**Thermovirga lienii** gen. nov., sp. nov., a novel moderately thermophilic, anaerobic, amino-acid-degrading bacterium isolated from a North Sea oil well

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A novel anaerobic, moderately thermophilic bacterium, strain Cas60314<sup>T</sup>, was isolated from hot oil-well production water obtained from an oil reservoir in the North Sea. The cells were Gram-negative, motile, straight rods. The salinity and pH growth optima were 2.0–3.0 % NaCl and 6.5–7.0, respectively. The optimum temperature was 58 °C. Strain Cas60314<sup>T</sup> had a fermentative type of metabolism and utilized proteinous substrates, some single amino acids and a limited number of organic acids, but not sugars, fatty acids or alcohols. Cystine and elemental sulfur were reduced to sulfide. The G+C content of the DNA was 46-6 mol%. On the basis of phenotypic and phylogenetic features, it is proposed that this isolate represents a novel genus and species with the name *Thermovirga lienii* gen. nov., sp. nov. within the family *Syntrophomonadaceae*.

The proposed type strain is strain Cas60314<sup>T</sup> (= DSM 17291<sup>T</sup> = ATCC BAA-1197<sup>T</sup>).

A wide range of micro-organisms have been recovered from samples obtained from oilfield environments (Birkeland, 2004; Magot et al., 2000). A limited number of the strains isolated are members of the order *Clostridiales*. These include (1) the thermophilic fermentative species *Anaerobaculum thermoterrenum* (Rees et al., 1997) isolated from the Redwash oilfield in Utah, (2) the mesophilic fermentative species *Fusibacter paucivorans* (Ravot et al., 1999) isolated from an offshore oil-producing well in the Emeraude oilfield in Congo, Central Africa, (3) the thermophilic nitrate-reducing species *Garcinella nitratireducens* (Miranda-Tello et al., 2003) isolated from a separator tank in the SAMIII oilfield in Tabasco, Gulf of Mexico, (4) the mesophilic sulfate-reducer *Dethiosulfovibrio peptidivorans* (Magot et al., 1997) isolated from an offshore oil-producing well in the Emeraude oilfield in Congo, Central Africa and (5) thermophilic sulfate-reducers from the genus *Desulfotomaculum*, such as *Desulfotomaculum thermocister-num* (Nilsen et al., 1996) isolated from a hot oil reservoir in the North Sea, and *Desulfotomaculum kuznetsovi*, which was initially isolated from an underground, thermal mineral water ecosystem (Nazina et al., 1988) and subsequently recovered from two well-field samples taken from unflooded oilfields in the Paris Basin (Magot et al., 2000). The low number of *Clostridiales* strains isolated from oil reservoirs, however, may not reflect their actual abundance in these environments. A recent study, using culture-independent techniques, indicated that some of the most abundant organisms in an oil-bearing formation in California were close relatives of the genera *Dethiosulfovibrio* and *Aminobacterium*, within the family *Syntrophomonadaceae*, and the genus *Acidaminococcus*, within the family *Acidaminococcaceae* (Orphan et al., 2000).

The family *Syntrophomonadaceae* is represented by 14 genera. Thermophilic organisms are found in eight of these genera (*Anaerobaculum*, *Anaerobranca*, *Caldicellulosiruptor*, *Carboxydocella*, *Syntrophothermus*, *Thermoaerobacter*, *Thermosyntrophus* and *Thermanaerovibrio*). Here, we describe a novel thermophilic anaerobe, strain Cas60314<sup>T</sup>, isolated from a Norwegian offshore oil well not injected with seawater. The isolate is proposed as a member of a novel genus and species within the family *Syntrophomonadaceae*, *Thermovirga lienii* gen. nov., sp. nov.

