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ARTICLE

Hydrogen is an energy source for hydrothermal vent symbioses

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The discovery of deep-sea hydrothermal vents in 1977 revolutionized our understanding of the energy sources that fuel primary productivity on Earth. Hydrothermal vent ecosystems are dominated by animals that live in symbiosis with chemosynthetic bacteria. So far, only two energy sources have been shown to power chemosynthetic symbioses: reduced sulphur compounds and methane. Using metagenome sequencing, single-gene fluorescence *in situ* hybridization, immunohistochemistry, shipboard incubations and *in situ* mass spectrometry, we show here that the symbionts of the hydrothermal vent mussel *Bathymodiolus* from the Mid-Atlantic Ridge use hydrogen to power primary production. In addition, we show that the symbionts of *Bathymodiolus* mussels from Pacific vents have *hupL*, the key gene for hydrogen oxidation. Furthermore, the symbionts of other vent animals such as the tubeworm *Riftia pachyptila* and the shrimp *Rimicaris exoculata* also have *hupL*. We propose that the ability to use hydrogen as an energy source is widespread in hydrothermal vent symbioses, particularly at sites where hydrogen is abundant.

Deep-sea hydrothermal vents and their associated chemosynthetic communities were discovered in 1977[1]. Since then, two energy sources have been shown to fuel primary production by the symbiotic bacteria that form the basis of the food chain in marine chemosynthetic ecosystems. In 1981, chemolithoautotrophic bacteria that use reduced sulphur compounds as an energy source were discovered in the gutless *Riftia pachyptila* tubeworms from hydrothermal vents in the Pacific[2,3]. Five years later, the first symbionts that use methane as an energy source were discovered in mussels from hydrocarbon seeps in the Gulf of Mexico[4,5]. Since then, a vast array of chemosynthetic ecosystems has been explored, and novel symbioses of phylogenetically diverse hosts and symbionts are constantly being described[6]. Despite this, no other source of energy for metazoan chemosynthetic symbioses has yet been found. This is remarkable, given that many potential sources of energy for chemosynthesis are available, such as hydrogen, ammonium, ferrous iron and manganese(II)[7], and free-living vent microbes able to use these energy sources are well known[8–10].

Some hydrothermal vents produce fluids with very high hydrogen concentrations due to the interaction of seawater with mantle-derived ultramafic rocks. Fluids originating from ultramafic-hosted vents are also characterized by high methane concentrations, whereas H_2S concentrations are rather low. In contrast, basalt-hosted vents produce fluids comparably high in H_2S but low in H_2 and CH_4. The Logatchev vent field at 14° 45′ N on the Mid-Atlantic Ridge (MAR) is located in a ridge segment characterized by ultramafic outcrops (Supplementary Fig. 2). Fluids venting at this site have the highest hydrogen concentrations ever measured in hydrothermal systems (19 mM in the end-member fluids)[11], which would provide a rich source of energy for chemosynthetic microbes. Hydrogen is a particularly favourable electron donor as the energy yield from hydrogen oxidation is much higher than from methane oxidation, sulphur oxidation and all other potential electron donors for chemolithoautotrophic growth under

standard conditions[12]. In fact, our thermodynamic model predicts that at the Logatchev vent field, aerobic hydrogen oxidation could provide up to 7 times more energy per kilogram of vent fluid than methane oxidation, and up to 18 times more energy per kilogram of vent fluid than sulphide oxidation (Supplementary Fig. 1).

At Logatchev, *Bathymodiolus puteoserpentis* mussels are by far the most abundant macrofauna[13]. They live in a dual symbiosis with methane-oxidizing and chemoautotrophic sulphur-oxidizing bacteria that are hosted in their gills[14–16]. Here we show that the sulphur-oxidizing symbiont of *B. puteoserpentis* uses hydrogen as an energy source. We also show that *Bathymodiolus* symbionts from basalt-hosted MAR vent fields can oxidize hydrogen. This ability is therefore not limited to the ultramafic-hosted vent fields and could also be powering chemosynthetic symbioses at basalt-hosted vent fields.

