Supporting Information for

Hydrogen and thiosulfate limits for growth of a thermophilic, autotrophic *Desulfurobacterium* species from a deep-sea hydrothermal vent

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This file includes:

- Figures S1 through S4
- Tables S1 and S2
- Experimental procedures
- References

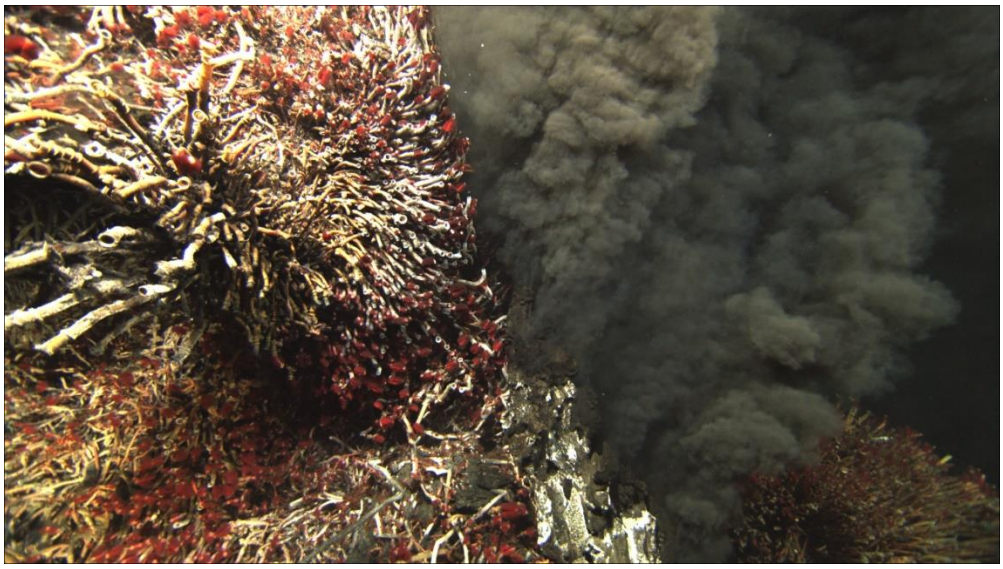

Fig. S1. Boardwalk hydrothermal vent sampling site showing the black smoker (bottom) that was the source of the 341°C hydrothermal fluid and the tubeworm mound (left side) that was the source of the 19°C fluid. The image is a video frame grab from ROV *Jason* dive J2-576.

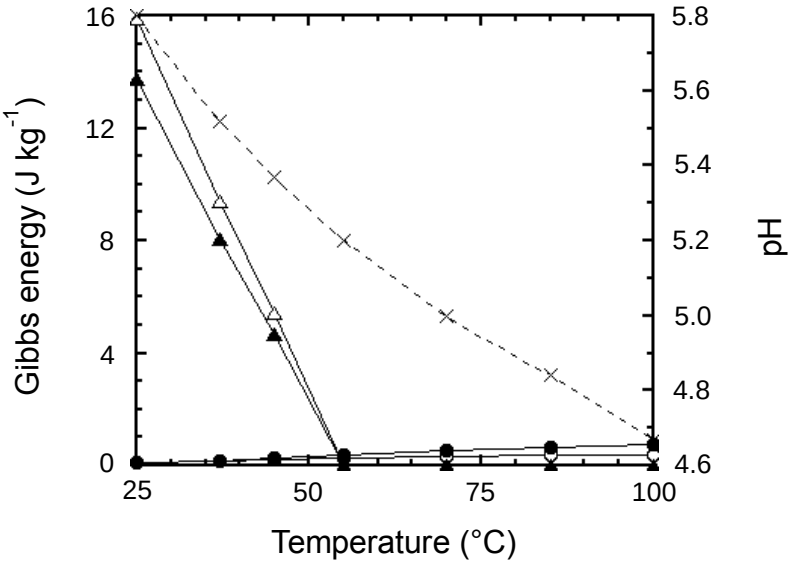

Fig. S2. Predicted catabolic energies (per kg of mixed fluid) available for hydrogenotrophic sulfate reduction (●), hydrogenotrophic methanogenesis (○), aerobic sulfide oxidation (▲), and aerobic methane oxidation (Δ) at varying temperatures in mixed abiotic hydrothermal-seawater solutions flowing from the Boardwalk edifice. The calculated pH for the mixed fluid is also shown (×).

