Abyssivirga alkaniphila gen. nov., sp. nov., an alkane-degrading, anaerobic bacterium from a deep-sea hydrothermal vent system, and emended descriptions of Natranaerovirga pectinivora and Natranaerovirga hydrolytica

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A strictly anaerobic, mesophilic, syntrophic, alkane-degrading strain, L81\(^T\), was isolated from a biofilm sampled from a black smoker chimney at the Loki’s Castle vent field. Cells were straight, rod-shaped, Gram-positive-staining and motile. Growth was observed at pH 6.2–9.5, 14–42 \(^\circ\)C and 0.5–6 % (w/w) NaCl, with optima at pH 7.0–8.2, 37 \(^\circ\)C and 3 % (w/w) NaCl. Proteinaceous substrates, sugars, organic acids and hydrocarbons were utilized for growth. Thiosulfate was used as an external electron acceptor during growth on crude oil. Strain L81\(^T\) was capable of syntrophic hydrocarbon degradation when co-cultured with a methanogenic archaeon, designated strain LG6, isolated from the same enrichment. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain L81\(^T\) is affiliated with the family Lachnospiraceae, and is most closely related to the type strains of Natranaerovirga pectinivora (92 % sequence similarity) and Natranaerovirga hydrolytica (90 %). The major cellular fatty acids of strain L81\(^T\) were \(\mathrm{C}_{15}:0\), anteiso-\(\mathrm{C}_{15}:0\) and \(\mathrm{C}_{16}:0\), and the profile was distinct from those of the species of the genus Natranaerovirga. The polar lipids were phosphatidylglycerol, diphosphatidylglycerol, three unidentified phospholipids, four unidentified glycolipids and two unidentified phosphoglycolipids. The G+C content of genomic DNA was determined to be 31.7 mol%. Based on our phenotypic, phylogenetic and chemotaxonomic results, strain L81\(^T\) is considered to represent a novel species of a new genus of the family Lachnospiraceae, for which we propose the name Abyssivirga alkaniphila gen. nov., sp. nov. The type strain of Abyssivirga alkaniphila is L81\(^T\) (\(=\)DSM 29592\(^T\)=JCM 30920\(^T\)). We also provide emended descriptions of Natranaerovirga pectinivora and Natranaerovirga hydrolytica.

Microbial degradation of hydrocarbons in marine environments is a natural process fuelled by hydrocarbon seepages from the ocean floor (Head et al., 2006; Muyzer & Stams, 2008). This means that we can expect to find hydrocarbon-degrading micro-organisms in a wide variety of marine environments across the globe. The total annual input of non-gaseous hydrocarbons to the marine environment has been estimated to 1 268 000 t, of which 47 % originates from natural seepages and 53 % is caused by human activity related to oil exploitation (Kvenvolden & Cooper, 2003).

Biodegradation of hydrocarbons is relevant to bioremediation of oil-polluted environments (Dubinsky et al., 2013; Mason et al., 2014) as well as to understanding the mechanisms involved in microbe-enhanced oil recovery (MEOR) technologies that are based on growth and activity of hydrocarbon-degrading bacteria and microbial consortia.
Bioremediation of hydrocarbons has been a particular focus of interest in the aftermath of the Deepwater Horizon oil spill in the Gulf of Mexico in 2010. Large volumes of crude oil were probably deposited on the deep-sea ocean floor as a result of extensive use of chemical dispersants (Schrope, 2013). Initial studies showed that the deep oil plume stimulated indigenous bacteria affiliated with known hydrocarbon degraders. The higher-than-expected biodegradation rates observed were partly attributed to adaptation of the microbial community to hydrocarbon input from natural oil-seeps in the Gulf of Mexico (Hazen et al., 2010).

Biodegradation of hydrocarbons by methanogenic consortia has a low energy yield compared with that of anaerobic respiration with nitrate or sulfate as terminal electron acceptors (Mbadinga et al., 2011), but may represent the dominant mode of anaerobic degradation of hydrocarbons in environments depleted in external electron acceptors (e.g. oil reservoirs, deep-sea sediments). A large fraction of the energy of the source molecule is left in the end metabolic product, methane, making the process interesting for MEOR. Biodegradation of C₂–C₅ hydrocarbons makes the gases richer in methane. This, in combination with a reduction in the alkane content of the oil during biodegradation, reduces the ability of the oil to dissolve gas. Biodegradation thus reduces the gas : oil ratio of the trapped oil, and may result in the exsolution of a dry gas cap enriched in methane and CO₂ (Jones et al., 2008; Larter et al., 2005; Wenger et al., 2002). This process may offer a way to extract energy otherwise trapped in non-extractable oil (Gieg et al., 2008; Gray et al., 2009; Jones et al., 2008). Based on the conversion rates estimated from methane-producing consortia published so far, the commercial potential of the process may be significant (Berdugo-Clavijo & Gieg, 2014; Gieg et al., 2008).

