Phorcysia thermohydrogeniphila gen. nov., sp. nov., a thermophilic, chemolithoautotrophic, nitrate-ammonifying bacterium from a deep-sea hydrothermal vent

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A novel hyperthermophilic, anaerobic, chemolithoautotrophic bacterium, designated strain HB-8T, was isolated from the tube of Alvinella pompejana tubeworms collected from the wall of an actively venting sulfide structure on the East Pacific Rise at 13° N. The cells were Gram-negative rods, approximately 1.0–1.5 μm long and 0.5 μm wide. Strain HB-8T grew between 65 and 80 °C (optimum 75 °C), 15 and 35 g NaCl l⁻¹ (optimum 30 g l⁻¹) and pH 4.5 and 8.5 (optimum pH 6.0). Generation time under optimal conditions was 26 min. Growth occurred under chemolithoautotrophic conditions with H₂ as the energy source and CO₂ as the carbon source. Nitrate and sulfur were used as electron acceptors, with concomitant formation of ammonium or hydrogen sulfide, respectively. The presence of lactate, formate, acetate or tryptone in the culture medium inhibited growth. The G+C content of the genomic DNA was 47.8 mol%. Phylogenetic analysis of the 16S rRNA gene and of the alpha subunit of the ATP citrate lyase of strain HB-8T indicated that this organism formed a novel lineage within the class Aquificae, equally distant from the type strains of the type species of the three genera that represent the family Desulfitubacteriaceae: Thermovibrio ruber ED11/3LLK8T, Balnearius lithothrophicum 175T and Desulfitubacterium thermodihydrophothrophum BSA1T. The polar lipids of strain HB-8T differed substantially from those of other members of the Desulfitubacteriaceae, and this bacterium produced novel quinones. On the basis of phylogenetic, physiological and chemotaxonomic characteristics, it is proposed that the organism represents a novel genus and species within the family Desulfitubacteriaceae, Phorcysia thermohydrogeniphila gen. nov., sp. nov. The type strain of Phorcysia thermohydrogeniphila is HB-8T (=DSM 24425T =JCM 17384T).

The phylum ‘Aquificae’ is generally considered, based on phylogenetic analyses of rRNA genes and concatenated monophyletic protein genes, to be one of the deepest- and earliest-branching phyla within the ‘Bacteria’ (Battistuzzi et al., 2004; Reysenbach, 2001a). Cultivated representatives of the phylum ‘Aquificae’ are thermophilic or hyperthermophilic bacteria isolated from marine and continental geothermal environments (Reysenbach, 2001a). This phylum includes three families (Aquificae, Desulfitubacteriaceae and Hydrogenothermaceae) within the single order Aquificales (Burggraf et al., 1992; L’Haridon et al., 2006; Reysenbach, 2001b). The family Aquificae is composed of five genera, Hydrogenobacter, Aquifex, Thermococcus, Hydrogenobaculum and Hydrogenivirga (Huber et al., 1998, 1992; Kawasumi et al., 1984; Nakagawa et al., 2004; Stöhr et al., 2001). The family Hydrogenothermaceae (Eder & Huber, 2002) is formed by the genera Hydrogenothermus, Persephonella and Sulfurimonas (Götz et al., 2002; Stöhr et al., 2001; Takai et al., 2003a). The family Desulfitubacteriaceae (L’Haridon et al., 2006) includes the genera Desulfitubacterium, Thermovibrio and Balnearius (Huber et al., 2002; L’Haridon et al., 1998; Takai et al., 2003b). All the members of the family Desulfitubacteriaceae are extremely

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and partial aclA gene sequence of strain HB-8T are HQ336972 and JN118840, respectively.

Five supplementary figures and a supplementary table are available with the online version of this paper.
thermophilic, strictly chemolithoautotrophic anaerobes that couple hydrogen oxidation with the reduction of sulfur, sulfite, thiosulfate and/or nitrate, and have been isolated from deep-sea hydrothermal vents (Alain et al., 2003; Huber et al., 2002; L’Haridon et al., 1998, 2006; Takai et al., 2003b; Vetriani et al., 2004).