Uptake hydrogenase genes in mussels

The key enzymes involved in hydrogen metabolism are hydrogenases, which catalyse the reaction: $H_2 \leftrightarrow 2H^+ + 2e^-$ (ref. 17). Enzymes of the group 1 NiFe hydrogenases are membrane-bound respiratory enzymes that channel electrons from hydrogen into the quinone pool, providing the link between hydrogen oxidation and energy production[17,18]. The large subunit of the membrane-bound uptake hydrogenase is encoded by the *hupL* gene. We amplified and sequenced this gene from symbiont-containing *B. puteoserpentis* gill tissues from Logatchev (Supplementary Fig. 3). The closest related sequence was the large subunit of the NiFe hydrogenase from the alphaproteobacterium *Oligotropha carboxidovorans* (79.6% amino acid identity). *O. carboxidovorans* can grow chemolithoautotrophically under aerobic conditions using either CO or H_2 as an electron donor[19]. Phylogenetic analyses based on multiple treeing methods placed the enzyme from *B. puteoserpentis* in a well-supported cluster with other group 1 NiFe hydrogenases, showing the genetic potential for hydrogen oxidation

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linked to energy generation in *B. puteoserpentis* endosymbionts. To examine whether the endosymbionts of mussels from hydrogen-poor habitats also have the genetic potential for hydrogen uptake, we tried to amplify the *hupL* gene from gill tissues of mussels from basalt-hosted vents and cold seeps that have fluids with low hydrogen concentrations. Indeed, we could amplify the *hupL* gene from mussels from basalt-hosted vents, including undescribed *Bathymodiolus* mussels (*Bathymodiolus* spp.) from vents on the southern MAR (Wideawake at 4° 48′ S, 5,600 km from Logatchev, and Lilliput at 9° 33′ S, 6,500 km from Logatchev), and *B.* aff. *thermophilus* from the Axial Dome vent on the Pacific–Antarctic Ridge at 37° 47′ S (Supplementary Figs 2, 3 and Supplementary Table 7), showing that the genetic potential for hydrogen oxidation is not restricted to mussel symbionts from ultramafic-hosted vent fields that have high hydrogen concentrations. Intriguingly, we could not amplify this gene from any of the cold seep mussels investigated (see Supplementary Discussion).

B. puteoserpentis symbionts use H_2

Given the genetic potential for hydrogen uptake in *Bathymodiolus* mussels, we incubated *B. puteoserpentis* mussel gill tissues from the ultramafic-hosted Logatchev vent field on the MAR with hydrogen at partial pressures of ~100 p.p.m. in the headspace (0.08 μM H_2 dissolved in the medium) and measured its consumption over time. Hydrogen was taken up rapidly by the symbiont-containing gill tissues at a rate of ~650 ± 200 nmol h^{-1} (g wet weight)$^{-1}$ ($n = 7$). In contrast, symbiont-free foot tissue did not consume hydrogen at rates above the negative controls—boiled gill tissue and seawater (Fig. 1 and Supplementary Table 3).

Because hydrogen uptake by bacteria is not necessarily coupled to CO_2 fixation[17,20], we incubated mussel gill tissues with hydrogen in seawater containing ^{14}C-bicarbonate to determine whether hydrogen is an energy source for autotrophic CO_2 fixation by *B. puteoserpentis* symbionts. Control gill tissues were incubated in the presence of sulphide or without an electron donor. [14]C uptake was stimulated by sulphide, known to be an energy source for the sulphur-oxidizing symbionts of *Bathymodiolus* mussels[21], and also by hydrogen (Fig. 1). The rates of carbon fixation with hydrogen were comparable to those supported by sulphide oxidation, which suggests that hydrogen could be fuelling autotrophy to the same extent as sulphide. We therefore conclude that hydrogen provides energy for the production of mussel biomass at the Logatchev hydrothermal vent field.

