Exilispira thermophila gen. nov., sp. nov., an anaerobic, thermophilic spirochaete isolated from a deep-sea hydrothermal vent chimney

Hiroyuki Imachi,¹ Sanae Sakai,^{1,2} Hisako Hirayama,¹ Satoshi Nakagawa,¹ Takuro Nunoura,¹ Ken Takai¹ and Koki Horikoshi¹

Correspondence

Hiroyuki Imachi
imachi@jamstec.go.jp

¹Subground Animalcule Retrieval (SUGAR) Program, Extremobiosphere Research Center, Japan Agency for Marine–Earth Science and Technology (JAMSTEC), Yokosuka, Kanagawa 237-0061, Japan

²Department of Environmental Systems Engineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2188, Japan

A novel thermophilic, anaerobic bacterium, strain RASEN^T, was isolated from a deep-sea hydrothermal vent chimney sample collected in the Iheya North field, Okinawa Trough, Japan, at a water depth of 982 m. The cells were motile, Gram-negative and helical with hooked ends, 0.23–0.28×15–27 µm in size with an approximate wavelength of 1.1–1.5 µm. Growth of the strain was observed at 37–60 °C (optimum 50 °C), in 2.5–3.5 % (w/v) NaCl (optimum 2.5–3 % NaCl) and at pH 6.0–7.5 (optimum pH 7.0). The strain grew on yeast extract only of the substrates examined in this study. The G + C content of the genomic DNA was 27.1 mol%. Major fatty acids for the strain were C_{16:0}, C_{18:1}(Δ9) *trans*, C_{18:0} and C_{18:1}(Δ9) *cis*. Based on comparative 16S rRNA gene sequence analysis, strain RASEN^T formed a deeply branching lineage within the phylum *Spirochaetes* and had only low levels of sequence similarity with other species of the phylum (range of similarity 72.1–80.6 %). Hence, we propose the name *Exilispira thermophila* gen. nov., sp. nov. The type strain of *Exilispira thermophila* is strain RASEN^T (=JCM 14728^T =NBRC 103205^T =KCTC 5595^T).

Members of the phylum *Spirochaetes* are Gram-negative, helical bacteria with free-living or host-associated lifestyles, some of which are pathogenic to man and other animals (Holt *et al.*, 1994; Paster & Dewhirst, 2000). They are widespread in association with animals and in natural environments, including extreme environments like the deep sea. Several rRNA gene sequences phylogenetically associated with the phylum *Spirochaetes* have been detected directly from deep-sea environments (e.g. Campbell & Cary, 2001; Inagaki *et al.*, 2006). However, only a single isolate, strain GS-2, possibly belonging to the genus *Spirochaeta*, has been obtained, from a deep-sea hydrothermal vent habitat in the Galapagos Rift (Harwood *et al.*, 1982). According to the report (Harwood *et al.*, 1982), the morphology and physiological properties of strain GS-2 resemble those of members of genus *Spirochaeta*, and thus the strain was identified as a possible member of the genus *Spirochaeta*. However, the phylogenetic position of strain GS-2 was not determined, and the strain has not been deposited in any culture collection and is no longer available.

We have successfully isolated an anaerobic, thermophilic spirochaete, designated strain RASEN^T, from a piece of hydrothermal vent chimney structure obtained from a deep-sea hydrothermal field in the Iheya North field, Okinawa Trough, Japan (27° 47.451' N 126° 53.803' E), at a depth of 982 m, via the remotely operated vehicle *Hyper-Dolphin* (cruise NT05-03, dive no. 392, 15 April 2005). Thermophiles are rare in the phylum *Spirochaetes*, and only two species have been characterized so far, *Spirochaeta thermophila* (Aksenova *et al.*, 1992; Rainey *et al.*, 1991), isolated from a marine hot spring, and *Spirochaeta caldaria* (Pohlschroeder *et al.*, 1994), obtained from a freshwater hot spring. In this report, we describe the detailed morphological, physiological characteristics of strain RASEN^T and propose a novel species of a new genus for this strain. This is the first formal description of an isolate of the phylum *Spirochaetes* from the deep-sea environment.