In this study, we report the isolation and characterization of a strain that represents a novel lineage within the Desulfurobacteriaceae. This strain is a thermophilic, chemolithoautotrophic, strictly anaerobic, hydrogen-oxidizing bacterium that was isolated from an active deep-sea hydrothermal vent on the East Pacific Rise.

Samples of microbial biofilms were collected during R/V Atlantis cruise AT 15-28 (January 2008) from an experimental microbial colonization device that was deployed on the wall of a diffuse-flow-venturing sulfide structure colonized by the tubeworm Alvinella pompejana (‘Jumeaux’ site; depth 2621 m, East Pacific Rise, 12° 48′ N 103° 56′ W). The experimental microbial colonization device was deployed using the manipulator of the DSV Alvin, collected 3 days later and stored in a box on the submersible’s working platform for the rest of the dive. At the time of recovery, white microbial biofilms covered the colonization device and a few tubes of the tubeworm Alvinella pompejana that were associated with the device. Subsamples were transferred to the ship’s laboratory and stored at 4°C under a dinitrogen atmosphere. Primary enrichment cultures were initiated shipboard by inoculating a fragment of a biofilm-covered A. pompejana tube into 10 ml modified SME medium (Stetter et al., 1983; Vetriani et al., 2004). The primary enrichments were incubated shipboard at 75°C. Back in the laboratory, aliquots (0.1 ml) of the original cultures were subsequently transferred to fresh medium and pure cultures were isolated by several consecutive series of dilutions to extinction. During the isolation procedures, the cultures were incubated at 75°C. The pure culture obtained using this procedure was designated strain HB-8T. Long-term stocks were prepared by adding 50 μl DMSO (Fisher Scientific) to 1 ml culture and were stored at −80°C.

Cells were routinely stained with 0.1% acidine orange and visualized with an Olympus BX 60 microscope with an oil-immersion objective (UPlanFl 100/1.3). Transmission electron micrographs were obtained as described previously (Vetriani et al., 2004). Biofilm samples were collected from the gas-liquid interphase of batch cultures, fixed in Trump’s EM fixative, washed in 0.2 M cacodylate buffer (EMS), dehydrated in ethanol, subjected to critical-point drying and sputter coating with Au/Pd and visualized on an AMRAY 1830I scanning electron microscope. Cells of strain HB-8T were rod-shaped, approximately 1.0–1.5 μm long and 0.5 μm wide, and divided by constriction (Fig. 1a). Cells stained Gram-negative. The presence of polar flagella was observed in electron micrographs of platinum-shadowed cells (Fig. 1b). Batch cultures of strain HB-8T formed a 5 μm-thick biofilm at the gas-liquid interphase (Fig. 1c) and the presence of endospores was not observed.

Growth rates (μ; h⁻¹) were estimated as μ = (ln N₂ − ln N₁) / (t₂ − t₁), where N₂ and N₁ are numbers of cells per ml at times (in h) t₂ and t₁. Generation times (tₕ; h) were calculated as tₕ = (ln 2)/μ. All growth experiments were carried out, in duplicate, in 10 ml modified SME medium supplemented with 0.2 ml 10% (w/v) potassium nitrate (yielding a final concentration of about 20 mM) under H₂/CO₂ (80:20; 200 kPa) unless stated otherwise. Quantitative determinations of nitrate, nitrite and ammonium were carried out spectrophotometrically using a Lachat QuickChem automated ion analyser according to the manufacturer’s specifications (Diamond, 1993).

