Persephonella marina gen. nov., sp. nov. and
Persephonella guaymasensis sp. nov., two novel, thermophilic, hydrogen-oxidizing microaerophiles from deep-sea hydrothermal vents

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INTRODUCTION

Currently, the only two obligately chemolithoautotrophic, thermophilic lineages within the bacterial domain are the Aquificales (Huber et al., 1992) and members of the recently described S²-reducing lineage represented by Desulfurobacterium thermolithotrophum (L’Haridon et al., 1998). Members of the Aquificales have been isolated from shallow marine (Huber et al., 1992; Nishihara et al., 1990) and terrestrial hydrothermal systems (Kryukov et al., 1983; Kawasumi et al., 1984; Shima & Suzuki, 1993; Huber et al., 1998; Kristjansson et al., 1985), from heated compost (Beffa et al., 1996) and, recently, from a deep-sea hydrothermal vent (Reysenbach et al., 2000a). On the basis of molecular ecological analyses, the Aquificales appear to be dominant members of communities in near-neutral terrestrial thermal springs in Japan (Yamamoto et al., 1998), Iceland (Hjörleifsdottir et al., 2001) and Yellowstone National...
The type genus of the *Aquificales* is *Aquifex*, originally isolated from a shallow marine hydrothermal system on the Kolbeinsey Ridge, Iceland (Huber et al., 1992). Close relatives of this genus include *Hydrogenobacter*, which has been isolated from terrestrial thermal springs in Japan (*Hydrogenobacter acidophilus* (Shima & Suzuki, 1993) and *Hydrogenobacter thermophilus* (Kawasumi et al., 1984)) and Iceland (*H. thermophilus* (Kristjansson et al., 1985)) and from shallow marine springs in Japan (*Hydrogenobacter halophilus* (Nishihara et al., 1990)), *Calderobacterium*, which was isolated from terrestrial thermal springs in Kamchatka (*Calderobacterium hydrogenophilum* (Saveleva et al., 1982; Kryukov et al., 1983)) and Yellowstone National Park (Takacs et al., 2001), and *Thermocrinis ruber*, the single representative of the genus *Thermocrinis* isolated from Octopus Spring, Yellowstone National Park (Huber et al., 1998). Recently, another novel, hydrogen-oxidizing thermophile, *Hydrogenothermus* (~Hyr~), was obtained from a sulfidic chimney called ‘Q-vent’. The minerals associated with the sulfide sample included pyrite, barite and anhydrite. The temperature measured at the orifice of the chimney was 133 °C. The sample obtained during Alvin dive 3521 in Guaymas Basin was taken from a flange structure (Delaney et al., 1988) east of Rebecca’s Roost at a depth of 2000 m. Cursory examination of the mineralogy of the sample suggested that it consisted of pyrrhotite, barite, some sphalerite and traces of pyrite. The highest temperature measured on the flange was 170 °C. Once collected, the samples were placed in an insulated box on the submersible’s basket. Upon reaching the ship, the rocks were immediately sectioned and ground under a N₂ atmosphere. The rock slurries were used as the inocula.

**Enrichment cultures and isolation.** The rock slurry (0.5 ml) was inoculated into 5 ml modified MSH medium (see below) with a final gas phase of CO₂/O₂/H₂ (20:1:26, by vol.). The medium was made under an atmosphere of CO₂ and, after inoculation, the appropriate volume of O₂ was added and the tubes were pressurized with H₂ (138 kPa). Enrichments were incubated for 2–3 days at 70 °C without agitation. Enrichment cultures were purified by three consecutive end-point dilutions and by plating the culture on modified MSH medium solidified with 0.8% (w/v) Gelrite (Sigma). Plates were incubated in anaerobic jars at 70 °C and for 5–7 days under an atmosphere of CO₂, H₂ and O₂ until colonies formed. For long-term storage, pure cultures in the modified MSH medium (below) containing 15% (v/v) glycerol were stored in two separate collections at −80 °C and in liquid nitrogen.

**Culture conditions.** Isolates EX-H1~T~ and EX-H2~T~ were routinely grown on modified MSH medium (Boone et al., 1989) which was prepared with anoxic distilled water and contained the following (l⁻¹): 29 g NaCl, 2 g NaOH, 0.5 g KCl, 1.36 g MgCl₂.6H₂O, 7 g MgSO₄.7H₂O, 2 g Na₂S.O₃.5H₂O, 0.4 g CaCl₂.2H₂O, 0.2 g NH₄Cl, 0.3 g K₂HPO₄, 3H₂O and 10 ml of a trace-element stock solution (adapted from Ferguson & Mah, 1983). Prior to autoclaving, the pH of the medium was adjusted to 6.0 with 3 M H₂SO₄. CO₂/O₂/H₂ (20:1:26, by vol.) was used as the headspace. The O₂ (21%, v/v) was added after autoclaving, and the tubes were pressurized with H₂ (138 kPa) after inoculation. Subsequently, all growth experiments were done anaerobically, with nitrate (10 mM) as the electron acceptor and a gas phase (138 kPa) of CO₂ and H₂ (20:27, v/v).