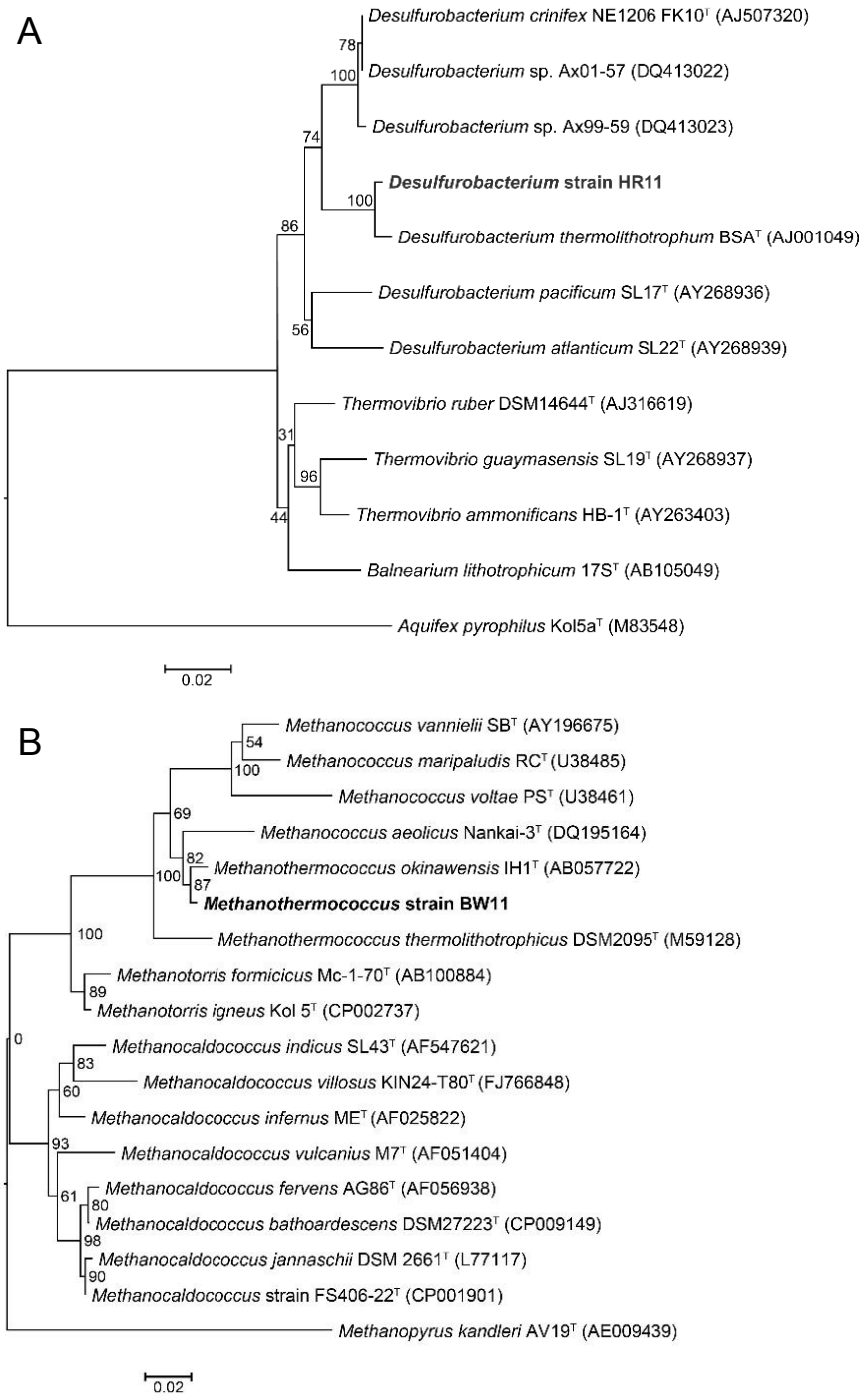

Fig. S3. Neighbour-joining trees showing the positions of (A) strain HR11 within the genus *Desulfurobacterium* (870 nt) and (B) strain BW11 within the genus *Methanothermococcus* (880 nt) based on sequences of the 16S rRNA gene. GenBank/EMBL/DDBJ accession numbers are included in parentheses. The topology of the tree was estimated by bootstraps based on 500 replications. Numbers at the branch point are the percentage support by bootstraps. Bar, 2% sequence divergence.