Methanogenic hydrocarbon degradation is a syntrophic metabolism, where the methanogenic archaeon serves as the electron acceptor for the reaction (Zengler et al., 1999). No single species can degrade higher hydrocarbons to methane gas, as the thermodynamics of the reaction require that intermediates, such as hydrogen, acetate and formate, are kept at low concentrations in order for the overall reaction to be favourable (Dolfing et al., 2008). A minimal methanogenic consortium consists of one species that degrades higher hydrocarbons to hydrogen, formate, acetate and CO₂, or a combination of these, and a methanogenic archaeon that converts one or more of these compounds to methane (Mbadinga et al., 2011; Schink & Stams, 2006). Hydrogenotrophic methanogenesis is the dominant pathway in enrichment cultures from sub-surface oil reservoirs (Bonch-Osmolovskaya et al., 2003; Dolfing et al., 2008; Mueller & Nielsen, 1996; Rozanova et al., 1995), and the majority of methanogens described are hydrogenotrophic (Dolfing et al., 2008).

Two syntrophic alkane-degraders have been described to date, Desulfatibacillum alkenivorans (Callaghan et al., 2008, 2012) and Desulfoglaeba alkanexedens (Davidova et al., 2006; Lyles et al., 2014). Both species are also capable of hydrocarbon degradation via sulfate reduction. No purely syntrophic hydrocarbon-degrader has been isolated so far (Cheng et al., 2014), to the best of our knowledge.

In order to understand better the nature and role of methanogenic hydrocarbon-degrading consortia in bioremediation and MEOR, it is important to have a clearer understanding of the diversity of micro-organisms that take part in the process and their biochemistry. Oil reservoirs have naturally been widely studied as a source of hydrocarbon-degrading organisms (Cheng et al., 2014; Jones et al., 2008; Larter et al., 2005), but other environments such as deep-sea hydrothermal vents also offer suitable habitats (He et al., 2013; Kleindienst et al., 2012; Rueter et al., 1994; Simonet, 1990; Teske et al., 2014). In this study, a hydrocarbon-degrading bacterium, strain L81T, was isolated from a methane-producing, hydrocarbon-degrading microbial consortium enriched from a black smoker at the Loki’s Castle vent field (LCVF). The LCFV is located on the Arctic Mid-Ocean Ridge (AMOR) at a depth of approximately 2400 m (Pedersen et al., 2010). High concentrations of ammonium and methane in the end-member fluids indicate that the system is sediment influenced. The sediment influence makes formation of higher hydrocarbons plausible in the LCFV, as in the Guaymas Basin (Bazylinski et al., 1989) and the Middle Valley vent fields at the Juan de Fuca Ridge (Adams et al., 2013). Measurements of complex hydrocarbons have not been performed in hydrothermal fluids from the LCFV. As a result of increased oil exploration activity in the Arctic regions, knowledge of the intrinsic potential for hydrocarbon degradation in the local environment is important in order to evaluate better the environmental impact of possible future oil spills. Strain L81T offers a new model for systematic analyses of a methanogenic hydrocarbon-degradation process indigenous to the LCFV.

The current paper describes strain L81T with regard to its characteristics as a hydrocarbon-degrader alone and in syntrophy with a methanogenic partner. The low 16S rRNA gene sequence similarity to other known bacterial genera suggested that strain L81T should also be described as representing the type species of a new genus within the family Lachnospiraceae.

A biofilm (10ROV9BS) was sampled in 2010 from a black smoker wall at the LCFV (73° 33′ N 8° 09′ E, depth 2349 m below sea level) using a 1 l hydraulic cylinder (biosyringe) as described previously (Dahle et al., 2015). Sample material was transferred to 50 ml anaerobic culture bottles with Met II growth medium amended with crude oil immediately upon arrival at the surface. The bottles were incubated at 30 and 60 °C.

