 70 °C with no growth observed at 80 °C (Table 1). Strain KI4\textsuperscript{T} grew optimally at 65 °C (Table 1). Strain KI3\textsuperscript{T} was catalase-positive, while strain KI4\textsuperscript{T} was catalase-negative, as determined by pipetting a 3 % hydrogen peroxide solution onto cell pellets in microfuge tubes, and observing oxygen bubble formation. API 20 NE test strip (bioMe`rieux) results revealed that both strains hydrolysed gelatin and were positive for urease, \(\beta\)-glucosidase and \(\beta\)-galactosidase.

Strains KI3\textsuperscript{T} and KI4\textsuperscript{T} were unable to use any of the following carbon sources for growth (at 25 mM each) at 65 °C with a basal salts medium supplemented with 0.01 % yeast extract: acetate, acetone, alanine, arabinose, aspartate, betaine, citrate, dimethylamine, ethanol, fructose, fumarate, galactose, galacturonate, gluconate, glucose, glucuronate, glutamate, glycerol, glycine, glycolate, inositol, 2-propanol, lactate, lactose, malate, malonate, mannitol, mannose, methanol, methylamine, proline, propionate, pyruvate, rhamnose, ribose, serine, succinate, sucrose, tartrate, trimethylamine, valine or xylose. Growth was assessed for 7 days and compared with controls containing 0.01 % yeast extract only. In addition to TM, strains KI3\textsuperscript{T} and KI4\textsuperscript{T} grew well in R2A medium (Reasoner & Geldreich, 1985). Differentiating traits are provided in Table 1.

GENIII Biolog microplates were used to compare carbon metabolism of strains KI3\textsuperscript{T} and KI4\textsuperscript{T}, and their closest phylogenetic neighbour, Thermomicrobium roseum DSM 5159\textsuperscript{T}, obtained from the German Resource Center for Biological Material at the DSMZ. Biolog plates were inoculated with cultures that were grown in TM for 4 days, harvested by centrifugation, washed and resuspended in Biolog inoculating fluid A at an OD600 of about 0.1 absorbance units. GENIII Biolog plates were wrapped with DuraSeal, placed in ziplock bags and incubated at 65 °C. Colour development at 590 nm was determined at intervals using a microplate reader (BioTek Instruments). Substrate utilization profiles were similar for strain KI3\textsuperscript{T} and Thermomicrobium roseum DSM 5159\textsuperscript{T}; however,
**Table 1. Differential characteristics between strains KI3^T and KI4^T and Thermomicrobium roseum**

Strains: 1, KI3T; 2, KI4T; 3, Thermomicrobium roseum DSM 5159^T. Data for Thermomicrobium roseum are from Jackson et al. (1973). Numbers in parentheses for CO uptake rates represent ±1 SE.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO uptake rate [nmol CO h(^{-1}) (mg protein(^{-1})]</td>
<td>6.7 (0.8)</td>
<td>–</td>
<td>4.1 (0.3)</td>
</tr>
<tr>
<td>Colony colour</td>
<td>Off white</td>
<td>Pink</td>
<td>Pink</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>66.0</td>
<td>60.7</td>
<td>64</td>
</tr>
<tr>
<td>Temperature (°C):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimum</td>
<td>70</td>
<td>65</td>
<td>70–75</td>
</tr>
<tr>
<td>Range</td>
<td>50–70</td>
<td>55–70</td>
<td>45–80</td>
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<tr>
<td>pH:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimum</td>
<td>7.0</td>
<td>6.7–7.1</td>
<td>8.2–8.5</td>
</tr>
<tr>
<td>Range</td>
<td>6.5–9.0</td>
<td>5.8–8.0</td>
<td>7.5–8.7</td>
</tr>
<tr>
<td>Growth with:</td>
<td></td>
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<tr>
<td>1% NaCl</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>2% NaCl</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Growth on complex media</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>0.5% Yeast extract</td>
<td>–</td>
<td>–</td>
<td>+</td>
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Thermomicrobium roseum DSM 5159^T was weakly positive for the metabolism of several amino sugars. Notably, strain KI4^T uniquely oxidized salicin, galactose, mannose and fucose (Table S2).