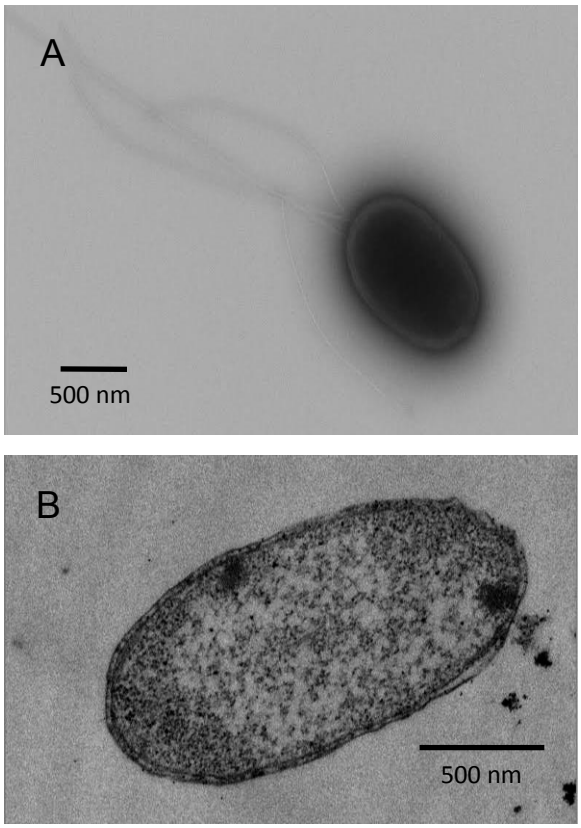

Fig. S4. Negative staining (A) and thin-section transmission electron micrographs of strain HR11. Bars, 500 nm.

Table S1. Characteristics of various global deep-sea hydrothermal vent sites and the presence or absence of *Desulfurobacterium* and *Methanococcales* species

	Boardwalk edifice, Endeavour Segment, Juan de Fuca Ridge	Hulk edifice, Endeavour Segment, Juan de Fuca Ridge	Axial Volcano, Juan de Fuca Ridge	Lucky Strike Field, Mid-Atlantic Ridge	Kilo Moana, Lau Basin	Mariner Field, Lau Basin	TOTO Caldera, Mariana Arc	Brothers Volcano, Kermadec Arc	Iheya North Field, Mid-Okinawa Trough	Kairei Field, Central Indian Ridge	Logatchev Field, Mid-Atlantic Ridge	Rainbow Field, Mid-Atlantic Ridge
Geologic setting[a]	MORB	MORB	MORB	MORB	VA	VA	VA	VA	VA	UM	UM	UM
Max. temp. (°C)	341	305	275	163-324	290-304	338-359	170	290	311	365	300-350	191-370
H_2S (mM) [b]	3.4	NA	37	2.4-3.4	3.5-3.9	6.1-19	14.6	7.9	4.0	4.0	0.3	1.8-3.3
H_2 (μM)[b]	79	165	600	25-71	220-498	33-179	~10	17	~200	2,500	5,900	12,300-16,900
H_2 (μM) at 72°C[c]	17	39	158	11-16	55-119	7-36	4	4	47	496	>1,400	>4,600
Desulfurobacterium	+	+	+	–	+	–	–	–	+	+	+	+
Methanococcales	+	+	+	–	+	+	–	–	+	+	+	+
References[d]	1	2,3	4-6	7	8	8	9	10	11	12	13	7

[a]Symbols and abbreviations: MORB, mid-ocean ridge basalt; VA, volcanic arc; UM, ultramafic; +, *Desulfurobacterium*-and *Methanococcales*- related 16S rRNA sequences found in the samples; n.d., none detected; NA, not available.