The sample used in this study was an oil/water mixture collected at the upper-riser of a Norwegian oil reservoir in the North Sea, the Troll C Reservoir, with the geographical position 60° 53’ 10-73” N 03° 36’ 41-41” E. The concentration of sodium in the production water was 164 g l<sup>-1</sup>. The sample was transported from the oil platform at ambient temperature and stored at room temperature prior to being used as an inoculum. The temperature of the water at the sampling site was 68 °C. The pH of the water at 18 °C was 6.8. The Troll C Reservoir has not been injected with seawater.
Enrichment and growth was performed using an anaerobically prepared basal medium containing the following components (\(1^{-1}\) distilled water): 20 g NaCl, 0.9 g MgCl\(_2\cdot6\)H\(_2\)O, 1.4 g MgSO\(_4\)\(\cdot7\)H\(_2\)O, 0.33 g KCl, 0.25 g NH\(_4\)Cl, 0.14 g CaCl\(_2\)\(\cdot2\)H\(_2\)O, 0.45 g KH\(_2\)PO\(_4\), 1.0 ml trace element solution SL-10 (Widdel et al., 1983) and 0.5 ml resazurin (0.02%). After being autoclaved in a dispenser (Lien & Beeder, 1997) the medium was reduced with 4 ml 0.5 M Na\(_2\)S under nitrogen gas; 10 ml vitamin solution (Balch et al., 1979) was added and the pH was adjusted to 6.8 with 1 M NaOH. The medium was dispensed into 50 ml serum bottles. For the enrichment of anaerobic Casamino acid-degrading micro-organisms, Casamino acids (Oxoid) were added from a stock solution to a final concentration of 0.3% (w/v). Enrichments were carried out at 37, 50, 57, 70 and 80 °C with 10% inoculum. Positive enrichments were serially diluted in medium solidified by 0.3% (w/v) Gelrite (Kelco) and incubated anaerobically at the same temperature as that used for the primary enrichment. Colonies from each dilution series were transferred to fresh liquid medium and analyzed further. The strain that is characterized here, strain Cas60314\(^7\), was obtained after three dilution series in solidified medium.

Morphological studies were performed with a light microscope (Eclipse E400; Nikon) equipped with a digital camera (Nikon). For electron microscopy, exponentially grown cells were fixed in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.0 for 45 min and postfixed in 1 % OsO\(_4\) for 20 min at room temperature. Samples were washed twice with 0.1 M sodium cacodylate buffer, postfixed in 1% Os\(_4\) for 45 min and rinsed twice with the buffer. Samples were dehydrated in an ethanol series (30, 50, 70, 96 and 100%). For scanning electron microscopy, samples were dried by critical point drying using a Polaron Range CPD 7501, mounted on scanning electron microscopy stubs, sputter-coated with carbon using a JEOL JFC-2300 HR and then observed with a field-emission scanning electron microscope (FeSEM 7400F) at an accelerating voltage of 1-0 kV. For transmission electron microscopy, samples were treated with propylene oxide as a transitional solvent, prior to infiltration with epoxy resin (Agar). Samples were thin-sectioned on an ultramicrotome (Ultracut S; Reichert) and electron microscopy was performed with a JEM 1230 microscope.

Growth was determined by direct cell counting with a light microscope (Labophot; Nikon) and a counting chamber (Thoma). All growth experiments were performed using Belco tubes with 10 ml medium. For physiological characterization with regard to pH, temperature, NaCl concentration and possible use of alternative electron acceptors, 0.3% peptone (Oxoid) and 0.02% yeast extract (Difco) were used as substrates. Gradients of pH and NaCl were used to obtain physiological data. The pH was measured at room temperature. Amorphous Fe(III) oxide was synthesized by neutralizing a solution of FeCl\(_3\) as described previously (Lovley & Phillips, 1986). The production of magnetite was tested for by holding a magnet close to the culture tube. The production of sulfide was measured as described by Cline (1969).

Substrates were tested with the following concentrations: 10 mM for glutamate, alanine, isoleucine, valine, arginine, serine, threonine, glycine and 2-oxoglutarate; 20 mM for pyruvate, methanol, ethanol, phenol, lactate, acetate, propionate, malate, succinate, citrate and formate; 25 mM for glucose, maltose, xylose, ribose, sucrose, arabinose, fructose, lactose, mannose and maltodextrin; and 0.3% (w/v) for xylan, cellulose, starch and chitin, peptone, meat extract, casein, tryptone and Casamino acids. The utilization of H\(_2\) was tested with a gas phase comprising N\(_2\)/H\(_2\) (1:1). The utilization of various substrates was tested in medium with or without 0.05% (w/v) yeast extract and 0.1% (w/v) cystine.