The optimal growth temperature for strain HB-8T was determined by incubating cultures at temperatures between 60 and 85°C (at 5°C intervals). Strain HB-8T grew at temperatures between 65 and 80°C, with optimal growth at 75°C. No growth was detected at 60 or 85°C (Fig. S1a, available in IJSEM Online). All other experiments were carried out at 75°C. The optimal salt requirement was determined by varying the concentration of NaCl between 5 and 45 g l⁻¹, at 5 g l⁻¹ intervals. HB-8T grew at NaCl concentrations between 15 and 35 g l⁻¹, with optimal growth at 30 g l⁻¹ (no growth was detected at 10 or 40 g NaCl l⁻¹; Fig. S1b). The optimal pH for growth was determined as described previously (Vetriani et al., 2004). Growth of strain HB-8T occurred between pH 4.5 and 8.5, with an optimum at pH 6.0 (Fig. S1c). Under optimal temperature (75°C), salt (30 g NaCl l⁻¹) and pH (pH 6.0) conditions, the generation time of strain HB-8T was 26 min (Fig. S1c).
Antibiotic resistance was tested in the presence of ampicillin, chloramphenicol, kanamycin and streptomycin (all 100 µg ml⁻¹). All antibiotics were added aseptically before incubation at 75 °C and an ethanol control was performed for chloramphenicol. Strain HB-8ᵀ was resistant to kanamycin and streptomycin, while it was inhibited by chloramphenicol and ampicillin. Strain HB-8ᵀ exhibited catalase activity, detected by the formation of gas bubbles after concentrated cells were resuspended in 70 µl of a 3 % solution of H₂O₂ at room temperature.

The effect of organic substrates on the growth of strain HB-8ᵀ was investigated by adding the following substrates to the medium under a H₂/CO₂ gas phase (80 : 20; 200 kPa): acetate, formate, lactate, peptone, tryptone, Casamino acids, (+)-D-glucose, sucrose (all at 2 g l⁻¹) and yeast extract (0.1 and 1 g l⁻¹). Lactate (2 g l⁻¹), acetate (2 g l⁻¹), formate (2 g l⁻¹) and tryptone (2 g l⁻¹) inhibited growth of HB-8ᵀ under a H₂/CO₂ gas phase. Casamino acids (2 g l⁻¹), glucose (2 g l⁻¹), sucrose (2 g l⁻¹) and yeast extract (1.0 and 0.1 g l⁻¹) did not affect growth of HB-8ᵀ under a H₂/CO₂ gas phase, but no growth was observed in the presence of the same compounds under N₂/CO₂ (80 : 20; 200 kPa). H₂ (100 %; 200 kPa) or N₂ (100 %; 200 kPa) gas phases, indicating that the organic substrates were not used as electron and/or carbon sources.

The ability of strain HB-8ᵀ to use alternative electron acceptors was tested by adding thiosulfate (4 mM), sulfite (4.1 mM), arsenate (5 mM), selenate (5 mM), sulfur (3%, w/v) or oxygen (0.5 %, v/v) to nitrate-depleted medium. Strain HB-8ᵀ did not grow when oxygen, arsenate, selenate, thiosulfate or sulfite were offered as electron acceptors. However, HB-8ᵀ was able to grow with elemental sulfur (3% w/v) as electron acceptor. Under these conditions, the generation time of strain HB-8ᵀ was 8.6 h, and about 4–10 mM hydrogen sulfide [measured as described by Vetriani et al. (2004)] was produced from the reduction of elemental sulfur. The ability of strain HB-8ᵀ to use elemental sulfur (3% w/v) and thiosulfate (4 mM) as electron donors was tested in nitrate-containing medium. However, strain HB-8ᵀ was unable to couple the oxidation of these reduced sulfur species to the reduction of nitrate. Therefore, we concluded that strain HB-8ᵀ was a strictly anaerobic, chemolithoautotrophic bacterium that used nitrate, hydrogen and carbon dioxide as the primary electron acceptor, electron donor and carbon source, respectively. Time-course measurements of nitrate, nitrite and ammonium and concomitant growth (carried out in 1 l SME medium supplemented with 35 mM potassium nitrate) showed that nitrate was reduced to ammonium and that nitrite did not accumulate in the culture medium (Fig. S2).