[b]End-member H_2 and CH_4 concentrations in hydrothermal fluids are based on an extrapolation of measured values to zero magnesium concentration.

[c]H_2 concentrations at 72°C are estimated assuming conserved mixing between 2°C seawater containing no H_2 and end-member hydrothermal fluid H_2.

[d]References: 1, this study; 2, Anderson *et al.* (2013); 3, Ver Eecke *et al.* (2012); 4, Huber *et al.* (2002); 5, Huber *et al.* (2003); 6, Butterfield *et al.* (2004); 7, Flores *et al.* (2011); 8, Flores *et al.* (2012); 9, Nakagawa *et al.* (2006); 10, Takai *et al.* (2009); 11, Nakagawa *et al.* (2005); 12, Takai *et al.* (2004); and 13, Perner *et al.* (2007).

Experimental procedures

Field sampling and chemical analyses

In July 2011, 19°C and 341°C hydrothermal fluids were collected within a meter of each other on top of the Boardwalk edifice (Fig. S1) at a depth of 2,134 m in the High Rise vent field along the Endeavour Segment of the Juan de Fuca Ridge (47.968°N 129.087°W). For the 341°C fluids, duplicate samples were drawn into Tedlar plastic bags with valves within rigid housings using the NOAA Hydrothermal Fluid and Particle Sampler (Butterfield *et al.*, 2004) and titanium gas-tight samplers (Edmond *et al.*, 1992). For the 19°C fluid, the sample was drawn into another Tedlar plastic bag. The sampler pumped vent fluid through a titanium nozzle and recorded the temperature of the fluid within the intake nozzle once every second during pumping. Samples were collected using the research submarine *Jason* II operated from the research vessel *Thomas G. Thompson*. Fluid samples were analyzed on board ship for pH, alkalinity, ΣH_2S, dissolved silica, and ΣNH_3. The gases were extracted from the gas-tight samplers using a shipboard gas extraction line and sealed in glass ampules for later analysis by gas chromatography. The extraction water (acidified with sulfamic acid) was analyzed for major elements on shore. Major and minor elements in the hydrothermal fluids were analyzed at the Pacific Marine Environmental Laboratory and at the University of Washington as described previously (Edmond *et al.*, 1992; Butterfield *et al.*, 1997; 2004).

Redox energy estimates

Four redox reactions were considered for microbial energy availability estimates (Table S2). Two represent aerobic respiration of inorganic electron donors (sulfide oxidation and methane oxidation) and two represent anaerobic respiration of H_2 and inorganic electron acceptors (sulfate reduction and methanogenesis).

Table S2. Inorganic redox reactions (from Amend *et al.*, 2011)

Metabolism	Reaction
Aerobic sulfide oxidation	$H_2S + 2O_2 \rightarrow SO_4^{2-} + 2H^+$
Aerobic methane oxidation	$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$
Hydrogenotrophic sulfate reduction	$4H_2 + SO_4^{2-} + 2H^+ \rightarrow H_2S + 4H_2O$
Hydrogenotrophic methanogenesis	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$

All compounds were in the aqueous phase. The compositions of the mixed hydrothermal solutions were calculated from those of the end-member vent fluid from Boardwalk and seawater using the REACT module with its default settings in the computer code Geochemist's Workbench™ as previously described (Jin and Bethke, 2005; Amend *et al.*, 2011). The seawater composition was as previously described (Amend et al., 2011), except O_2 (70 µmol kg^{-1}) that was based on O_2 measurements at 2,200 m depth within the Endeavour Segment axial valley (Richard Thomson, personal communication). The reaction energetics of H_2 oxidation coupled with the reduction of

thiosulfate or sulfur reduction could not be calculated since thiosulfate is not in the Geochemist's Workbench database and concentrations of thiosulfate and sulfur/polysulfide in vent systems are unknown.