Enrichment, growth and isolation were performed under anoxic conditions on Met II growth medium modified from
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Whitman et al. (2006), containing the following components (per litre distilled water): 22 g NaCl, 0.14 g CaCl₂, 2H₂O, 0.34 g KCl, 0.5 g NH₄Cl, 5.5 g MgCl₂, 6H₂O, 0.14 g K₂HPO₄, 3H₂O, 0.2 g yeast extract, 10 ml trace element solution (DSMZ medium 141) and 0.5 ml resazurin solution (0.02 %). Following autoclaving for 20 min at 121 °C and cooling to below 60 °C under flushing with N₂/CO₂ (80 : 20), 10 ml iron solution [0.2 g (NH₄)₂Fe(SO₄)₂·6H₂O, 0.2 g cysteine hydrochloride, 10 ml vitamin solution (Whitman et al., 2006), 30 ml 1.0 M NaHCO₃ and 5 ml 0.5 M Na₂S] were added to the medium. The medium was adjusted to pH 7.2 with 1 M HCl and then dispensed into 30 ml anaerobic culture-tubes, containing 15 ml medium and 15 ml N₂ headspace at 1 bar. The tubes were sealed with butyl rubber stoppers and crimp seals. Growth substrates were added from anoxic stock solutions to final concentrations of 20 mM, prior to inoculation with 1 ml sample water. Tubes intended for growth experiments with H₂ as substrate were given a headspace containing H₂ and CO₂ (80 : 20) prior to inoculation.

A 10⁻⁹ dilution series for isolation of single colony was made in 10 ml serum bottles containing 4.5 ml Met II medium with 20 mM formate as substrate. One millilitre of a 4 % anoxic agar solution was added to each bottle as a gelling agent. Three white, fluffy colonies, designated L81T, L82 and L83, were picked from the 10⁻⁸ dilution after incubation at 30 °C for 5 days. The strains were further purified by dilution to extinction in liquid medium amended with 10 % (w/v) glycerol. Similar dilutions were also performed at 60 °C with H₂/CO₂ (80 : 20) as growth substrate and Geltrite as a gelling agent. One colony, designated LG6, was isolated from this dilution. Purity of the cultures was assessed by phase-contrast microscopy, sequencing of the 16S rRNA gene and DGGE analysis. All bands from the DGGE were sequenced, and cultures with a unique sequence present were deemed pure cultures.

16S rRNA gene sequences were determined after amplification by PCR, using primers Prba8f (5'–AGAGTTT-GATCCGTGCTCAG–3') and Hr (5'–AAGGAGGTGATCC–AGCGCA–3') (Giovannoni, 1991). The 16S rRNA gene sequence obtained was compared with available sequences in the GenBank database (http://www.ncbi.nlm.nih.gov) by BLAST (Altschul et al., 1990) and by analysing the 16S rRNA gene sequence with the ARB software package (Ludwig et al., 2004). To reconstruct a phylogenetic tree, 16S rRNA gene sequences from genera suggested to be the closest relatives by both BLAST and ARB were downloaded and aligned with the SINA aligner (Pruesse et al., 2012). The maximum-likelihood tree in Fig. 1 was reconstructed using RAxML (Stamatakis, 2014) and visualized with Dendroscope (Huson & Scornavacca, 2012). The genomic DNA G+C content of strain L81T was determined by HPLC by the Identification Service of the DSMZ, Braunschweig, Germany.

The substrate spectrum and temperature, salinity and pH optima of strain L81T were determined under anoxic conditions using a modified Met II medium, where the yeast extract and iron solution were omitted; this medium was designated Met IV. The presence of iron sulfide had no effect on the growth of the strain, and its omission made spectrophotometric measurements of growth possible. All growth experiments were performed in triplicate and repeated three times. Natranaerovirga hydrolytica DSM 24176T and Natranaerovirga pectinivora DSM 24629T were grown in parallel with strain L81T. Growth was determined as an increase in OD₆₀₀ and by total cell counts. The amounts of hydrogen, methane and CO₂ were determined using an HP 6890 GC, as described by Dahle & Birkeland (2006). All growth experiments, except determination of the temperature range, were performed at 37 °C. Glucose was used as a substrate in all growth experiments, except for substrate-utilization and hydrocarbon-degradation tests. For testing substrate utilization, a fresh overnight culture that had been transferred at least five times in Met IV with 0.02 % (w/v) yeast extract was used as the inoculum. Cultures were transferred three times on the same substrate before growth was determined. All substrate-utilization experiments were performed both with the substrate as sole carbon and energy source and with the addition of 0.02 % (w/v) yeast extract. All substrates were also tested with 20 mM thiosulfate added as an external electron acceptor. For the determination of the pH range and optimum, three different buffers were used: phosphate/CO₂ (Standard Met IV medium), HEPES (20 mM, pKₐ 7.48) and MOPS (20 mM, pKₐ 7.20). The range of NaCl concentration for growth was measured in Met IV with modified salinities. Two solutions of the medium were prepared; one containing no NaCl and one with 100 g NaCl l⁻¹. Intermediate NaCl concentrations were obtained by mixing appropriate volumes of the two solutions.