For CO uptake assays, strains KI3^T and KI4^T, and Thermomicrobium roseum DSM 5159^T were initially grown at 65 °C in TM in gastight serum bottles containing 100 p.p.m. CO. Cell suspensions were harvested by centrifugation, and cell pellets were washed and resuspended in a minimal version of TM (TM-min) lacking yeast extract and tryptone. Aliquots (5 ml) of washed cells were transferred to triplicate 60 cm\(^3\) serum bottles that were then sealed with gastight neoprene stoppers. All samples were incubated with shaking (100 r.p.m.). CO (final concentration 100 p.p.m.) was added to the headspaces, which were assayed as described previously (King, 1999). Cell protein contents were determined using a bicinchoninic acid assay kit (Pierce Protein Research Products; Thermo Scientific) after terminating uptake assays. The mean (±SE) CO uptake rate of strain KI3^T [6.7±0.8 nmol CO h\(^{-1}\) (mg protein\(^{-1}\)] was similar to that of Thermomicrobium roseum DSM 5159^T (4.1±0.3); strain KI4^T did not oxidize CO. Neither strain KI3^T nor Thermomicrobium roseum DSM 5159^T was able to grow with CO as a sole carbon and energy source as determined from assessing growth in TM-min with a 30% CO and 5% CO\(_2\) headspace.

Sequences for the large subunit of CO dehydrogenase genes from Thermomicrobium roseum DSM 5159^T, S. thermophilus DSM 2079^T and Rhodothermus marinus DSM 4252^T were obtained from GenBank. Sequences were aligned using CLUSTAL W in MEGA 5.1, and conserved regions were used to design primers. OligoCalc (Kibbe, 2007) and in silico PCR (Bikandi et al., 2004) were employed to aid primer selection. The forward primer coxlF (5'-GCCG[A/G]TCAAGTGGATCGA-3’) and the reverse primer coxlR (5'-GGCGTGATCG-GGGATGTCGAT-3’) amplified a 1500 bp region of the coxl gene, which encompassed the diagnostic active site motif. The coxl gene was amplified using these primers in 25 μl volumes using the GoTaq Green Master Mix (Promega). Amplification conditions consisted of initial denaturation for 3 min at 94 °C, followed by 30 cycles of denaturation for 45 s at 94 °C, annealing for 1 min initiated at 65 °C followed by 0.4 °C stepwise decreases in annealing temperature to a final value of 53 °C, and elongation for 110 s at 72 °C. A final elongation for 10 min at 72 °C completed the thermocycler program. The coxl PCR products were sequenced, edited and assembled as described above.

The coxl gene sequence from strain KI3^T was translated into its predicted amino acid sequence and aligned using Muscle (Edgar, 2004) in MEGA 5.1 against reference coxl gene sequences obtained from the GenBank and IMG databases. Maximum-likelihood, maximum-parsimony and neighbour-joining phylogenetic trees were created in MEGA 5.1 (Tamura et al. 2011) using 1000 bootstrap replicates. The coxl gene sequences from strain KI3^T and Thermomicrobium roseum DSM 5159^T shared 83.3% nucleotide similarity and 97.5% amino acid similarity, and formed a well-supported clade with S. thermophilus DSM 20745^T (Fig. 3). These results support the inclusion of KI3^T in the genus Thermomicrobium based on the previously described taxonomic cut-off of 90% coxl amino acid sequence similarity for members of the same genus (King & Weber, 2008).