Symbionts from basalt–hosted vents use H_2

We examined whether the symbionts of *Bathymodiolus* mussels from basalt-hosted vent fields on the southern MAR at Comfortless Cove and Lilliput with low *in situ* hydrogen concentrations could also consume hydrogen. Hydrogen concentrations measured in discrete samples of the diffuse fluids from these vents were typically below 0.1 μM (refs 22,23) as opposed to Logatchev, which had up to 154 μM, on the basis of discrete sampling (Supplementary Table 3). We incubated gill tissues of *B.* spp. mussels and measured hydrogen consumption over time as described earlier. Our results show that mussels from these vents were also able to take up hydrogen (Fig. 1 and Supplementary Table 3). However, the rates at which the symbiotic gill tissues of the southern MAR mussels consumed hydrogen were 20- to 30-fold lower than those of mussels from Logatchev.
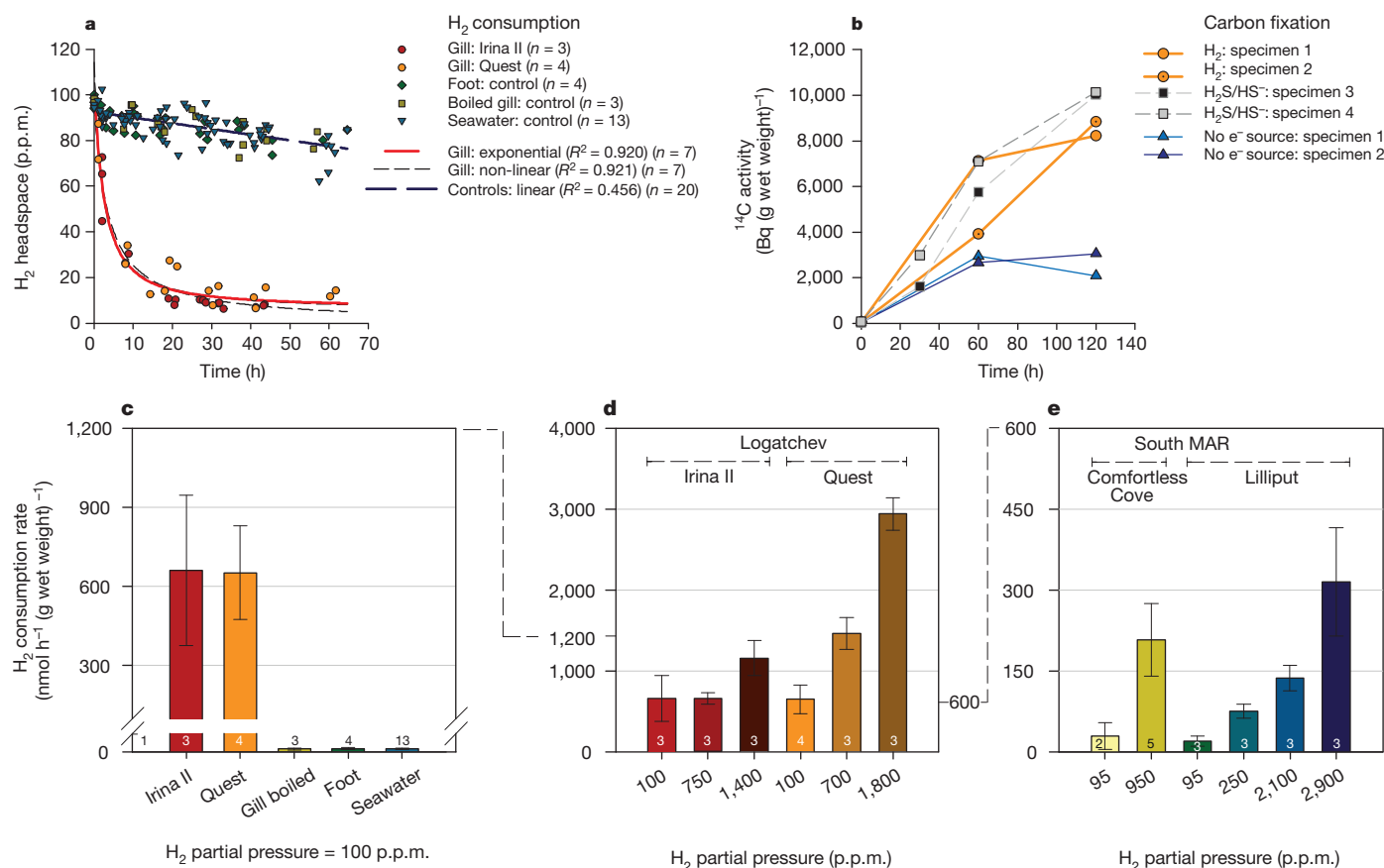
Figure 1 | Hydrogen consumption in *Bathymodiolus* gills. a, b, Hydrogen was consumed rapidly by *B. puteoserpentis* gills (Logatchev) and carbon fixation was stimulated after incubation with ^{14}C-bicarbonate in the presence of hydrogen or sulphide. Each data point is one distinct measurement. **c**, Consumption rates of gills from two sites at Logatchev were in the same range. **d, e**, Rates from Logatchev and the southern MAR fields (*B.* spp.; Comfortless Cove, 4° 48′ S and Lilliput, 9° 33′ S) increased with increasing hydrogen partial pressures. Rates were roughly 20–30-fold higher at the hydrogen-rich Logatchev field than at the hydrogen-poor southern MAR vents. Error bars represent one standard deviation.