Catalase activity was tested by adding 15 % (w/v) H₂O₂ to a cell pellet collected from 1 ml exponential phase culture; the presence or absence of gas generation was then observed (Mahon et al., 2015).

The membrane characteristics of strain L81T in exponential-phase cultures were assessed by the KOH string test (Powers, 1995), Gram-staining, peptidoglycan analysis (Schumann, 2011) and transmission electron microscopy (TEM). Analysis of cell-wall sugars was performed by the DSMZ according to protocol 3 of Schumann (2011). Polar lipids, peptidoglycan and cellular fatty acid composition were analysed by the DSMZ from freeze-dried cells grown under optimal conditions.

The morphology of the strain was evaluated by phase-contrast microscopy (Zeiss Axioscope 40), scanning electron microscopy (SEM) (Zeiss Supra 55VP) and TEM (JEOL 2100). Samples for electron microscopy were prepared as described by Dahle & Birkeland (2006). Motility was assessed by phase-contrast microscopy (Zeiss Axioscope 40).

To determine whether strain L81T was capable of being the hydrocarbon-degrading partner in a methanogenic, hydrocarbon-degrading consortium, the strain was co-cultured.
with the methanogenic strain LG6 or *Methanoplanus limicola* DSM 2279T (Wildgruber *et al.*, 1982) in Met IV medium containing 10 μl sterile crude oil ml⁻¹ or 1 μl hexadecane ml⁻¹ as the only carbon and energy source. A co-culture of strains L81T and LG6 was grown on Met II medium with glucose, and methane production...
was observed under these conditions. This co-culture was used as the inoculum for the hydrocarbon-degradation experiments. The cultures were transferred three times on Met IV with added crude oil, with an incubation time of 6 months, before the hydrocarbon fraction was analysed. Sterile Met IV medium with added crude oil or hexadecane and inoculated medium without hydrocarbons were used as controls. Crude oil-degradation experiments were performed in triplicate. The hexadecane-degradation experiment was performed with 20 parallels. The ability of strain L81T to degrade hydrocarbons with an external electron acceptor was assessed by adding 20 mM nitrate, sulfur, sulfate or thiosulfate to Met IV medium with added crude oil.

Degradation of higher hydrocarbons was determined by whole-oil gas chromatography as described by Barman Skaare et al. (2007), except that the detector temperature was set to 350 °C, the initial hold time at 30 °C oven temperature was 15 min and the oven temperature was increased to 60 °C at a rate of 0.7 °C min⁻¹, with no hold time at 60 °C, and then to 320 °C at a rate of 4 °C min⁻¹ with a hold time of 25 min at 320 °C. Production of methane and CO₂ was measured on a Hewlett Packard 6890 GC with a HayeSep R column. Helium was used as a carrier gas. The settings were: oven temperature, 35 °C; front inlet temperature, 50 °C; flow, 30 ml min⁻¹; detector temperature, 150 °C; detector reference flow, 56 ml min⁻¹.

Three bacterial strains designated L81T, L82 and L83 and one archaeon designated strain LG6 were isolated from agar dilutions using Met II medium with formate. Sequencing of the 16S rRNA gene and growth experiments showed the three bacterial strains to be identical. Strain L81T was chosen as a representative strain. Strain LG6 showed 100 % 16S rRNA gene sequence similarity with the type strain of Methanothermococcus okinawensis (Takai et al., 2002) and was not characterized further.

Phylogenetic analysis of the 16S rRNA gene sequence of strain L81T suggested that it represents a novel species from a new genus within the order Clostridiales, most closely related to members of the family Lachnospiraceae (Fig. 1). The highest 16S rRNA gene sequence similarity with the type strain of a described species was 92 %, with N. pectinivora (Sorokin et al., 2012).