The effect of various concentrations of oxygen on growth was investigated in medium that was not reduced with sulfide and by exchanging portions of the gas phase with air.

GC was used to determine the production of ethanol and fatty acids (HP 5890; Hewlett Packard) and of hydrogen and CO\(_2\) (HP 6890; Hewlett Packard). The HP 5890 was equipped with an HPLC column (ZB-WAX; Phenomenex) and a flame-ionization detector. The oven temperature was programmed to remain at 65 °C for 1 min before rising to 150 °C in increments of 8 °C min\(^{-1}\). Helium (60 ml min\(^{-1}\)) was used as the carrier gas. The HP 6890 apparatus was equipped with a HayeSep R (Hewlett Packard) packed column and a thermal conductivity detector (Hewlett Packard). The oven temperature was 125 °C. Argon (30 ml min\(^{-1}\)) was used as the carrier gas. Genomic DNA was isolated using the cetyltrimethylammonium bromide method as modified by Lien et al. (1998). The 16S rRNA gene sequence was selectively amplified using a PCR with universal primers 5'-GAG TTT GAT CCT GGC TCA G-3' and 5'-GAA AGG AGG TGA TCC AGC C-3', as modified by Loffler et al. (2000). The PCR was performed with an initial denaturation at 94 °C for 3 min, followed by 30 cycles of annealing at 50 °C for 30 s, extension at 72 °C for 1·5 min and denaturation at 94 °C for 30 s and, finally, an extension cycle at 72 °C for 10 min. After purification with a PCR purification kit (Stratagene), the PCR products were sequenced according to the protocol of the ABI Prism BigDye Terminator kit (Perkin Elmer). The 16S rRNA gene sequence obtained from strain Cas60314\(^7\) was compared with other sequences in the GenBank database (Benson et al., 2005), using BLAST (Altschul et al., 1997) to identify its closest relatives. Alignments for phylogenetic analysis were made by using CLUSTAL X (Thompson et al., 1997). A phylogenetic reconstruction was produced using PHYLIP software (version 3.63) with the Jukes–Cantor distance matrix (Jukes & Cantor, 1969) and the neighbour-joining algorithm (Saitou & Nei, 1987). Confidence in the tree topology was determined by using 100 bootstrapped trees (Felsenstein, 1985).
Growth occurred in enrichments with Casamino acids at 37, 50 and 57 °C after 3–7 days. No growth was observed in enrichments at 70 or 80 °C after 3 weeks incubation. Each enrichment was serially diluted in medium solidified with Gelrite, and round, white colonies 0.5–1 mm in diameter developed after 2–5 days. Single colonies were obtained from all positive enrichments, and all strains analysed had identical 16S rRNA gene sequences. One of the isolates, originating from the enrichment at 57 °C, was studied further. After two additional dilutions in solidified medium, the strain, now designated Cas60314^T, was considered pure.

Cultural and morphological characteristics are given in the description of the novel genus and species. The cells of strain Cas60314^T were motile only in the early exponential growth phase. The cells were straight rods 0.4–0.8 µm in diameter and 2–3 µm in length (see Supplementary Fig. S1a available in IJSEM Online). They appeared as single cells or in chains of two to five cells. During growth, strain Cas60314^T formed aggregates of up to several hundred cells (see Supplementary Fig. S1b in IJSEM Online). Scanning electron microscopy revealed the presence of flagella (Fig. 1a). The Gram reaction was negative and a two-layered cell-wall structure typical of Gram-negative cells was observed in ultrathin sections (Fig. 1b). Spore formation was not observed.

Optimal growth was observed under strictly anaerobic conditions, but strain Cas60314^T could also grow in medium not supplemented with sodium sulfide as a reducing agent. No growth was observed in unreduced medium under an N2/air (20:1, v/v) gas phase. On the basis of these observations, strain Cas60314^T should be regarded as an obligately anaerobic organism.

Strain Cas60314^T was able to grow with 100% H₂ in the headspace, with peptone as the substrate.

Table 1 shows the end products from some of the substrates utilized by strain Cas60314^T. In addition, propionate was formed when succinate was used as substrate and cystine as an electron acceptor. In this case, however, no growth could be detected.