Results of phylogenetic, chemotaxonomic, physiological and biochemical analyses (Table 1) indicate that strain KI3^T represents a novel species within the genus Thermomicrobium, for which the name Thermomicrobium carboxidum sp. nov. is proposed. The low levels of 16S rRNA gene sequence similarity to Thermomicrobium roseum DSM 5159^T, together with phenotypic and genotypic differences, indicate that
strain KI4\(^T\) represents a novel species of a new genus, for which the name *Thermorudis peleae* gen. nov., sp. nov. is proposed.

**Description of Thermomicrobium carboxidum** sp. nov.

*Thermomicrobium carboxidum* (carb.o’xi.dum. L. n. carbo charcoal, carbon; Gr. adj. oxys sharp, acid; N.L. neut. adj carboxidum pertaining to carbon monoxide).

Cells are aerobic, Gram-stain-positive, non-motile, non-spore-forming rods often found in pairs (0.7 µm width, 1.0–2.0 µm length). Grows at 50–70 °C (optimum 70 °C) and pH 6.5–9.0 (optimum pH 7.0). NaCl is not required for growth; growth is inhibited by 3 % NaCl. Catalase-positive. Positive for urease, aesculin hydrolysis, gelatinase and β-galactosidase. Negative for indole production from tryptophan, arginine dihydrolase, fermentation and nitrate reduction. Oxidizes CO. Grows on complex media.

The type strain, KI3\(^T\) (=DSM 27067\(^T\)=ATCC BAA-2535\(^T\)), was isolated from a geothermally heated biofilm at the Kilauea Iki crater (Hawai‘i, USA). The DNA G+C content of the type strain is 66.0 mol%.

**Description of Thermorudis** gen. nov.

*Thermorudis* (Gr. adj. thermos hot; L. n. fem. rudis a small stick; N.L. fem. n. *Thermorudis* a small, thermophilic stick).

Cells are Gram-stain-positive, non-motile, non-spore-forming rods. Grows as an aerobic, thermophilic heterotroph. 12-methyl C\(_{18}:0\) is the major fatty acid. The type strain is *Thermorudis peleae*.

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**Fig. 3.** Neighbour-joining phylogeny of inferred coxL amino acid sequences based on 452 aa positions. Filled circles indicate nodes that were supported by bootstrap values (1000 replications) greater than 70 % with the neighbour-joining, maximum-likelihood and maximum-parsimony tree-building algorithms. Grey circles indicate nodes supported by bootstrap values greater than 70 % by two of the three tree-building algorithms examined. Bar, 10 % sequence dissimilarity. Accession numbers are given in parentheses. The outgroup consisted of form II coxL gene sequences from *Rhodobacter sphaeroides* (accession no. YP_352939), *Mesorhizobium loti* (BAB48572), *Roseobacter denitrificans* (YP683179) and *Rhodobacter sphaeroides* (YP352939). A, Firmicutes; B, Deinococcus–Thermus; C, Crenarchaeota; D, Bacteroidetes; E, Chloroflexi; F, Proteobacteria; G, Actinobacteria; H, Euryarchaeota.
Description of Thermorudis peleae sp. nov.

Thermorudis peleae (pe.î.e’a. N.L. fem. gen. n. peleae belonging to Pele, the Hawaiian goddess of fire, intended to mean volcanic).

Cells are often found in pairs or short chains (1.0 μm width, 1.5–2.0 μm length). Forms pink-pigmented colonies. Grows at 55–70 °C (optimum 65 °C) and pH 5.8–8.0 (optimum near pH 7.0). NaCl is not required for growth; growth is inhibited by 1% NaCl. Catalase-negative. Positive for urease, aesculin hydrolysis, gelatinase and β-galactosidase. Negative for indole production from tryptophan, arginine dihydrolase, fermentation and nitrate reduction. Grows on complex media.

The type strain, KT49 (= DSM 27169T = ATCC BAA-2536T), was isolated from a geothermally heated biofilm at the Kilauea Iki crater (Hawaii, USA). The DNA G+C content of the type strain is 60.7 mol%.

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