**Determination of growth.** Growth was determined either by direct counting in a Thoma counting chamber (depth
Growth parameters and requirements. All growth parameters were tested with H₂ as the electron donor and NO₃⁻ as the electron acceptor. The effect of pH on growth was determined using 10 mM MES (pH 4.5–5.5) and 10 mM HEPES (pH 7–8). The carbonate buffer in the medium provided sufficient buffering between pH 5–5 and pH 7. The pH was adjusted prior to autoclaving and was periodically checked after autoclaving. NaCl requirements were determined with concentrations of NaCl ranging from 0 to 8% (w/v). O₂ tolerance and requirements were determined by injecting defined volumes of pure O₂ into culture tubes. O₂ concentrations from 0 to 12% (v/v) were tested.

Minimum, maximum and optimal growth temperatures were calculated by applying the square-root equation as described by Franzmann et al. (1997). The following electron acceptors were added to a minimal medium (modified MSH medium without Na₃S·O₅ but with 4 g l⁻¹ MgSO₄·7H₂O to ensure an adequate sulfur source: 138 kPa H₂ as the electron donor) as follows: SO₄²⁻, SO₂⁻, NO₃⁻, Fe⁴⁺ at 10 mM; NO₂⁻ at 1 mM; S²⁻ at 4% (w/v, added after autoclaving); O₂ at 21% (v/v, added after autoclaving). Electron donors were provided at final concentrations of 10 mM (formate, propionate, succinate and Fe⁴⁺), 15 mM (NH₄Cl), or 1 mM (acetate). O₂ was used as the electron acceptor, except in the case of Fe⁴⁺, which was tested with NO₂⁻ as the electron acceptor. Controls contained no electron acceptor or no electron donor. Organic carbon sources were tested at 2 g l⁻¹ final concentration (yeast extract, peptone, formate, lactate) or at 5 g l⁻¹ final concentration (glucose, fructose, sucrose, lactose, sorbitol, dextrose and starch). In all experiments, the gas phase was CO₂. Cultures were transferred at least twice on the same substrate combination to ensure that growth was not due to substrate carry-over from the inoculum.

Sulfide production was determined qualitatively by adding a drop of a saturated lead citrate solution to the culture. A black-brown precipitate indicated the presence of sulfide. Sulfide production was determined qualitatively by adding a drop of a saturated lead citrate solution to the culture. A black-brown precipitate indicated the presence of sulfide. N₂ production was determined by GC with a Gow-Mac 400 G/C gas chromatograph using helium as the carrier gas.

Light- and electron microscopy. Cultures were observed and counted using an Olympus BX60 light microscope fitted with an Optronics Engineering CCD camera. Gram staining was carried out using standard procedures. For electron microscopy, samples were shipped overnight to the laboratory of T. J. B. in 2% (v/v) glutaraldehyde. The cells were washed in 1 mM HEPES buffer (pH 6–8) before being suspended in 2% (w/v) OsO₄ for 2 h at 22 °C. The cells were washed as before and enrobed in 2% (w/v) Noble agar, cut into 1 mm³ blocks and stained with 2% (w/v) aqueous uranyl acetate for 2 h. Blocks were then dehydrated through an ethanol series and embedded in LR White embedding medium. Once cured, the blocks were sectioned and stained with uranyl acetate and lead citrate before being viewed in a transmission electron microscope. Negative stains were made of glutaraldehyde-fixed cells with 2% (w/v) uranyl acetate as the stain on Formvar- and carbon-coated 200-mesh TEM grids. Electron microscopy was performed using either Philips EM300 or LEO 912AB apparatus under standard operating conditions at either 60 or 80 kV with liquid nitrogen anti-contaminators in place.