A continuous stretch of 1427 nt was determined for the 16S rRNA gene sequence of strain Cas60314^T and was compared with sequences in the GenBank database. The highest levels of similarity were found with respect to members of the family Syntrophomonadaceae in the order Clostridiales. In a phylogenetic analysis, strain Cas60314^T was placed in a distinct branch within this family (Fig. 2). The 16S rRNA gene nucleotide sequence similarity between strain Cas60314^T and its closest relative, Aminobacterium colombiense, was 86%.

Strain Cas60314^T can be distinguished from its closest relatives (shown in Table 2) by its substantially different G+C content (relative to the genera Dethiosulfovibrio and Thermanaerovibrio), by its clearly different temperature optimum (relative to the genera Aminobacterium, Aminomonas and Dethiosulfovibrio), by its halophilic nature (relative to the genera Aminobacterium, Aminomonas and Anaerobaculum), by its inability to reduce thiosulfate (relative to the genera Dethiosulfovibrio and Anaerobaculum), by its different amino-acid-utilization pattern (relative to the genera Aminobacterium and Aminomonas), by its inability to utilize glycerol, fumarate and malate (relative to the genus Anaerobaculum), by its inability to ferment sugars (relative to the genera Thermanaerovibrio and Anaerobaculum) and by the lack of a requirement for yeast extract for growth on peptone, Casamino acids or casein (relative to the genera Aminobacterium and Aminomonas).

With the methods used in the analysis of metabolic products, we were unable to distinguish between isovaleric acid and 2-methylbutyric acid. On the basis of the chemical structure of leucine and isoleucine, however, it seems most likely that strain Cas60314^T converts leucine to isovaleric acid and isoleucine to 2-methylbutyric acid. This would be...
Table 1. End products from some of the substrates utilized by *Thermovirga lienii* Cas60314<sup>T</sup>

Unless otherwise specified, all experiments were performed in medium supplemented with yeast extract (0.05 %, w/v) and incubated for 2–3 weeks at 58 °C. Cystine was added to a final concentration of 0.1 % (w/v). The result for each experiment is based on a comparison between test vials and a control vial without substrate. All substrates degraded by *Thermovirga lienii* Cas60314<sup>T</sup> are listed in the species description. ND, Not determined.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Products formed</th>
<th>Mean cell density in stationary phase [cells ml&lt;sup&gt;−1&lt;/sup&gt;] × 10&lt;sup&gt;8&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Ethanol</td>
<td>Acetate</td>
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<tr>
<td>Peptone†</td>
<td>+</td>
<td>+</td>
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<td>Casamino acids†</td>
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<td>+</td>
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<tr>
<td>Casein†</td>
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<td>+</td>
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<tr>
<td>Serine</td>
<td>+</td>
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<tr>
<td>2-Oxoglutarate</td>
<td>−</td>
<td>−</td>
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<td>Pyruvate</td>
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<td>Alanine + cystine</td>
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<td>Glutamate + cystine</td>
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<td>Valine + cystine</td>
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<td>Isoleucine + cystine</td>
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<td>Leucine + cystine</td>
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<td>Arginine + cystine</td>
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<td>Serine + cystine</td>
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<tr>
<td>2-Oxoglutarate + cystine</td>
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<tr>
<td>Pyruvate + cystine</td>
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*Formation of isovalerate or 2-methylbutyrate could not be distinguished by the methods used in this study.
†Medium not supplemented with yeast extract.

Fig. 2. Dendrogram, based on 16S rRNA gene sequences, indicating the position of *Thermovirga lienii* among members of the family *Syntrophomonadaceae*. *Anaerococcus hydrogenalis* was used as the outgroup. Bootstrap values from 100 replications are shown at branching points. Only values above 95 are reported. Bar, 10 substitutions per 100 bases.
Table 2. Comparison of Thermovirga lini strain Ca6031T and its closest phylogenetic relative within the family Syntrophomonadaceae

<table>
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<th>Characteristic</th>
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<td>G: C content</td>
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<td>Temperature range (optimum):</td>
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<td>Optimum:</td>
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*Only in the presence of cysteine.
*Only in the presence of thiosulfate.
*Only in co-culture with a methanogenic organism.
*Only in the presence of thiosulfate.
in agreement with what is observed for *Aminobacterium colombiense* (Baena et al., 1998). Also as observed for *Aminobacterium colombiense*, strain Cas60314<sup>T</sup> converts alanine and serine to acetate, while glutamate and valine are converted to propionate and isobutyrate, respectively. The production of fatty acids that are each one carbon shorter than the corresponding amino acids indicates that the amino acids are degraded via oxidative deamination, with the oxo-acid intermediate being decarboxylated.