The vent chimney sample was brought to the sea surface in a sample box. Immediately after the sample was retrieved from the vehicle, a portion of the fractured chimney was suspended with 20 ml MJASi synthetic seawater medium (described below) under an atmosphere of N₂/CO₂ (80 : 20 v/v). The suspended sample was used for the primary

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enrichment culture. MJASi medium was used for enrichment, isolation and maintenance of strain RASEN^T; the medium consisted of the following (l⁻¹): NaCl, 30 g; K₂HPO₄, 0.09 g; KH₂PO₄, 0.07 g; CaCl₂·2H₂O, 1.06 g; MgCl₂·6H₂O, 4.18 g; KCl, 0.33 g; NH₄Cl, 0.535 g; NaHCO₃, 2 g; Na₂S·9H₂O, 0.3 g; cysteine hydrochloride, 0.3 g; trace element solution, 10 ml; vitamin solution (Balch *et al.*, 1979), 10 ml; and resazurin solution (1 ml; 1 mg ml⁻¹). The trace element solution contained (l⁻¹): nitrilotriacetic acid, 0.15 g; MnCl₂·4H₂O, 0.53 g; CoCl₂·6H₂O, 0.42 g; ZnCl₂, 0.09 g; CuCl₂·2H₂O, 0.007 g; AlCl₃, 0.006 g; H₃BO₃, 0.01 g; Na₂MoO₄·2H₂O, 0.001 g; SrCl₂·6H₂O, 0.01 g; NaBr, 0.01 g; KI, 0.01 g; FeCl₂·4H₂O, 0.1 g; NiCl₂·6H₂O, 0.025 g; Na₂SeO₃, 0.0002 g; and Na₂WO₄·2H₂O, 0.0003 g. Enrichment cultures were incubated anaerobically at 55 °C. Isolation was done using a roll-tube method as described below. For this method, solid medium was prepared by adding purified agar (agar noble; Difco) to the MJASi medium at a final concentration of 20 g l⁻¹. After isolation of the strain, all cultivations were performed at 50 °C in 50 ml serum vials containing 20 ml medium (pH at 25 °C, 7.2) under an atmosphere of N₂/CO₂ (80:20 v/v) without shaking, unless mentioned otherwise. The serum vials were sealed with butyl rubber stoppers and aluminium crimp seals.

An aliquot (1 ml) of the suspended slurry that had been passed through a 0.2 µm-pore-size cellulose acetate filter disc (DISMIC-13CP; Advantec) was inoculated into MJASi medium containing 0.1 % yeast extract and 10 mM sucrose. The culture was incubated anaerobically at 55 °C. Cell growth was observed after 2 months of incubation in the primary enrichment culture, but growth was obtained within 2–4 days of incubation in subsequent cultures (2.5 % v/v inoculum). After six successive transfers, we conducted roll-tube isolation. Small colonies that were yellowish, lens-shaped and approximately 0.5 mm in diameter were formed after 1 month of incubation. This roll-tube isolation was repeated twice, and the purified strain, designated strain RASEN^T, was obtained. The isolated strain did not utilize sucrose; consequently we cultured it in medium with 0.1 % yeast extract as sole energy source for further physiological and genetic studies.

Growth and substrate utilization were determined by direct cell counts after staining with 4',6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980) and monitoring the increase in turbidity by optical density at 400 nm. Neutralized substrates were added to vials containing basal medium from stock solutions prior to inoculation. All incubations for substrate tests were performed using growing cultures (10 % v/v inoculum) at 50 °C for over 2 weeks. Effects of pH, temperature and NaCl concentration on growth of strain RASEN^T were determined in cultures grown on 0.1 % yeast extract (Difco) medium and inoculated with cells in the exponential growth phase. For determination of the optimum pH for growth, the pH of

the yeast extract medium was adjusted at room temperature to 5.5–8.5 with HCl or NaOH solutions under a 100 % N₂ atmosphere prior to inoculation. During the incubation, the pH of the medium was monitored routinely. To determine the temperature range for growth, cultures were incubated at 25–80 °C (pH 7.2). NaCl requirements were determined with varying concentrations from 0 to 6 % (w/v) NaCl in MJASi medium. Effects of antibiotics on growth were evaluated by supplementing the medium with each antibiotic at a final concentration of 50 µg ml⁻¹. All measurements were performed in triplicate, and the specific growth rate was calculated based on direct cell counts with DAPI staining.