Effect of H₂ concentration on consumption

Some hydrogen-oxidizing microorganisms are known to induce hydrogenase expression only in the presence of hydrogen, and some express this enzyme constitutively at a low level, but increase its expression upon incubation with hydrogen[24]. To investigate the effect of dissolved hydrogen concentration on hydrogen consumption rates, we incubated gill tissues from the Logatchev and southern MAR venting sites at partial pressures of up to 3,000 p.p.m. (~2.3 µM dissolved H_2). Hydrogen consumption rates in mussels from Logatchev, Comfortless Cove and Lilliput increased by 135, 21 and 8 nmol h^{-1} (g wet weight)$^{-1}$, respectively, for each 100 p.p.m. increase in partial pressure (Fig. 1). Thus, hydrogen uptake is clearly stimulated by increasing hydrogen concentrations.

The sulphur–oxidizing symbiont uses H₂

Many phylogenetically diverse microorganisms can grow autotrophically on hydrogen. *Bathymodiolus* mussels from the MAR host two types of chemosynthetic gammaproteobacteria: a sulphur oxidizer and a methane oxidizer[14] (Fig. 2). In addition, these mussels have a gammaproteobacterial parasite that infects gill nuclei[25]. Hydrogen is known to provide energy for chemo- and photolithoautotrophic
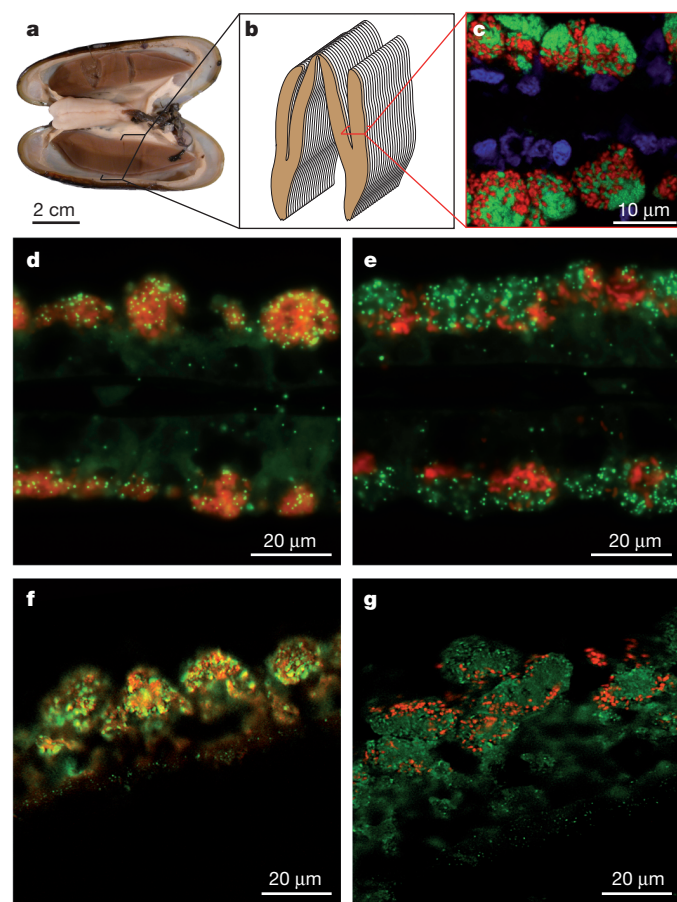
Figure 2 | The sulphur-oxidizing symbiont has the gene for hydrogen uptake, which it expresses. a, *B. puteoserpentis* with brown, symbiont-containing gills. **b**, Morphology of a single gill, each composed of many filaments (modified from ref. 48). **c**, FISH of a cross-section through a single gill filament (host cell nuclei, blue; sulphur-oxidizing symbiont, green; methane-oxidizing symbiont, red). **d, e**, *hupL* geneFISH (signals in green) with 16S rRNA FISH (signals in red) for the sulphur-oxidizing symbiont (**d**) and the methane-oxidizing symbiont (**e**). The negative control gave no gene signals (Supplementary Fig. 7). **f, g**, HupL immunohistochemistry (signals in green) with 16S rRNA FISH (signals in red) for the sulphur-oxidizing symbiont (**f**) and the methane-oxidizing symbiont (**g**). Yellow colours in **d** and **f** appear where green and red signals overlap.

growth of some free-living sulphur-oxidizing bacteria[26,27]. Some free-living methane-oxidizing bacteria can also oxidize hydrogen in addition to methane[28]. Lastly, hydrogen has been shown to be a key determinant of the virulence of pathogenic *Helicobacter pylori*[29]. Accordingly, it was unclear which of the three types of bacteria associated with *Bathymodiolus* mussels could be using the hydrogen. To investigate this further, we used three molecular methods allowing us to link identity with function at the DNA and protein level: genome sequencing of the mussel symbionts, single-gene fluorescence *in situ* hybridization (geneFISH) and immunohistochemistry, the latter two combined with 16S rRNA FISH.