Lipid extraction and analysis. The cell samples (one wet and one freeze-dried) were transferred into a pre-cleaned 5 ml vial before extraction with dichloromethane/methanol (2 ml each, v/v) under ultrasonication for 15 min. The extract was concentrated under N₂ (99.9% purity, with molecular sieve contaminant trap) at room temperature. The extraction and concentration procedures were repeated three consecutive times. The residual cell membranes were subjected to reductive hydrolysis at 300 °C for 30 h. The extracts from this procedure were also prepared and analysed as below. The analyses of the total extracts were performed by using GC-MS. Identification of compounds bearing carboxylic acid or hydroxyl functionalities were facilitated by derivatizing aliquots of the extract with N,O-bis(trimethylsilyl)trifluoroacetamide. Both undervatized and derivatized extracts were analysed. The GC-MS analyses were performed on a Hewlett Packard 6890 gas chromatograph coupled to a 5973 Mass Selective Detector using a DB-5 (HP Agilent Technology) fused silica capillary column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness) and helium as the carrier gas. The GC was temperature-programmed from 65 °C (2 min initial time) to 300 °C at 6 °C min⁻¹ (isothermal for 20 min final time). The mass spectrometer was operated in the electron-impact mode at 70 eV ion-source energy. Data were processed with a Hewlett Packard Chemstation. Identification of homologous compounds was facilitated by comparison of GC retention times and mass spectra with authentic standards.

DNA isolation. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide method (Ausubel et al., 1994). Cells pellets were resuspended in 567 µl 1 x TE buffer (10 mM Tris/HCl, pH 8.0; 1 mM EDTA), 7.5% Chelex 100 (Sigma), 0.05 M EDTA (pH 7.0), 1% (w/v) SDS and 200 µg Proteinase K, and incubated with slow rotation for 1 h at 50 °C. Chelex was removed by centrifugation. Then 100 µl 5 M NaCl and 80 µl 10% (w/v) cetyltrimethylammonium bromide in 0.7 M NaCl were added to the cell lysate and the sample incubated for 30 min at 65 °C. The DNA was extracted with chloroform/isoamyl alcohol (24:1), phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol. The DNA was precipitated with an equal volume of 2-propanol, washed with 70% (v/v) ethanol and resuspended in 10 mM Tris (pH 8.0).

For the determination of the DNA base composition, DNA was extracted from 100 ml of culture by using the Qiagen Blood and Cell Culture Midi kit according to the manufacturer’s protocol, with the bacterial lysis buffer modification.

DNA base composition. The G + C content of the genomic DNA was determined by melting-point analysis (Marmur & Doty, 1962) and HPLC. The G + C content of Escherichia coli DNA (57 mol%) was used as a standard.

PCR amplification and sequencing of the small-subunit (16S) rRNA genes. The 16S rRNA genes were amplified by a PCR from genomic DNA by using the Bacteria-specific primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and the universal primer 1492R (5'-GGTACCTTGTTACGACTT-3'). Reaction conditions contained 50 mM KCl, 30 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 12.5 mM each dATP, dCTP, dGTP, dTTP, 0.05% (w/v) 1gepol (Sigma), 1 U Taq Polymerase (Promega) and 20 pmol each primer. PCR incubation conditions were as previously described (Takacs et al., 2001). PCR products were purified using the PCRpure Spin kit (Intermountain Scientific). To check for potential polymorphisms in the 16S rRNA genes, the purified PCR

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products were used as templates for sequencing. Subsequently, to confirm the direct sequencing results, PCR products were cloned into the pCR2.1 vector by using the TOPO-TA cloning Kit (Invitrogen). Plasmid DNA was prepared from isolated colonies by using an alkaline-lysis method (Perfect Prep Kit; $5'\rightarrow 3'$) and the DNA was used as a template for sequencing. Sequencing reactions were performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit and an ABI 310 Genetic Analyzer according to the manufacturer’s protocol. The complete sequence on both strands was obtained using vector-specific primers and a suite of 16S rDNA-specific primers to generate an overlapping set of sequences that were assembled into one contiguous sequence using the AutoAssembler Program (Applied Biosystems). The following primers were used for sequencing: 8F, 1492R, 357F (5’-CTCTACGGAGGCAGCAG-3’), 704F (5’-GTAGCAGTGAAATGCGTAAG-3’), 1114F (5’-GCAACGAGCCCAACC-3’), 1391R (5’-GACGGGCGGTGTGTRCA-3’ with R = A or G), 519R (5’-ATTACCGCGGCTGCTGG-3’), 907R (5’-CCGTCAGATTTCTTTRAGTTT-3’ with R = A or G) and the vector-specific primers M13F (5’-GTAAAACGACGGCCAGT-3’) and M13 R (5’-CAGGAAACAGCTATGAC). The fidelity of the assembled sequences was confirmed using secondary-structure diagrams.