Cell morphology was examined under a fluorescence microscope (Olympus BX51F) with a colour CCD camera system (Olympus DP71). The Gram-staining reaction was performed by Hucker's method (Doetsch, 1981). Transmission electron microscopy of negatively stained cells was carried out as described by Zillig *et al.* (1990). Cells grown at 50 °C in the exponential growth phase were negatively stained with 2 % (w/v) uranyl acetate and observed under a JEOL JEM-1210 electron microscope. For ultrathin sectioning, cells were prefixed with 4 % para-formaldehyde in MJASi medium at 50 °C for 2 h and then post-fixed in 2 % osmium tetroxide in 0.1 M PBS at 4 °C for 2 h. The fixed cells were stained with 1 % uranyl acetate for 90 min at 4 °C, dehydrated in a graded ethanol series and embedded in Epon 812 (TAAB). Cells were sectioned with an ultramicrotome (UltracutS; Leica) and observed using a JEOL JEM-1210 electron microscope.

The G + C content of the genomic DNA was determined by HPLC as described previously (Nakagawa *et al.*, 2003). Fatty acids of the cells were converted to methyl esters using HCl/methanol (Takai *et al.*, 2003) and identified by GC-MS (TRACE GC ULTRA + DSQ system; Thermo electron). Comparative 16S rRNA gene sequence analysis was performed as described elsewhere (Imachi *et al.*, 2006). Bootstrap resampling analysis for 1000 replicates was performed with the neighbour-joining, maximum-parsimony and maximum-likelihood methods to estimate the confidence of tree topologies as described previously (Sekiguchi *et al.*, 2006). 16S rRNA gene sequence similarities were calculated using the Calculate Matrix function of the ARB program with the Jukes and Cantor correction (Jukes & Cantor, 1969; Ludwig *et al.*, 2004). Carbohydrates such as sucrose and glucose were determined by HPLC using an SCR101-H column (Shimadzu; eluent, H₂O; column temperature, 60 °C) and a refractive index detector (Shimadzu RID-10A). Short-chain fatty acids, alcohols, H₂, carbon dioxide and other substances such as succinate, fumarate and lactate were measured as described previously (Imachi *et al.*, 2006).

Cells of strain RASEN^T were helical and 0.23–0.28 × 15–27 µm in size, with a wavelength of 1.1–1.5 µm (Fig. 1a, b). The cells had hooked ends and often formed spherical bodies in late-exponential phase cultures (Fig. 1a). Electron

Fig. 1. Micrographs of strain RASEN^T. (a) Phase-contrast micrograph of cells grown on yeast extract medium. The cells have hooked ends. Arrows indicate spherical bodies. Bar, 20 μ m. (b) Transmission electron micrographs of a negatively stained cell with periplasmic flagella (PF). Bar, 1 μ m. (c) Cross-section of cells, showing the outer membrane (OM), two periplasmic flagella (PF) and the peptidoglycan–cytoplasmic membrane complex (P-CM). Bar, 100 nm.

microscopy revealed that the strain possessed a periplasmic space containing two periplasmic flagella, a peptidoglycan–cytoplasmic membrane complex and an outer membrane (Fig. 1b, c). The arrangement of periplasmic flagella was 1–2–1 (Fig. 1b, c). The cells were highly motile, with wavy movement and/or rotation. The cells were Gram-reaction

negative and did not pass through a 0.2 μ m pore-size filter after isolation of the strain, suggesting that the cells might have a larger diameter under artificial cultivation conditions.