Genomic DNA was extracted from cells of strain HB-8ᵀ, and the 16S rRNA gene and a 909 bp fragment of the aclA gene (encoding the alpha subunit of ATP citrate lyase) were selectively amplified from the genomic DNA by PCR and sequenced as described previously (Vetriani et al., 1999, 2004; Voordeckers et al., 2008). Nucleotide sequences of the 16S rRNA gene and amino acid sequences derived from the aclA gene sequence were aligned automatically using CLUSTAL_X and the alignments were refined manually using S E A V E W (Galtier et al., 1996; Thompson et al., 1997).

Neighbour-joining trees were constructed as described previously (Pérez-Rodríguez et al., 2010; Voordeckers et al., 2008). Maximum-likelihood trees were constructed using PhyML (Gouy et al., 2010) and the Jukes and Cantor nucleotide substitution model for the 16S rRNA gene (Jukes & Cantor, 1969) or the LG amino acid substitution model for ATP citrate lyase (Le & Gascuel, 2008). Pairwise nucleotide similarity values were calculated using the EzTaxon web-based tool (http://www.eztaxon.org/). The DNA G+C content of HB-8ᵀ was determined by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) by HPLC analysis of deoxyribonucleosides as described by Mesbah et al. (1989).

![Fig. 2. Maximum-likelihood phylogenetic tree inferred from 16S rRNA gene sequences showing the position of Phorcysia thermohydrogeniphiphila gen. nov., sp. nov. HB-8ᵀ relative to representative strains of the family Desulfurobacteriaceae. Bootstrap values based on 100 replications are shown at branch nodes. Bar, 0.1 % substitutions.](image-url)
Phylogenetic analysis of the 16S rRNA gene sequence placed strain HB-8\(^T\) within the class Aquificae (Fig. 2). Both maximum-likelihood and neighbour-joining phylogenetic analyses showed that strain HB-8\(^T\) represented a novel phylogenetic lineage related to members of the family Desulfurobacteriaceae (Figs 2 and S3). However, the two methods of phylogenetic inference provided slightly different results: while the maximum-likelihood method of tree reconstruction placed strain HB-8\(^T\) consistently within the family Desulfurobacteriaceae (Fig. 2), the neighbour-joining tree suggested that the novel lineage diverged prior to the differentiation of its closest cultured relatives (Fig. S3). In line with the phylogenetic analyses, pairwise nucleotide similarity values indicated that the 16S rRNA gene from strain HB-8\(^T\) had a similar degree of identity to those from representatives of the three genera of the Desulfurobacteriaceae. The identity values were 95.5\% to Thermovibrio ruber ED11/13LLK\(^T\), 95.4\% to Balneamicrobium lithotrophicum 175\(^T\) and 94.9\% to Desulfurobacterium thermolithotrophum BSA\(^T\), all within the range (90–96\%) accepted as an indication of genus-level differentiation (Gillis et al., 2001).

Since members of the family Desulfurobacteriaceae are known to fix CO\(_2\) via the reverse citric acid cycle (Hügler et al., 2007; L’Haridon et al., 2006), we further explored the phylogeny of the ATP citrate lyase of strain HB-8\(^T\), a key enzyme in this cycle. Both maximum-likelihood and neighbour-joining phylogenetic analyses of the amino acid sequence derived from the aclA gene sequence placed the ATP citrate lyase of strain HB-8\(^T\) consistently on a discrete lineage (Figs 3 and S4). When we compared the intragenus identities of the amino acid sequences of the ATP citrate lyase of members of the family Desulfurobacteriaceae, we found that the sequences of the enzymes of strains within the genus Desulfurobacterium were 98–99\% identical. Intergenus comparisons of the ATP citrate lyase sequences of T. ruber and B. lithotrophicum, T. ruber and D. thermolithotrophum and B. lithotrophicum and D. thermolithotrophum showed 96, 90 and 90 \% identity, respectively. However, the ATP citrate lyase of strain HB-8\(^T\) was 89, 87 and 91\% identical to those of the type strains of T. ruber, B. lithotrophicum and D. thermolithotrophum, respectively, which is well below the observed intragenus range (98–99\%).