Phylogenetic analysis of the 16S rRNA genes. The sequences were manually aligned to related sequences obtained from the Ribosomal Data Project (Maidak et al., 2000) and recent submissions to GenBank. Only homologous nucleotides were included in the phylogenetic analysis. The secondary structure was used as a guide to ensure that only homologous regions were compared. Of the 1463 nucleotides sequenced, 1271 nucleotides were included in the analysis. Using a subset of the Aquificales sequences, and on the basis of unequivocal sequence alignments and secondary-structure comparisons, 1403 homologous nucleotides were used to construct similarity matrices by pairwise analysis with the Jukes–Cantor correction (Jukes & Cantor, 1969). The phylogenetic relationships were determined using maximum-likelihood analysis, using fastDNAm (Olsen et al., 1994), which is part of the ARB software package (http://www.mikrobiologie.tu-muenchen.de/pub/ARB/) (Ludwig & Strunk, 1996) and PAUP 4.0. Alternative methods such as maximum-parsimony and neighbour-joining produced similar results. The bootstrap data represent 100 samplings using maximum-likelihood analysis.

RESULTS AND DISCUSSION

The novel isolates, EX-H1T and EX-H2T, are the first chemolithoautotrophic and thermophilic ‘Knallgas’ bacteria to be isolated from deep-sea hydrothermal vents.

Enrichment and isolation

Enrichments were obtained under microaerophilic and nitrate-reducing conditions from hydrothermal vent chimney samples from the M, Q and Io vents (for site descriptions, see Shank et al., 1998) at 9° N and from vents at Guaymas Basin. On the basis of the initial 16S rRNA gene sequence, an enrichment obtained from Q vent and from Rebecca’s Roost, Guaymas Basin, were chosen for further study. Colonies that were flat, white and 2–3 mm in diameter appeared after 7 days in.

Table 1. Comparison of physiological characteristics of strain EX-H1 and strain EX-H2 with the chemolithoautotrophic members of the Aquificales

<table>
<thead>
<tr>
<th>Character</th>
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<th>6</th>
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<th>9</th>
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<tbody>
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<td>Location</td>
<td>Deep-sea hydrothermal vent, East Pacific Rise</td>
<td>Deep-sea hydrothermal vent, Guaymas Basin, Mexico</td>
<td>Deep-sea hydrothermal vent, Guaymas Basin, Mexico</td>
<td>Marine spring, Vulcano, Italy</td>
<td>Marine spring, field, Japan</td>
<td>Marine spring, Kamchatka, Russia</td>
<td>Terrestrial spring, Yellowstone National Park, Usa</td>
<td>Terrestrial spring, Iceland</td>
<td>Terrestrial spring, Russia</td>
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<td>Temp. opt. (°C)</td>
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<td>pH opt.</td>
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<td>Salinity opt. (%)</td>
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<td>Salinity range (%)</td>
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</table>

| Strains (references) | 1, Strain EX-H1 | 2, Strain EX-H2 | 3, Aquificpsychrophila (Huber et al., 1998); 4, Thermocrinis ruber (Huber et al., 1992); 5, Caldarhabdlexigens marinus (Visai & Suzuki, 1993); 6, Thermophilic acidilithicus (Shima & Suzuki, 1998); 7, Hydrogenothermus marinus (Shima & Suzuki, 1998); 8, Hydrogenothermus marinus (Shima & Suzuki, 1998); 9, Hydrogenothermus marinus (Shima & Suzuki, 1998). |
cubation on Gelrite plates. The isolates were designated strains EX-H1<sup>T</sup> and EX-H2<sup>T</sup>. Successful transfer of both strains required a 10% mid-exponential phase inoculum.

**Morphology**

Like some members of the *Aquificales* (Table 1), both strain EX-H1<sup>T</sup> and strain EX-H2<sup>T</sup> are small, highly motile rods 2–4 µm long and 0.3–0.4 µm wide. Cells grown with S<sup>+</sup> as the electron donor and O<sub>2</sub> as the electron acceptor were distinctly smaller than cells grown with any other electron donor/acceptor combination.

Cell aggregates were rarely observed. Cultures of high cell density and cell pellets exhibited an apricot-pink coloration. Both strains stained Gram-negative and did not form spores.

Ultrathin sections of strain EX-H1<sup>T</sup> revealed that the cell wall structure was typical of Gram-negative bacteria (Fig. 1, top), although it was difficult to see the peptidoglycan layer as a distinct entity (Fig. 1, top, middle). This is not unusual, since the peptidoglycan layer can be in close apposition with, and not easily differentiated from, the inner face of the outer membrane in many Gram-negative genera. In addition, internal darkly staining stacked membranes were frequently observed at the periphery of the cytoplasm (Fig. 1, middle). Once elongated, they were seen at a variety of locations within the cytoplasm, many aligned to the long axis of the cell (Fig. 1, top). It is possible that these membranous structures were...
initially formed from pre-existing plasma membrane and were excised once elongated. Although internal membranes are often used to align enzymes for complex metabolic reactions, the function of these membranous structures is not yet known. Similar membranous structures have not previously been reported in the *Aquificales*. Negative stains of cells revealed that thin, flexible, pili-like filaments extended from the cell surface of many of the cells (Fig. 1, bottom).