The reaction path mimics the incremental titration of small aliquots of seawater into hot vent fluid with re-evaluation of the chemical speciation of the mixed solution at each step. All minerals were allowed to precipitate and dissolve in the model during mixing except quartz, tridymite, cristobalite, chalcedony, and talc (Jin and Bethke, 2005). Differences from Jin and Bethke (2005) were that talc precipitation was inhibited but hematite precipitation was permitted. H_2, H_2S, CH_4 and NH_4^+ were decoupled from redox reactions, but all other redox reactions were allowed. Values of Gibbs energy (ΔG_r) for the catabolic reactions were computed using the activities of relevant species as previously described (Amend et al., 2011). The amount of energy available (J) from catabolic reactions at 25°C, 37°C, 45°C, 55°C, 70°C, 85°C and 100°C in a kg of mixed fluid was calculated by multiplying the calculated Gibbs energy for the reaction at each temperature by the concentration of the reactant that was in limiting supply (Amend *et al.*, 2011). For comparison, the mixing reaction was also run for hydrothermal fluids and seawater as described by McCollom and Shock (1997) where all mineral precipitations were inhibited and H_2 and O_2 were in redox equilibrium. Under these modeling assumptions, O_2 was only depleted in the mixed hydrothermal fluid above 200°C and H_2 was depleted below 145°C, which means the fluids would be incapable of supporting anaerobic hydrogenotrophy at thermophilic growth temperatures.

Isolation of new thermophile strains

Hydrothermal fluid from the 19°C sample was immediately used to inoculate modified Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) medium 282 (see below) and DSM medium 399 (Jones *et al.*, 1983; Burggraf *et al.*, 1990) for the growth of autotrophic sulfur reducers and methanogens. The samples were incubated shipboard at 55°C until they became turbid. Growth was confirmed using phase-contrast light microscopy, headspace analysis using gas chromatography, and sulfide production using the methylene blue method (Chen and Mortenson, 1977). Cells in the modified DSM 282 medium were predominantly rods that produced H_2S and very little CH_4, while those in the DSM 399 medium were predominantly cocci that produced CH_4. On shore, purification of the strains from the 19°C hydrothermal fluid was performed by three rounds of 10-fold dilution-to-extinction incubations at 55°C using their original enrichment medium. They were identified through the partial sequencing of their 16S rRNA gene that had been amplified by PCR (see below). The result was the purification of *Desulfurobacterium* strain HR11 from the modified DSM 282 medium and *Methanothermococcus* strain BW11 from the DSM 399 medium. They were deposited in the DSM culture collection and assigned the collection numbers DSM 100454 and DSM 100453, respectively.

Growth conditions

The growth medium for all laboratory experiments, except where amended (see below), was DSM medium 282 (Jones *et al*., 1983) that was modified by the addition of 0.1% (wt vol^{-1}) $Na_2S_2O_3$, the removal of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and the use of 0.64 mM dithiothreitol (DTT) as the reducing agent instead of $Na_2S \cdot 9H_2O$ and cysteine-HCl. The

medium was pH balanced to 6.00 ± 0.05. Static cultures were grown in 10 ml of medium contained within Balch tubes sealed with butyl rubber stoppers with 2 atm of H_2/CO_2 (80:20 ratio) headspace and incubated in a forced-air incubator. Strain HR11 was incubated at 72°C unless otherwise indicated.

Monod kinetics

For the H_2 and $Na_2S_2O_3$ limitation experiments, a 2-L bioreactor stirred at 300 rpm with controls for gas flow, incubation temperature (72°C ± 0.1°C), and pH (6.0 ± 0.1 by the automatic addition of 0.25 mM HCl) was prepared with 1.5 L of modified DSM 282 medium. The reactor was degassed through a submerged fritted bubbler with a mixture of CO_2 (7.5 mL of gas min^{-1}), H_2, and N_2. For the H_2 limitation experiments, the H_2 gas flow rate and H_2 concentration were varied for different growth kinetics experiments. A H_2/N_2 tank mixture (5:95 ratio) was used in place of pure H_2 to attain H_2 concentrations below 20 µM. N_2 was added to balance the total gas flow at 70 mL min^{-1}. The aqueous H_2 concentration in the reactor at all H_2 flow rate settings was measured by drawing ~ 20 mL of fluid from the bottom of the reactor directly into anoxic 60-mL serum bottles with 20 mL of gas removed and measuring the headspace in the bottle with a gas chromatograph equipped with a thermal conductivity detector. For the $Na_2S_2O_3$ limitation experiments, the given concentration is for the initial concentration in the reactor. The reactor was inoculated with a logarithmic growth-phase culture of strain HR11. During growth, samples were drawn from the reactor and cell concentrations were determined using phase-contrast light microscopy and a Petroff-Hausser counting chamber. Specific growth rates (µ) were estimated using a best-fit curve through the exponential portion of growth. Each growth kinetic experiment was run in duplicate.