The genomic DNA G+C content of strain HB-8\(^T\), determined by HPLC analysis of the deoxyribonucleosides, was 47.8 mol\%, which was similar to that of T. ruber but higher than that of B. lithotrophicum and D. thermolithotrophum (Table 1).

Chemotaxonomic analyses of strain HB-8\(^T\), which included cellular fatty acid composition, polar lipids and respiratory quinones, were carried out by the Identification Service and Dr Brian Tindall (DSMZ) on 200 mg freeze-dried cells grown to early stationary phase under optimal culture conditions by the authors. The major cellular fatty acids of strain HB-8\(^T\) were analysed as the methyl ester derivatives using the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID) and an Agilent model 6890N GC

### Table 1. Differentiating features of Phorcysia thermohydrogeniphila gen. nov., sp. nov. HB-8\(^T\) and the type strains of Thermovibrio ruber, Balneamicrobium lithotrophicum and Desulfurobacterium thermolithotrophum

<table>
<thead>
<tr>
<th>Characteristic</th>
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<td>Range</td>
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<td>20–47</td>
<td>8–56</td>
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<tr>
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<td>30</td>
<td>32</td>
<td>30</td>
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<tr>
<td>pH for growth</td>
<td>Range</td>
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<td>5–7</td>
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<td>6</td>
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<td>+</td>
<td>–</td>
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<tr>
<td></td>
<td>Sulfite</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Thiosulfate</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Shortest generation time (min)</td>
<td>26</td>
<td>60</td>
<td>80</td>
<td>135</td>
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</table>

Strains: 1, Phorcysia thermohydrogeniphila sp. nov. HB-8\(^T\) (data from this study); 2, T. ruber ED11/13LLK\(^T\) (data from Huber et al., 2002); 3, B. lithotrophicum 175\(^T\) (Takai et al., 2003b); 4, D. thermolithotrophum BSA\(^T\) (L’Haridon et al., 1998). All four strains use sulfur as an electron acceptor.

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Phorcysia thermohydrogeniphila gen. nov., sp. nov.

Fig. 3. Maximum-likelihood phylogenetic tree inferred from amino acid sequences deduced from the nucleotide sequence of a fragment of the aclA gene (encoding the alpha subunit of the ATP citrate lyase), showing the position of Phorcysia thermohydrogeniphila gen. nov., sp. nov. HB-8\(^T\) relative to representative strains of the families Desulfurobacteriaceae and Hydrogenothermaceae. Bootstrap values based on 100 replications are shown at branch nodes. Bar, 5% estimated substitutions.
The two predominant polar lipids of strain HB-8T, identified by their staining behaviour on TLC plates (Tindall, 1990a, b), were phosphatidylethanolamine and phosphatidylglycerol, while minor phospholipids included phosphatidylglycerol and phosphatidylaminopentanetetrol (Fig. S5). The polar lipid profile of strain HB-8T differentiated this bacterium from other members of the family Desulfovibrioaceae, whose major polar lipids were shown to be phosphatidylglycerol and phosphatidylaminopentanetetrol (L’Haridon et al., 2006).

Analysis of the respiratory lipoquinones of strain HB-8T by TLC followed by HPLC of the eluted products (Tindall, 1990a, b) revealed that strain HB-8T synthesized novel quinones, unlike any known references. The retention times of the quinones of strain HB-8T were significantly longer than those of any of the quinones analysed to date from members of the Aquificales (B. J. Tindall, personal communication). This indicates that the isoprenoid side chains of the novel quinones are likely to be longer than those of the quinones previously identified in other members of the Aquificales.

When compared with its closest relatives T. ruber, B. lithotrophicum and D. thermolithotrophum, strain HB-8T showed the shortest generation time, the broadest pH range and the highest DNA G+C content (Table 1). Furthermore, strain HB-8T differed from B. lithotrophicum and D. thermolithotrophum in its ability to use nitrate and from D. thermolithotrophum in its inability to use sulfate and thiosulfate as alternative electron acceptors (Table 1).