**Table 2.** Electron donors and acceptors utilized by strain EX-H1<T> T, strain EX-H2<T> T and chemolithoautotrophic members of the order *Aquificales*

<table>
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<th>Electron donor</th>
<th>Electron acceptor</th>
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*Sulfur is required for growth.*

**DNA base composition and 16S rRNA phylogeny**

The *G+C* content of the DNA was 38.5 mol% for strain EX-H1<T> T and 37.4 mol% for strain EX-H2<T> T. This is consistent with that of other members of the *Aquificales*, whose *G+C* content varies from 35 mol% for *Hydrogenobacter acidophilus* to 40 mol% for *Aquifex pyrophilus* and 47.5 mol% for *T. ruber*. Additionally, EX-H1<T> T and EX-H2<T> T form a phylogenetically distinct lineage (Fig. 2) and are about 85% similar to *A. pyrophilus* (and 83% similar to *Hydrogenobacter thermophilus*), only 96% similar to each other, and 92–94% similar to environmental sequences that fall within this lineage. They are 94.5% similar to the recently described lineage *Hydrogenothermus*
The temperature optima and ranges for the novel isolates were more moderate compared to *A. pyrophilus* (Table 1). Strain EX-H1<sup>T</sup> grew at 55–80 °C, although growth at 80 °C was poor and could be observed only microscopically. The temperature growth range for strain EX-H2<sup>T</sup> was 60–75 °C. Strains EX-H1<sup>T</sup> and EX-H2<sup>T</sup> grew fastest at 73 °C (doubling time about 5.0 ± 2 h) and 70 °C (doubling time about 7 ± 8 h), respectively (Fig. 3). Growth of both strains was optimal at pH 6, although growth was observed between pH 4-7 and pH 7-5 (Fig. 4). Both strains exhibited growth at 1–4.5% NaCl (w/v) but grew fastest at 2-5% NaCl (w/v) (Fig. 5).

**Growth parameters and requirements**

With the exception of *Hydrogenothermus marinus*, all marine *Aquificales* isolates, including EX-H1<sup>T</sup> and EX-H2<sup>T</sup>, are able to grow with O<sub>2</sub> and NO<sub>3</sub> as electron acceptors and with H<sub>2</sub> as the electron donor (Table 2). Cell yields for strains EX-H1<sup>T</sup> and EX-H2<sup>T</sup> when grown in the presence of O<sub>2</sub> or NO<sub>3</sub> were approximately the same, ranging from about 9 × 10<sup>7</sup> to 1 × 10<sup>8</sup> cells ml<sup>−1</sup>. Nitrogen gas was evolved during NO<sub>3</sub> reduction. Growth of both EX-H1<sup>T</sup> and EX-H2<sup>T</sup> was optimal at oxygen concentrations between 2 and 3% (v/v). Very poor growth was observed at oxygen concentrations of 9% (EX-H1<sup>T</sup>) and 11% (EX-H2<sup>T</sup>). Growth was enhanced in the presence of SO<sub>4</sub><sup>2−</sup>, which suggests that this may be required for biosynthesis of certain cellular compounds under hydrogen-oxidizing conditions. However, both strains could use S<sup>0</sup> or S<sub>2</sub>O<sub>3</sub><sup>2−</sup> as an electron donor with O<sub>2</sub>, but only strain EX-H1<sup>T</sup> could use S<sup>0</sup> as an electron acceptor when H<sub>2</sub> was the electron donor. Sulfide was the product of S<sup>0</sup> reduction by EX-H1<sup>T</sup>. Inconsistent growth was observed when SO<sub>4</sub><sup>2−</sup> and acetate were provided as electron acceptors. Cell yields with these substrates were lower, and sulfide was detected in the cultures where SO<sub>4</sub><sup>2−</sup> was the electron acceptor. This growth could not be sustained after five transfers. Neither EX-H1<sup>T</sup> nor EX-H2<sup>T</sup> was able to grow with acetate in the absence of CO<sub>2</sub>. Both strains were strictly chemolithoautotrophic, and no growth was observed for either strain on formate, propionate, succinate, yeast extract, peptone, glucose, fructose, lactose, sucrose, dextrose, sorbitol or starch in the presence or absence of CO<sub>2</sub>.