The anaerobic bacterium strain L81T was isolated from the wall of a black smoker chimney at the LCVF. Strain L81T was mesophilic, and grew at 15–42 °C, with optimum growth at 37 °C. No growth was observed at 14 °C after 28 days, and cells lysed above 42 °C. The isolate grew in the presence of 5–60 g NaCl l⁻¹, with an optimum at 30 g l⁻¹. The doubling time at 37 °C, pH 7.2 and 30 g NaCl l⁻¹ was 120 min. Mid-exponential-phase cells grown on glucose at pH 7.0 were rod-shaped, 0.5 µm wide and 2–5 µm long (Fig. 2). In late-exponential-phase cultures, long chains of cells were present. In stationary phase, the majority of the rods became spherical, with a diameter of approximately 1.0 µm. Coccolid cells divided from rod-shaped cells, and coccoid cells were dividing. TEM images indicated a diminished cell wall in the coccoid cells. This suggests that the coccoid cells are metabolically active, partly cell-wall-deficient cells that are formed as a response to unfavourable growth conditions. As the cells still stained Gram-positive, they cannot be completely cell wall deficient (L-form) (Dienes & Weinberger, 1951).

In late-stationary phase, cells with diminished cytoplasmic content were commonly observed by light microscopy. Rod-shaped cells were observed to be motile, but coccoid cells displayed no motility. Terminal, round, spore-like structures were observed in late exponential phase, but cultures showed no other sign of having spores. The strain did not show the temperature, desiccation or oxygen tolerance characteristic of spores. The morphology of the strain was observed to be highly variable, and pH appeared to be the determining factor for the morphology. Cells were rod-shaped, as described above, between pH 7.0 and 8.1. Below pH 7, coccoid cells were dominant, and above pH 8.1, longer rods and rods forming long chains were dominant. The optimal pH range was 7.0–8.2. During fermentative growth with glucose, the medium in the culture bottles dropped to pH 5.8 in stationary-phase cultures. Cell morphology changed during growth in response to the decreasing pH of the growth medium. In rod-shaped cells grown at pH 7.2, an internal membrane structure was observed in the majority of the cells on TEM micrographs (Fig. 2). One structure was observed for each cell. The internal membrane structure was located centrally within the cell, running lengthwise. The function of this structure in this strain is unknown. The KOH string test yielded negative results, and cells stained Gram-positive in all stages of growth, indicating the presence of a Gram-positive cell envelope structure (Gram, 1884; Powers, 1995).

Strain L81T had a very low membrane peptidoglycan content. This was unexpected for a Gram-positive strain, but was confirmed by repeating the analysis with cells harvested from different cultures. Peptidoglycan analysis showed the presence of diaminopimelic acid, but the low peptidoglycan content prohibited more detailed analysis of the diaminopimelic acid isomer, or the peptidoglycan structure.

Whole-cell fatty acids were predominantly branched, saturated fatty acids. The major cellular fatty acids of strain L81T were C₁₅:₀ (10.4 %), anteiso-C₁₅:₀ (13.0 %) and C₁₆:₀ (15.6 %) (Table 1). The described members of the genus Natranaerovirga have an approximately equal distribution of saturated and unsaturated fatty acids, and palmitylic acid is strongly dominant, with 46.9 and 57.8 % in the type strains of N. pectinivora and N. hydrolytica, respectively (Sorokin et al., 2012). The fatty acid profile of strain L81T is thus markedly different from those of the closest described relatives. Strain L81T does not grow under the conditions used to obtain cell mass from the type strains of N. pectinivora and N. hydrolytica; some variation would therefore be expected based on differences in growth conditions. The following polar lipids were registered in
Fig. 2. (top) SEM of mid-exponential phase culture of strain L81T grown on Met IV with glucose. (bottom) TEM showing dividing cells of strain L81T in mid-exponential phase, grown on Met IV with glucose. A single internal membrane structure can be seen in two of the cells (labelled with arrows).
L81T: phosphatidylglycerol, diphosphatidylglycerol, three unidentified phospholipids, four unidentified glycolipids and two unidentified phosphoglycolipids (Fig. S1, available in the online Supplementary Material). The dominant whole-cell sugar identified was ribose, with trace amounts of glucose and galactose. The glucose registered probably originated from the growth medium.