The strain was strictly anaerobic; growth was inhibited completely in the presence of trace quantities of oxygen (0.1% v/v O₂). We tested many substrates, but the strain grew only on yeast extract. The major end products from yeast extract were hydrogen and acetate. Other end products were also detected in the liquid phase, but they were minor components that were not identifiable by HPLC. The following substrates did not support growth with or without 0.01% yeast extract (substrates added at a final concentration of 20 mM unless indicated): Casamino acids (0.1%), tryptone (0.1%), peptone (0.1%), crotonate, H₂/CO₂ (80:20 v/v; ~100 kPa in the headspace) plus acetate (1 mM), betaine (10 mM), glucose, sucrose, arabinose, fructose, galactose, raffinose, xylose, starch (5 g l⁻¹), pectin (5 g l⁻¹), maltose, cellobiose, lactate, formate plus acetate (1 mM), acetate, propionate, butyrate, malate, fumarate, succinate, pyruvate, benzoate (5 mM), ethanol (10 mM), trimethylamine (10 mM), glycerol (5 mM), *N*-acetylglucosamine (5 mM), chitin (4 g l⁻¹), amino acids (all at final concentrations of 10 mM: alanine, arginine, asparagine, cystine, glutamine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine). In addition, the following inorganic substances were not reduced in the presence of 0.1% yeast extract: sulphate, sulphite (2 mM), thiosulphite and elemental sulphur. No Stickland reaction was observed, using glycine or betaine as electron acceptors and alanine, valine, leucine or isoleucine as electron donors.

Strain RASEN^T grew between 37 and 60 °C with optimum growth at 50 °C. The pH range for growth of the strain was estimated to be pH 6.0–7.5, with optimum growth at pH 7.0. The optimum NaCl concentration for growth was 2.5–3% (w/v). No growth occurred below 2.0 or above 4.0% NaCl. Under the optimum conditions (pH 7.0, 50 °C, 3% NaCl), the cell density was approximately 1 $\times$ 10⁸ cells ml⁻¹ and the doubling time was about 3.5 h. To test the effect of yeast extract concentration on the growth of strain RASEN^T, we cultivated the strain in medium with 0.05–2% yeast extract. However, no significant difference in growth rate or yield was observed.

Strain RASEN^T tolerated ampicillin, but chloramphenicol, kanamycin, neomycin, rifampicin and vancomycin inhibited cell growth completely. The DNA G+C content of RASEN^T was 27.1 mol%. Fatty acid methyl ester analysis showed that the strain contained C_{16:0} (29%), C_{18:1}(Δ 9) *trans* (22%), C_{18:0} (14%) and C_{18:1}(Δ 9) *cis* (14%) as the major fatty acids; minor fatty acids were C_{16:1} (7%), iso-C_{17:0} (3%), C_{22:1} (3%), C_{18:0} 2-OH (2%), C_{14:0} (2%), C_{20:0} (1%), C_{17:0} (1%), C_{19:0} (1%) and C_{15:0} (1%).

For strain RASEN^T, 1456 bp of the 16S rRNA gene sequence was determined. Comparative 16S rRNA gene sequence analysis revealed that strain RASEN^T was

affiliated with a deeply branched lineage of the phylum *Spirochaetes* (Fig. 2). The closest cultured relative of strain RASEN^T was *Spirochaeta africana* (Zhilina *et al.*, 1996) (80.6% 16S rRNA gene sequence similarity to the type strain).

Phenotypic and genetic analyses in this study indicated that strain RASEN^T should be considered a member of the phylum *Spirochaetes*. In particular, the cell morphology and structure of strain RASEN^T are very similar to those observed in almost all *Spirochaetes* species, i.e. the cells are helical and possess the unique cell structure composed of an outer membrane enclosing periplasmic flagella and a peptidoglycan–cytoplasmic membrane complex (Fig. 1). Also, spherical body formation in a late-exponential phase culture (Fig. 1a) has been reported for other members of the genera *Spirochaeta* and *Treponema* (e.g. Fracek & Stolz, 1985; Schrank *et al.*, 1999; Zhilina *et al.*, 1996). Moreover, the cells of strain RASEN^T had hook-shaped ends. Similar cell structures have also been found for members of the genus *Leptospira* (Adler & Faine, 2006; Faine, 1984).

Various physiological features of strain RASEN^T differ from those of other members of the phylum *Spirochaetes*, e.g. sensitivity to oxygen, lifestyle, substrate utilization, optimum growth temperature and resistance to rifampicin (Table 1). Regarding the lifestyle of strain RASEN^T, we consider that the strain is derived from a free-living microbial population in the chimney habitat. The chimney sample for isolation contained no animal individuals and the original inoculum passed through 0.2 µm filtration, indicating no specific physical association with hydrothermal vent animals. However, we cannot exclude the possibility of a symbiotic lifestyle of the strain with vent animals. The most striking physiological feature of strain RASEN^T is its very narrow range of substrate utilization for growth; we only observed growth on yeast extract. This