The significant phylogenetic difference between *A. pyrophilus* and the novel isolates is not reflected in many physiological differences (Table 2). All members of the *Aquificales* are able to use O<sub>2</sub> as an electron acceptor, with H<sub>2</sub> as the favoured electron donor. Only the marine isolates, *A. pyrophilus*, *Hydrogenobacter acidophilus*, EX-H1<sup>T</sup> and EX-H2<sup>T</sup> are capable of using NO<sub>3</sub> as an electron acceptor. However, most of the genera share the ability to use hydrogen, thiosulfate and sulfur as electron donors. Strains EX-H1<sup>T</sup> and EX-H2<sup>T</sup> are capable of tolerating slightly higher O<sub>2</sub> concentrations than *A. pyrophilus*. However, batch-culture conditions are not optimal for studying this relationship, and these experiments should be repeated under continuous-culture conditions.

**Lipid composition**

The major lipid components of strains EX-H1<sup>T</sup> and EX-H2<sup>T</sup> were fatty acids, both free and methyl esters, various glycerol derivatives and phosphoric acid (Table 3). The main fatty acids of EX-H1<sup>T</sup> and EX-H2<sup>T</sup> were C<sub>16</sub> acids (32.3 and 38.9%, respectively) and C<sub>20</sub> acids (27.1 and 36.8%, respectively). Palmitic (C<sub>16</sub>) acid was present in a low amount (0.71 and 0.66%, respectively). Only saturated and monounsaturated fatty acids were detectable. The ratios of saturated to total unsaturated acids were as follows: C<sub>18:0</sub>/C<sub>18:1</sub> =
Table 3. Relative concentrations of the major compounds detected in the EX-H1T and EX-H2T cell cultures

<table>
<thead>
<tr>
<th>Compound</th>
<th>Composition</th>
<th>M&lt;sub&gt;r&lt;/sub&gt;</th>
<th>Relative concentration (as trimethylsilyl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoric acid</td>
<td>H&lt;sub&gt;3&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>98</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 3·02 17·62</td>
</tr>
<tr>
<td>Glyceric acid</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>106</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 0·71 0·66</td>
</tr>
<tr>
<td>Methyl hexadecanoate</td>
<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;31&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>270</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 5·22 0·82</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
<td>180</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 10·12 14·93</td>
</tr>
<tr>
<td>Methyl octadecenoate</td>
<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;31&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>296</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 6·43 15·04</td>
</tr>
<tr>
<td>Methyl octadecanoate</td>
<td>C&lt;sub&gt;15&lt;/sub&gt;H&lt;sub&gt;31&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>296</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 5·22 1·04</td>
</tr>
<tr>
<td>Octadecenoic acid</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;32&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>282</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 35·81</td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;32&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>284</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 0·15</td>
</tr>
<tr>
<td>Methyl eicosanoate</td>
<td>C&lt;sub&gt;21&lt;/sub&gt;H&lt;sub&gt;43&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>324</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 0·14 0·08</td>
</tr>
<tr>
<td>Methyl eicosanoate</td>
<td>C&lt;sub&gt;21&lt;/sub&gt;H&lt;sub&gt;43&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>326</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 1·30 0·19</td>
</tr>
<tr>
<td>Glycerol glucopyranoside</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;H&lt;sub&gt;34&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
<td>254</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 2·99 2·92</td>
</tr>
<tr>
<td>Eicosanoic acid</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;41&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>310</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 0·71 0·66</td>
</tr>
<tr>
<td>2-Monoolein</td>
<td>C&lt;sub&gt;21&lt;/sub&gt;H&lt;sub&gt;43&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>356</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 27·1 36·8</td>
</tr>
<tr>
<td>2-Monostearin</td>
<td>C&lt;sub&gt;21&lt;/sub&gt;H&lt;sub&gt;43&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>358</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 0·29 0·05</td>
</tr>
<tr>
<td>1-Monoolein</td>
<td>C&lt;sub&gt;21&lt;/sub&gt;H&lt;sub&gt;43&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>356</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 3·42 3·38</td>
</tr>
<tr>
<td>1-Monostearin</td>
<td>C&lt;sub&gt;21&lt;/sub&gt;H&lt;sub&gt;43&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>358</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 0·73 0·74</td>
</tr>
<tr>
<td>2-Eicosenoil glycerol</td>
<td>C&lt;sub&gt;22&lt;/sub&gt;H&lt;sub&gt;45&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>384</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 0·14 0·08</td>
</tr>
<tr>
<td>1-Eicosenoil glycerol</td>
<td>C&lt;sub&gt;22&lt;/sub&gt;H&lt;sub&gt;45&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>384</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 9·92 9·51</td>
</tr>
<tr>
<td>1-Eicosanoyl glycerol</td>
<td>C&lt;sub&gt;23&lt;/sub&gt;H&lt;sub&gt;47&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>386</td>
<td>EX-H1&lt;sup&gt;T&lt;/sup&gt; 9·92 9·51</td>
</tr>
</tbody>
</table>

1·02 and 2·08 and J<sub>20:0</sub>/J<sub>20:1</sub> = 0·29 and 0·05 for EXH1<sup>T</sup> and EX-H2<sup>T</sup>, respectively. Monoacylglycerols were minor, and di- and triacylglycerols and alkylglycerols were not detected.