Because of the various possibilities for contamination in the sampling procedure, and the possibility of contamination of an entire reservoir in the drilling or oil-recovery processes, it is not straightforward to determine whether organisms isolated from oil-reservoir samples are natural inhabitants of these habitats. In some cases, as in the case of *Desulfovibrio longus*, an indigenous nature can even be excluded (Magot et al., 1992). Strain Cas60314<sup>T</sup> was isolated from a reservoir that is not injected with seawater. This eliminates one common source of contamination of oil wells, and strengthens the view that strain Cas60314<sup>T</sup> is indigenous to the oil reservoir.

On the basis of its phenotypic characteristics as well as its distinct phylogenetic position, strain Cas60314<sup>T</sup> is considered to represent a novel genus and species, for which the name *Thermovirga lienii* gen. nov., sp. nov. is proposed.

**Description of Thermovirga gen. nov.**


Cells occur singly or in chains and have the ability to form aggregates. Motile. Gram-negative. No spores are formed. Growth is anaerobic. Cells are thermophilic and slightly halophilic. Able to ferment proteinous substrates, some single amino acids and a limited number of organic acids, but not sugars, fatty acids or alcohols. Able to reduce cystine and elemental sulfur to hydrogen sulfide. The type species is *Thermovirga lienii*.

**Description of Thermovirga lienii sp. nov.**

*Thermovirga lienii* (lien.i’i. N.L. gen. n. lieni’i named in honour of the Norwegian microbiologist Professor Torleiv Lien, for his important contribution in the study of anaerobes from petroleum reservoirs).

Cells are motile and typically 2–3 μm in length and 0·4–0·8 μm wide. Cells occur singly or in chains and can form aggregates of up to several hundred cells. The temperature range for growth is 37–68 °C (optimum, 58 °C; see Supplementary Fig. S2 available in IJSEM Online). The pH range for growth is 6·2–8·0 (optimum, pH 6·5–7·0). The NaCl range for growth is 5–80 g 1<sup>−1</sup> (optimum, 20–30 g 1<sup>−1</sup>). Substrates that can be utilized by strain Cas60314<sup>T</sup> in medium not supplied with yeast extract are as follows: peptone, Casamino acids, meat extract, tryptone and casein. Yeast extract is required for fermentation of pyruvate, serine and 2-oxoglutarate. Substrates that can be utilized only in medium supplied with yeast extract and cystine are as follows: glutamate, alanine, leucine, isoleucine, valine and arginine. The following substrates are not fermented in the presence of yeast extract, but were not tested in medium supplied with cystine: xylose, ribose, sucrose, arabinose, fructose, lactose, mannose, maltodextrin, xylene, cellulose, starch and chitin. Strains that cannot be utilized in the presence of yeast extract and in the presence or absence of cystine are as follows: H<sub>2</sub>, glucose, maltose, threonine, glycine, methanol, ethanol, phenol, lactate, acetate, propionate, malate, succinate, citrate and formate. Strain Cas60314<sup>T</sup> does not perform the Stickland reaction when alanine is provided as electron donor and serine or arginine is provided as electron acceptor. Cystine stimulates growth. Cystine and elemental sulfur, but not thiosulfate, are reduced to hydrogen sulfide. Amorphous Fe(III) does not stimulate growth, and, in its presence, magnetite is not formed. The G+C content of the genomic DNA is 46·6 mol% (HPLC).

The type strain, Cas60314<sup>T</sup> (=DSM 17291<sup>T</sup> = ATCC BAA-1197<sup>T</sup>), was isolated from production-water samples from an oil reservoir in the North Sea.

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**References**


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