very limited utilization pattern of heterotrophic substrates has not been reported among other species of the phylum *Spirochaetes*, although substrate utilization patterns have not been determined for members of the genera *Cristispira* and *Brevinema*. Additionally, only one species among the phylum *Spirochaetes*, *Spirochaeta coccoides*, has been reported to grow on yeast extract as a sole energy source (Dröge *et al.*, 2006). Sensitivity to rifampicin is also a unique and important trait that characterizes strain RASEN^T. Almost all species of the phylum *Spirochaetes* tolerate rifampicin, and rifampicin has often been used as a selective agent for isolation of spirochaetes (Norris *et al.*, 2006; Leschine *et al.*, 2006; Adler & Faine, 2006; Stanton, 2006). Moreover, the thermophilic growth exhibited by strain RASEN^T is rare amongst members of the phylum *Spirochaetes*, most of which are mesophiles as discussed earlier.

In addition to the physiological characteristics, the phylogenetic analysis clearly supported the conclusion that strain RASEN^T was distinct from other members of the phylum *Spirochaetes*. Our 16S rRNA gene sequence-based phylogenetic analysis showed that strain RASEN^T formed a deeply branching lineage and had low sequence similarity with other members of the phylum *Spirochaetes* (range of similarity 72.1–80.6%) (Fig. 2). These values strongly suggested that the strain should be a member of a new, independent genus. Additionally, the sequence of strain RASEN^T was distantly related to clones retrieved from deep-sea environments. Almost all these clones were affiliated with the genus *Spirochaeta* (e.g. Campbell & Cary, 2001; Inagaki *et al.*, 2006); thus strain RASEN^T and these clones were phylogenetically distinct at the genus level.

Based on these phenotypic and phylogenetic properties, we propose a novel genus and species with the name *Exilispira*

Fig. 2. Phylogenetic tree of the phylum *Spirochaetes* based on comparative analyses of 16S rRNA gene sequences, showing the placement of strain RASEN^T. The tree was calculated based on a distance matrix analysis of 16S rRNA gene sequences (neighbour-joining). Two 16S rRNA gene sequences of members of the genus *Fibrobacter* (*Fibrobacter intestinalis* NR9^T, GenBank accession no. M62695, and *Fibrobacter succinogenes* subsp. *succinogenes* S85^T, M62696; not shown) were used to root the tree. Bootstrap support (only values >50% are shown) was obtained from the neighbour-joining (first value), maximum-parsimony (second value) and maximum-likelihood (third value) methods based on 1000 replicates. Bar, 0.1 nucleotide changes per sequence position.

Table 1. Differential characteristics between strain RASEN^T and other genera in the phylum *Spirochaetes*

Data for strain RASEN^T are from this study. Data for other genera were taken from Holt *et al.* (1994), Paster *et al.* (1991) and the following sources: *Treponema* (Norris *et al.*, 2006; Smibert, 1984; Wyss *et al.*, 2004; Nordhoff *et al.*, 2005; Graber & Breznak, 2004; Graber *et al.*, 2004), *Spirochaeta* (Leschine *et al.*, 2006; Canale-Parola, 1984a, b; Hoover *et al.*, 2003; Dröge *et al.*, 2006), *Borrelia* (Caimono, 2006; Kelly, 1984; Güner *et al.*, 2004; Masuzawa *et al.*, 2001; Fukunaga *et al.*, 1996; Richter *et al.*, 2004; Le Fleche *et al.*, 1997; Baranton *et al.*, 1992; Canica *et al.*, 1993; Wang *et al.*, 1997; Karimi *et al.*, 1979), *Cristispira* (Breznak, 1984), *Brevinema* (Defosse *et al.*, 1995), *Brachyspira* (Stanton, 2006; Hampson & La, 2006) and *Leptospira/Leptonema* (Adler & Faine, 2006; Faine, 1984; Levett *et al.*, 2006; Perolat *et al.*, 1998; Ramadass *et al.*, 1992). +, Positive; –, negative; v, dependent on the species; ND, no data available.