Reductive hydrous pyrolysis of the cell membranes (of both EX-H1<sup>T</sup> and EX-H2<sup>T</sup>) yielded primarily fatty alcohols (n-alkanols) ranging from C<sub>15</sub> to C<sub>20</sub>, with a strong even carbon number predominance and a chain-length maximum of C<sub>18</sub>. No isopenoideal or hopanoid products were detectable. These lipid compositions are consistent with the high-temperature ecological niche of these strains (Huber et al., 1992).

Ecological niche and evolutionary implications

Microaerobic environments are among the most poorly studied and understood areas, yet they provide a competitive edge for microorganisms that are able to grow as microaerophiles and anaerobes. Such microaerobic niches also exist at deep-sea hydrothermal vents, so the presence of hydrogen-oxidizing microaerophiles, such as EX-H1<sup>T</sup> and EX-H2<sup>T</sup>, is not all that surprising. The porous chimney structures form as high-temperature hydrothermal fluid, rich in reduced metals, mixes with cold, oxygenated sea water. As the chimneys form, fluid flow continues both vertically and horizontally, mixing occurring by both advection and diffusion (Tivey, 1993). The vent structures that form under these different fluid chemistries and modes of mixing differ in their mineralogy and, ultimately, in the associated microbial diversity. In these highly mixed environments, chemical gradients can be very steep, associated microbial diversity. In these highly mixed environments, chemical gradients can be very steep, associated microbial diversity.
abilities to use sulfur as an electron acceptor (only slightly different), their doubling times, and their respectively), their oxygen tolerances (which are possibly different), the chemolithoautotrophic Aquificales are important members of high-temperature, deep-sea hydrothermal vent niches, providing organic carbon for deep-sea vent heterotrophs such as the Thermococcales (Canganella et al., 1998; Godfrey et al., 1996, 1997) and the Thermotogales (Takai & Horikoshi, 2000).

The deeply rooted position of the Aquificales may reflect its ancestral origins, though this remains controversial (e.g. Klenk et al., 1999). One might speculate that the broad suite of electron donors and acceptors that are used by the novel strains reported here is a history of their evolution through time. Hydrothermal systems were present on early Earth, and it is possible that ancestral relatives of strain EX-H1T evolved in these systems as the atmosphere on Earth changed from an anoxic one (more than 3.5 billion years ago) to an oxic one (about 2.8 billion years ago). Anaerobic, chemolithoautotrophic thermophiles were able to respond to this dramatic change in the Earth’s atmosphere by acquiring the ability to use other electron acceptors such as nitrate, thiosulfate and oxygen.

Extant relatives, such as EX-H1T, therefore have the ability to grow anaerobically using S\(^0\), but also by using more oxidized compounds such as nitrate and thiosulfate, and they grow microaerophilically. Their metabolic plasticity may therefore be a reflection of their ability to evolve with the changes in the early atmosphere on Earth.

Both EX-H1T and EX-H2T were obtained from similar environments, namely sulfide chimney structures from deep-sea hydrothermal vent fields, yet they differ physiologically and phylogenetically. The isolates differ from each other in their 16S rRNA sequences (96% similarity), their DNA G+C contents (mol%), their optimum temperatures for growth (73 and 70 °C respectively), their oxygen tolerances (which are slightly different), their doubling times, and their abilities to use sulfur as an electron acceptor (only EX-H1T has this ability). Whether these differences are a reflection of the environments from which they were isolated or a biogeographical difference needs to be assessed. However, the chemistry of the hydrothermal fluids and the mineralogy of the rocks at 9°N and Guaymas Basin differ significantly. The hydrothermal fluids of Guaymas Basin have higher pH values (pH 5.9), whereas the end-member hydrothermal fluids at 9°N are below pH 3.5. Ammonia concentrations are higher in Guaymas Basin hydrothermal fluids, whereas the sulfide concentrations are lower than those measured for fluids from 9°N (Von Damm et al., 1995; Von Damm, 2000). These differences result in very different mineralogy of the sulfides. Common minerals in Guaymas Basin sulfides are pyrrhotite, barite and chalcopyrite, whereas 9°N sulfides are rich in pyrite, chalcopyrite and anhydrite. Additionally, these chemical and mineralogical differences may select for distinctly different microbial populations.