Yeast extract, glucose, glycerol, maltose, peptone, butyrate, lactose, pectin, galacturonic acid and polygalacturonic acid allowed good growth, typically to a cell density of $10^9$ cells ml$^{-1}$ within 24 h (Table 2). Growth on fructose, galactose, lactate and ribose required addition of 0.2 g yeast extract l$^{-1}$. Methanogenic hydrocarbon degradation was observed in co-culture with Methanothermococcus okinawensis LG6. Degradation of higher hydrocarbons was observed in pure cultures of strain L81T with thiosulfate added as an electron acceptor (Fig. 3). Addition of thiosulfate did not enhance growth on other substrates. Because of the high sulfide content of the medium, it was not possible to quantify thiosulfate reduction to sulfide. Addition of nitrate, sulfur or sulfate as external electron acceptors did not have any effect on growth. No growth was observed with acetate, formate, methane, methylamine, 2-methylamine, propionate or H$_2$/CO$_2$. Addition of acetate to a final concentration of 20 mM inhibited growth. Although strain L81T was isolated on Met II medium with formate as growth substrate, it did not grow on formate in pure culture. It was later discovered that the amount of yeast extract added to the Met II medium was sufficient to support growth of strain L81T, and yeast extract was therefore probably the actual substrate for isolation of the strain. No growth was observed in unreduced growth media.

Major fermentation products when grown on glucose were H$_2$ and CO$_2$. Strain L81T was catalase-negative. The capacity for hydrocarbon degradation is the most interesting feature of strain L81T. Both straight and branched alkanes, ranging from n-C$_5$ to n-C$_{25}$, were degraded (Fig. 3), via both thiosulfate reduction and methanogenesis in co-culture with Methanothermococcus okinawensis LG6.

Table 1. Cellular fatty acid compositions of strain L81T and the type strains of species of Natranaerovirga

The main cellular fatty acids are shown. Data for the reference strains were taken from Sorokin et al. (2012). Values are percentages of total fatty acids.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>L81T C$_{13}$ : 1 at 12–13</th>
<th>N. pectinivora AP3T C$_{13}$ : 1 at 12–13</th>
<th>N. hydrolytica APP2T C$_{13}$ : 1 at 12–13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.8</td>
<td>6.3</td>
<td>4.7</td>
</tr>
<tr>
<td>iso-C$_{14}$ : 0</td>
<td>4.7</td>
<td>10.4</td>
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<td>C$_{14}$ : 0</td>
<td>7.5</td>
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</tr>
<tr>
<td>iso-C$_{15}$ : 3-OH</td>
<td>2.4</td>
<td>0.9</td>
<td>2.0</td>
</tr>
<tr>
<td>a15</td>
<td>11.5</td>
<td>15.6</td>
<td>1.1</td>
</tr>
<tr>
<td>anteiso-C$_{16}$ : 107</td>
<td>1.7</td>
<td>16.9</td>
<td>4.8</td>
</tr>
<tr>
<td>C$_{16}$ : 07c</td>
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<td>15.6</td>
<td>4.8</td>
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<tr>
<td>C$_{16}$ : 09</td>
<td>1.7</td>
<td>15.6</td>
<td>4.8</td>
</tr>
<tr>
<td>C$_{16}$ : 3-OH</td>
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<td>15.6</td>
<td>4.8</td>
</tr>
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<tr>
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<td>0.5</td>
<td>0.5</td>
</tr>
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<td>iso-C$_{17}$ : 07</td>
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<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>C$_{18}$ : 03-OH</td>
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<td>17.5</td>
<td>3.7</td>
</tr>
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<td>3.7</td>
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<td>1.5</td>
<td>17.5</td>
<td>3.7</td>
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</table>

Table 2. Comparison of differential characteristics of strain L81T and the type strains of species of Natranaerovirga