Phylogenetic analyses of both the 16S rRNA gene and the ATP citrate lyase of strain HB-8T, along with its unique polar lipid and lipoquinone profiles, reinforce the differences between this strain and other members of the family Desulfovibrioaceae. Overall, the phylogenetic, physiological and chemotaxonomic characteristics of strain HB-8T suggest that this bacterium can be classified in a novel genus, for which we propose the name Phorcysia gen. nov.

The type species is Phorcysia thermohydrogeniphila sp. nov.

Thermophilic, strictly anaerobic, hydrogen-oxidizing chemolithoautotrophs such as P. thermohydrogeniphila are likely to be important primary producers in high-temperature deep-sea hydrothermal vents. The respiratory metabolism of P. thermohydrogeniphila, along with that of other recently isolated chemolithoautotrophs (Miroshnichenko & Balnearium, 2006), suggests a widespread distribution of nitrate respiration in microorganisms from deep-sea hydrothermal vents. In particular, the very short generation time that we observed for P. thermohydrogeniphila, along with its ability to form biofilms and to grow chemolithoautotrophically by coupling hydrogen oxidation to nitrate or sulfur reduction, makes it extremely well adapted to the conditions found in active, high-temperature deep-sea vents.

Description of Phorcysia gen. nov.

Phorcysia (Phor.cys’ia. N.L. fem. n. Phorcy sia named after Phorcys, son of Neptune, father of Medusa and the other Gorgons, who was changed after death into a sea-god, referring to the habitat of the type species).

Cells are rod-shaped with several polar flagella. Cells stain Gram-negative and do not form spores. Thermophilic, strictly anaerobic and chemolithoautotrophic. Growth by reduction of nitrate and formation of ammonium. Major cellular fatty acids are C₁₈:₁₀7c and C₁₈:₀ and the predominant polar lipids are phosphatidylethanolamine and phosphatidylglycerol. On the basis of phylogenetic analyses of the 16S rRNA gene and the ATP citrate lyase, the genus Phorcysia forms a separate lineage with the phylum Aquificae, and is related to the genera Thermovibrio, Desulfovibrio and Balnearium. Known members of the genus Phorcysia inhabit marine geothermal environments. The type species of the genus is Phorcysia thermohydrogeniphila.

Description of Phorcysia thermohydrogeniphila sp. nov.

Phorcysia thermohydrogeniphila [ther. mo. hy. dro. ge. ni’ phi. la. Gr. fem. n. thermē heat; N.L. n. hydrogenum hydrogen; N.L. fem. adj. philus -a -um (from Gr. adj. philos -ē -on) friend, loving; N.L. fem. adj. thermohydrogeniphila heat- and hydrogen-liking, referring to its ability to grow lithotrophically on H₂ at elevated temperature].

Displays the following properties in addition to those given for the genus. Cells are 1.0–1.5 μm long and 0.5 μm wide. Catalase-positive. Growth occurs between 65 and 80 °C, 15 and 35 g NaCl l⁻¹ and pH 4.5 and 8.5. Optimal growth conditions are 75 °C, 30 g NaCl l⁻¹ and pH 6.0 (shortest generation time 26 min). Growth occurs in the presence of H₂ and CO₂ with nitrate or elemental sulfur as electron acceptors and the formation of ammonium and hydrogen sulfide, respectively. The following are not utilized as electron acceptors: oxygen, arsenate, selenate, thiosulfate and sulfite. Acetate, lactate and formate inhibit growth under a H₂/CO₂ gas phase. No chemo-organotrophic growth occurs in the presence of Casamino acids, glucose, sucrose or yeast extract.

The genomic DNA G+C content of the type strain is 47.8 mol%. Major cellular fatty acids are C₁₈:₁₀7c and C₁₈:₀, with smaller amounts of C₁₆:₀ and C₂₀:₁₀7c. Resistant to kanamycin and streptomycin; sensitive to chloramphenicol and ampicillin (each at 100 mg ml⁻¹).

The type strain is HB-8T (=DSM 24425T =JCM 17384T), which was isolated from an active deep-sea hydrothermal vent on the East Pacific Rise at 12° 48′ N 103° 56′ W.
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