Characteristic	Strain RASEN ^T	<i>Treponema</i>	<i>Spirochaeta</i> *	<i>Borrelia</i>	<i>Cristispira</i>	<i>Brevinema</i>	<i>Brachyspira</i>	<i>Leptospira/Leptonema</i>
Relationship to O ₂	Strictly anaerobic	Strictly anaerobic†	Strictly anaerobic	Microaerophilic	ND	Microaerophilic	Microaerophilic	Aerobic
Lifestyle	Free-living	Host-associated	Free-living	Host-associated	Host-associated	Host-associated	Host-associated	Free-living/host-associated
Pathogenic species	–	v	–	+	ND	+‡	v	v
Cell size (µm)	0.23–0.28 × 15–27	0.1–0.7 × 3–20	0.2–0.5 × 5.5–50	0.18–0.5 × 4–30	0.5–3 × 20–100	0.2–0.3 × 4–5	0.2–0.45 × 2–11	0.1–0.2 × 6–24
Periplasmic flagella (<i>n</i>)	2	1–16	1–2	2–30	>100	2	8–30	2
DNA G + C content (mol%)	27.1	37–54	45–65.6	27–32.4	ND	34–36	24.6–27	33.5–53
Optimum growth temperature (°C)	50	30–39	15–68	30–37	ND	30–34	36–42	28–30
Energy sources								
Carbohydrates	–	v	+	+	ND	ND	+	–
Amino acids	–	v	–	ND	ND	ND	v	–
Resistance to rifampicin	–	+	+	+	ND	ND	+	+

**Spirochaeta coccoides* is a facultatively anaerobic, host-associated, coccoid bacterium, and the cells have no spiral form and are non-motile (Dröge *et al.*, 2006).

†*Treponema pallidum* may be microaerophilic or a facultative anaerobe (Smibert, 1984).

‡*Brevinema andersonii* may be pathogenic (Defosse *et al.*, 1995).

thermophila gen. nov., sp. nov. to accommodate strain RASEN^T.

Description of *Exilispira* gen. nov.

Exilispira (Ex.i.li.spi'ra. L. adj. *exilis* slender; L. fem. n. *spira* helix; N.L. fem. n. *Exilispira* slender helix, referring to the cell shape).

Strictly anaerobic and thermophilic organisms. Cells are helical with a cell structure typical of spirochaetes, i.e. the cells possess periplasmic flagella, a peptidoglycan–cytoplasmic membrane complex and an outer membrane. Cells are highly motile. Gram reaction is negative. Can use yeast extract for growth. Phylogenetic position is in a deeply branched lineage of the phylum *Spirochaetes*. The type species is *Exilispira thermophila*.

Description of *Exilispira thermophila* sp. nov.

Exilispira thermophila (ther.mo'phi.la. Gr. fem. n. *therme* heat; Gr. adj. *philos* loving; N.L. fem. adj. *thermophila* heat-loving).

Displays the following properties in addition to those described for the genus. Cells have hooked ends, are 0.23–0.28 µm wide and 15–27 µm long and exhibit wavy movement and/or rotation. Growth occurs at 37–60 °C (optimum 50 °C), at pH 6.0–7.5 (optimum pH 7.0) and in the presence of 2.5–3.5 % NaCl (optimum 2.5–3 %). Grows only on yeast extract of the substrates examined. The following substrates are not utilized: Casamino acids, tryptone, peptone, crotonate, H₂/CO₂ plus acetate, betaine, glucose, sucrose, arabinose, fructose, galactose, raffinose, xylose, starch, pectin, maltose, cellobiose, lactate, formate plus acetate, acetate, propionate, butyrate, malate, fumarate, succinate, pyruvate, benzoate, ethanol, trimethylamine, glycerol, *N*-acetylglucosamine, chitin, amino acids, sulphate, sulphite, thiosulphite and elemental sulphur. No Stickland reaction is observed. The major cellular fatty acids are C_{16:0}, C_{18:1}(Δ9) *trans*, C_{18:0} and C_{18:1}(Δ9) *cis*. The G+C content of the DNA of the type strain is 27.1 mol%. The 16S rRNA gene sequence of the type strain exhibits 80.6 % similarity to that of *Spirochaeta africana*.

The type strain is strain RASEN^T (=JCM 14728^T =NBRC 103205^T =KCTC 5595^T), which was isolated from a deep-sea hydrothermal vent chimney at the Iheya North field, in the Okinawa Trough, Japan (27° 47.451' N 126° 53.803' E; depth 982 m).

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