+ , Growth; –, no growth; ND, no data available.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>L81T</th>
<th>N. pectinivora DSM 24629T</th>
<th>N. hydrolytica DSM 24176T</th>
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<tr>
<td>Spores</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Substrates</td>
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</tr>
<tr>
<td>Hydrocarbons</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lactate</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Maltose</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Ribose</td>
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<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Methylamine</td>
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<td>–</td>
</tr>
<tr>
<td>Yeast extract</td>
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<tr>
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<td>Peptone</td>
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<td>Propionate</td>
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<tr>
<td>Formate</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>for growth (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>42</td>
<td>43*</td>
<td>45*</td>
</tr>
<tr>
<td>Minimum</td>
<td>14</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pH optimum</td>
<td>7–8.2</td>
<td>9.5–9.7*</td>
<td>10*</td>
</tr>
<tr>
<td>Salinity range [ % (w/w) NaCl]</td>
<td>0.5–6.0</td>
<td>1.2–1.46*</td>
<td>1.2–2.05*</td>
</tr>
<tr>
<td>Genomic DNA G+C content (mol%)</td>
<td>31.7</td>
<td>32.0*</td>
<td>30.7*</td>
</tr>
</tbody>
</table>

*Data from Sorokin et al. (2012).
After 6 months of incubation, up to 20 μmol methane was produced by a 15 ml culture. Co-cultures of L81T and *Methanoplanus limicola* DSM 2279T on Met IV medium with added hexadecane produced up to 32 μmol methane in a 15 ml culture after 3 months of incubation. No methane was detected in the controls. The mechanism of alkane activation in strain L81T has not yet been determined, but the disappearance of such a wide range of alkanes suggested the presence of more than one activating enzyme, or co-activation of straight and branched alkanes by the same enzyme. Previously, co-activation of otherwise non-metabolized straight-chain n-alkanes and cycloalkanes together with the preferred n-alkanes (Wilkes *et al.*, 2003) and otherwise non-metabolized aromatic hydrocarbons with the preferred n-alkanes (Rabus *et al.*, 2011) has been demonstrated experimentally. When the genome of strain L81T was investigated for the presence of potential alkane-activating enzymes, two glycyl-radical enzymes related to the alkylsuccinate synthases (AssA1, WP_043807415.1; AssA2, WP_028315530.1) postulated to activate n-alkanes via fumarate addition in *Desulfatibacillum alkenivorans* AK-01 (Callaghan *et al.*, 2008, 2012) were identified (unpublished data). The two AssA sequences in strain L81T showed 66 % amino acid sequence identity to each other and 24–25 % identity to AssA1 and AssA2 from *Desulfatibacillum alkenivorans* AK-01. Both enzymes contained the conserved cysteine and glycine residues required for alkane activation (Callaghan *et al.*, 2008; Khelifi *et al.*, 2014). The glycy1-radical enzymes were both followed immediately by a protein annotated as pyruvate–formate lyase D (PflD), which is a pyruvate–formate lyase-activating enzyme. The two PflD sequences in strain L81T showed 41 % amino acid sequence identity to each other, and 34–35 and 40–42 % identity to AssD1 (WP_012610859.1) and AssD2 (WP_015946970.1) from *Desulfatibacillum alkenivorans*. Both PflD sequences contain two cysteine-rich regions that would be involved in iron–sulfur cluster binding. This is a feature unique to alkyl- and benzylsuccinate synthases, and not found in activating enzymes for Pfls (Leuthner *et al.*, 1998). This supports the idea that the two identified glycyl-radical enzymes represent AssA, and the activating enzymes represent AssD. Amino acid sequences are provided in Fig. S2.

Strain L81T produced an emulsifying agent that affected the water-solubility of crude oil. During growth of the strain, the crude oil changed from a uniform layer to a collection of tiny droplets suspended in the water phase. The emulsifier probably promotes hydrocarbon degradation by increasing the oil–water surface area, and possibly facilitating uptake of oil droplets into the cell. Cells were observed to be positioned with one end in direct contact with oil droplets. In cultures amended with both crude oil and glucose, hydrocarbon degradation did not start until the glucose was depleted, as expected. After glucose depletion, a larger fraction of hydrocarbons were degraded in these cultures compared...
with cultures without glucose. This was probably due to the larger number of cells and larger amount of emulsifier in the culture at the onset of hydrocarbon degradation.

Based on differences in 16S rRNA gene sequences and morphological characteristics, metabolic differences and differences in fatty acid composition compared with the closest relatives, we propose L81T as the type strain of a novel species and genus within the family Lachnospiraceae, for which the name Abyssisirva alkaniphila gen. nov., sp. nov. is proposed. Emended descriptions of Natranaerovirga pectinivora and Natranaerovirga hydrolytica are also provided. A simple system as presented by strain L81T coupled to a methanogen such as Methanothermococcus okinawensis offers a unique opportunity to study the syntrophic hydrocarbon-degradation process. Further studies of this system will include a genome analysis of strain L81T and further growth experiments aimed at better understanding the degradation process and determining rates of methane production with regard to the commercial potential of strain L81T.