The novel thermophilic isolates form a distinct bacterial lineage within the Aquificales (Fig. 2) and may represent a novel family. Unlike the hyperthermophile A. pyrophilus whose optimum temperature for growth is 85 °C, EX-H1T and EX-H2T are moderate thermophiles and share comparable optimal growth temperatures with other moderate thermophilic Aquificales such as Hydrogenobacter and Calderobacterium. On the basis of the phylogenetic position of EX-H1T and EX-H2T and their distinct physiological characteristics, we propose that these isolates belong to a new genus Persephonella, where Persephonella marina is the type species of this genus. We further propose that EX-H1T is the type strain of Persephonella marina and EX-H2T is the type strain of a novel species Persephonella guaymasensis.

While this manuscript was under revision, the description of Hydrogenothermus and the proposal of name changes within the Aquificales were published (Stoehr et al., 2001). Consequently, we included information regarding this new description that was critical to the evaluation of our proposed genus.

**Description of Persephonella gen. nov.**

*Persephonella* (per.se.pho.nel’la. Gr. fem. n. Persephonella little Persephone, after the Greek mythological goddess, who spent half of each year in the Underworld and the other half on Earth).

Cells are short, motile rods (0.3–0.4 μm × 2–4 μm), arranged singly or in pairs. Growth is fastest at 70–73 °C and growth occurs at 55–80 °C. The salinity at which growth was optimal is 2.5%, and the salinity range for growth is 1–4.5%. The pH range is 4.7–7.5, the fastest growth occurring at pH 6. Microaerophilic. Obligately chemolithoautotrophic. H\(_2\), S\(^0\) or S\(_2\)O\(_3\)^2– can serve as an electron donor, and O\(_2\), NO\(_3\), or S\(^0\) can serve as an electron acceptor. 16S rRNA phylogeny indicates that the genus *Persephonella* belongs to the order Aquificales. Isolated from deep-sea hydrothermal vent chimneys. The type species is *Persephonella marina*.

**Description of Persephonella marina sp. nov.**

*Persephonella marina* (ma.ri’na. L. fem. adj. marina from the sea).

Short, motile rods (0.3–0.4 μm × 2–4 μm), arranged singly or in pairs. The best growth is observed at 73 °C, the growth range being 55–80 °C. Grows fastest at pH 6; growth occurs at pH 4.7–7.5. The optimum salinity is 2.5% (w/v) and growth occurs between 1 and 4.5% NaCl. Growth is inhibited by 9% (v/v) oxygen. Doubling time of 5.02 h (with NO\(_3\) as electron acceptor and H\(_2\) as electron donor). Chemolithoautotrophic. Utilizes H\(_2\), thiosulfate or S\(^0\) as an electron donor, and O\(_2\), NO\(_3\) or S\(^0\) as an electron acceptor. N\(_2\) is formed from NO\(_3\)\(^–\), and H\(_2\)S is formed from S\(^0\). The G+C
content of the DNA is 38.5 mol\%. Isolated from a deep-sea hydrothermal vent chimney at 9° N 104° W on the East Pacific Rise. The type strain is EX-H1T (= DSM 14350T = OCM 794T). The GenBank accession number for the 16S rRNA sequence for EX-H1T is AF188332.

Description of *Persephonella guaymasensis* sp. nov.

*Persephonella guaymasensis* (gua.y.mas.en’sis. N.L. gen. n. guaymasensis of the Guaymas Basin, Mexico, to depict the place of isolation).

Short, motile rods 0.3–0.4 \( \mu m \) and 2–4 \( \mu m \) long. Grows fastest at 70 °C; growth occurs at 55–75 °C. The optimal pH for growth is 6, the growth range being at pH 4.7–7.5. Grows fastest at 2.5% NaCl (w/v) and between 1 and 4.5% NaCl. Capable of growth at 11% \( O_2 \) (v/v). Doubling time of 7.8 h (with \( NO_3^- \) as the electron acceptor and \( H_2 \) as the electron donor). Utilizes \( H_2 \), thiosulfate or \( S_2O_3^- \) as an electron donor and \( O_2 \) or \( NO_3^- \) as an electron acceptor. \( N_2 \) is formed from \( NO_2^- \). The G+C content of the DNA is 37.4 mol\%. Isolated from a deep-sea hydrothermal vent chimney in Guaymas Basin, Mexico (27° N 111° W). The type strain is *Persephonella guaymasensis* EX-H2T (= DSM 14351T = OCM 975T). The GenBank accession number for the 16S rRNA sequence for EX-H2T is AF385630.

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