Cell characteristics

For kinetic experiments, at least 10 Balch tubes were inoculated concurrently with a logarithmic growth phase culture grown under the same experimental conditions. At various time points, at least two tubes were permanently removed from incubation. Cell concentrations were determined using a Petroff-Hausser counting chamber and phase-contrast light microscopy. Specific growth rates were calculated using a best-fit exponential curve to the logarithmic portion of cell growth. The amount of H_2S produced was determined spectrophotometry by adding 0.1 N NaOH to each tube and then using the methylene blue method. Confidence intervals (95%) were calculated for growth rates as described previously (Zar, 1996).

Desulfurobacterium strain HR11 was grown at pH 4 (no buffer), pH 5 and 6 (5 mM MES buffer), pH 7 and 8 (20 mM PIPES buffer), and pH 8.5 and 9 (30 m $NaHCO_3$ buffer) to determine the effect of pH on growth. It was also grown on 0-5% (wt vol^{-1}) NaCl to determine the effect of salt on growth. Elemental sulfur (0.2% wt vol^{-1}), 10 mM $NaSO_3$, 20 mM Na_2SO_4, 20 mM ferric citrate, 100 mmol L^{-1} amorphous Fe(III) (oxy)hydroxide, and 20 mM KNO_3 were tested separately in place of $Na_2S_2O_3$ as terminal electron acceptors. Yeast extract (0.2% wt vol^{-1}), 10 mM maltose, 10 mM tryptone, 10 mM sodium acetate, and 10 mM sodium formate were tested separately as carbon and electron donors using 2 atm of N_2/CO_2 (80:20 ratio) and H_2 only in the headspace. All tests were done in duplicate, and positive cultures were transferred three times on the same substrate to confirm growth.

Phylogenetic analyses

DNA was extracted from strain HR11 and BW11 using a genomic DNA extraction kit (Qiagen) and their 16S rRNA genes were amplified using the polymerase chain reaction and sequenced. The primers used were 8f (5'-AGA GTT TGA TCC TGG CTC A-3') and 1492r (5′-TAC CTT GTT ACG ACT T-3′). Phylogenetic trees were constructed using 16S rRNA gene sequences from the Ribosomal Database Project (Cole *et al.*, 2007) in MEGA 5 (Tamura *et al.*, 2011) using a neighbor-joining algorithm. Both sequences are deposited in GenBank under accession numbers KR023948-KR023949.

Electron microscopy

For negative staining of whole mounted cells, 10 ml of culture within a sealed Balch tube were fixed by adding 0.2 ml of 50% glutaraldehyde with gentle mixing and incubating at room temperature for 1 h. An aliquot (3 ml) of the fixed culture was then removed from the sealed Balch tube, processed, and applied to plasma-treated carbon films (ca. 0.5 nm thickness) on 400 mesh copper grids. The grids were stained with 3% NH_4OH and 2% aqueous uranyl acetate and viewed with a JEOL-100S transmission electron microscope.

For thin section microscopy, 10 ml of culture within a sealed Balch tube were fixed by adding 0.4 ml of 50% glutaraldehyde with gentle mixing and incubating at room temperature for 2 h. An aliquot (3 ml) of the fixed culture was then removed from the sealed Balch tube, post fixed, and enrobed by resuspension in a minimal volume of 2% type IX agarose to create a non-friable unit rich in cells. The agarose was then gelled and cut into 1 mm blocks with a razor blade. These blocks were then rinsed in dH_2O, dehydrated, infiltrated with Ellis-Spurrs low-viscosity epoxy resin formulation (Ellis, 2006). Polymerized blocks were sectioned on a diamond knife set at 60 nm thickess. Sections were stained with 2% aqueous uranyl acetate and alkaline lead citrate (5 mg ml^{-1} in 0.1 N NaOH). Sections were viewed on a JEOL 100S transmission electron microscope.

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