Description of Abyssisirva gen. nov.

Abyssisirva (A.bys.si.vir’ga. L. gen. n. abyssi from the deep; L. fem. n. virga a rod; N.L. fem. n. Abyssisirva a rod-shaped organism from the deep).

Cells are motile and rod-shaped under optimal growth conditions. Variable morphology in form of viable dividing cocoid cells budding from rods, to larger spherical bodies in stationary growth phase and below pH 7. Chain-forming longer rods are predominant above pH 8.1. Formation of spores is not observed. Cells are mesophilic, obligately anaerobic chemo-organoheterotrophs, able to ferment carbohydrates, peptides and aliphatic hydrocarbons. The 16S rRNA gene sequence places the genus within the family Lachnospiraceae. The type species is Abyssisirva alkaniphila.

Description of Abyssisirva alkaniphila sp. nov.

Abyssisirva alkaniphila (al.ka.ni.phil’la. N.L. neut. n. alkanum alkane, aliphatic hydrocarbon; N.L. adj. philus loving; N.L. fem. adj. alkaniphila loving alkanes).

Displays the following characteristics in addition to those given for the genus. Rod-shaped, motile cells are 0.5 × 2–5 µm in the exponential phase. Cells are usually single, but can form long chains. In the stationary phase, the majority of cells have a spherical form, which is probably related to reduced pH resulting from accumulation of fermentation products. No growth is observed below 15 °C or above 42 °C. A minimum of 0.5 % (w/w) NaCl is required for growth, and no growth is observed at above 6 % (w/w) NaCl. Optimal growth conditions are 37 °C, pH 7.0–8.2 and 3 % (w/w) NaCl. The doubling time at optimal growth on glucose is 120 min. Requires a reduced growth medium. Yeast extract, glucose, glycerol, maltose, peptone, pectin, galacturonic acid, polygalacturonic acid and short-chain and higher alkanes can serve as growth substrates. Growth is observed on fructose, galactose, lactate and ribose in the presence of yeast extract. Thiosulfate can serve as an electron acceptor during hydrocarbon degradation. Acetate inhibits growth. Major fermentation products are H2 and CO2. Methane, formate, H2/CO2, propionate, caproate, palmitate, methylamine and 2-methylamine do not support growth. Main whole-cell fatty acids are C15:0, anteiso-C15:0 and C16:0. The main whole-cell sugar is ribose, with trace amounts of galactose.

The type strain is L81T (DSM 29592T=JCM 30920T), isolated from the LCVF at AMOR. The DNA G+C content of the type strain is 31.7 mol%.

Emended description of Natranaerovirga hydrolytica

Originally described as non-motile, utilizing only galacturonic acid, glucuronic acid, polygalacturonates and fructose as growth substrates, and with a pH range of 8.2–10.6 (Sorokin et al., 2012). In our experiments, motile, grows well at pH 7.2 and is able to utilize pectin, lactose, acetate, butyrate and formate, in addition to galacturonic acid, polygalacturonic acid and fructose. Growth on acetate, butyrate and formate was weak, and required the addition of 20 mM thiosulfate to the medium.

Emended description of Natranaerovirga pectinivora

Originally described as non-motile, utilizing only galacturonic acid and its polymers as growth substrates, and with a pH range of 8.0–10.5 (Sorokin et al., 2012). In our experiments, motile, grows well at pH 7.2 and is able to utilize glucose, glyceral, lactose, maltose, ribose, methylamine, acetate, butyrate, formate and propionate in addition to galacturonic acid, polygalacturonic acid and pectin. Growth on acetate, butyrate, formate and propionate required the addition of 20 mM thiosulfate to the medium. Growth on acetate, butyrate and formate was weak.

Acknowledgements

This work was supported by the Norwegian Research Council (projects 208491 and 179560). We acknowledge the crew on the R/V G. O. Sars and the ROV pilots from ARVUS Remote Systems for all support during the cruise to the LCVF in 2010. Frida-Lise Daæe is thanked for assistance with sample preparations. Bente Thorbjørnsen and Rikke Helen Ulvøen are thanked for technical assistance. Håkon Dahl is thanked for assistance with phylogeny.

References