In the genomes of hydrogen-oxidizing bacteria, the genes for the large and small subunits of membrane-bound NiFe hydrogenases are often found clustered together with the genes necessary for biosynthesis, maturation and processing[17]. We used metagenomics to investigate hydrogen oxidation in the genome of *Bathymodiolus* symbionts from the southern MAR (Lilliput). We found a hydrogenase operon with 19 open reading frames on a 726-kb genome fragment (Supplementary Fig. 4). The large subunit gene from this genome fragment was 99.6% identical to the *hupL* gene amplified from the Wideawake (southern MAR) mussels using polymerase chain reaction (PCR), and 83% identical to the gene from *B. puteoserpentis* from Logatchev on the northern MAR. Homologues of all structural genes and those necessary for hydrogenase synthesis, assembly and function in the chemolithoautotrophic hydrogen oxidizer *Cupriavidus necator*[30] were present on our genome fragment. The southern MAR *Bathymodiolus* symbiont therefore has all of the genetic components required for hydrogen uptake. On the same genome fragment, we found the key genes for sulphur oxidation via the reverse dissimilatory sulphite reductase (rDsr) and Sox pathways, as well as for CO_2 fixation by the Calvin–Benson–Bassham (CBB) cycle (Supplementary Fig. 4), indicating that this fragment is from the sulphur-oxidizing symbiont of *Bathymodiolus*.

To establish further that it is the sulphur-oxidizing symbiont that uses hydrogen, we used geneFISH, a method in which simultaneous detection of single genes and rRNA enables the linking of function and identity at the single-cell level[31]. We detected the *hupL* gene in the symbiont-containing gill tissue of *B. puteoserpentis* (Fig. 2). GeneFISH signals from probes designed to target the *hupL* gene amplified by PCR from *B. puteoserpentis* overlapped with the 16S rRNA signals from the previously described sulphur-oxidizing symbiont (Fig. 2). This further suggests that the *hupL* gene we amplified is from the sulphur-oxidizing symbiont of *B. puteoserpentis*.

To show that the sulphur-oxidizing symbiont expresses the uptake hydrogenase, we combined immunohistochemistry with FISH of the 16S rRNA of the symbionts of *B. puteoserpentis*, thus linking identity and function at the level of gene expression. Using a polyclonal antiserum against the membrane-bound hydrogen-uptake NiFe hydrogenase from *C. necator* (74% amino acid sequence identity to the *B. puteoserpentis* symbiont HupL; Supplementary Fig. 5), we were able to detect hydrogenase expression in single symbiont cells in *B. puteoserpentis* (Fig. 2). Signals from the anti-hydrogenase antibody were seen in gill bacteriocytes of *B. puteoserpentis* where they overlapped with the FISH signals from the sulphur-oxidizing symbiont, but not with those from the methane-oxidizing symbiont.
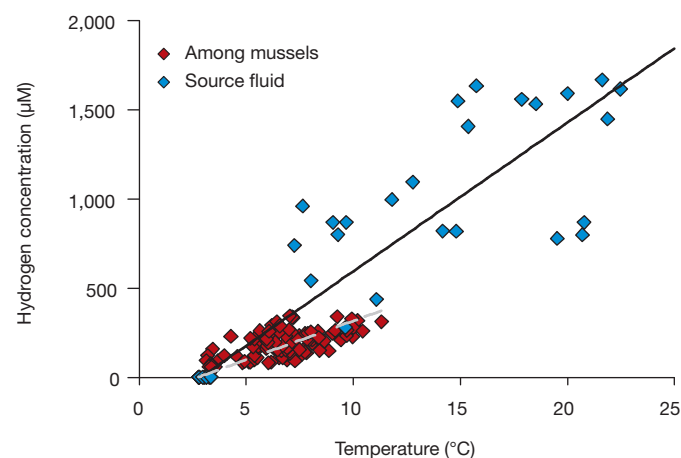
Environmental significance of H₂ use

One of the major challenges in hydrothermal vent research is to link physiological experiments, performed on board the research vessel at atmospheric pressure, with processes occurring under *in situ* temperature and hydrostatic pressure. We deployed an *in situ* mass spectrometer (ISMS) to measure hydrogen concentrations in the mussel habitat at Logatchev, and compared these with temperature as a conservative tracer for mixing of the hydrothermal fluids with seawater. Simultaneous measurements of hydrogen concentrations and temperature were made in two different settings; first, in an area of

focused flow, directly at the source where the fluids exit the seafloor. Here, the fluids had not been exposed to *B. puteoserpentis* mussels or other macrofauna. Second, we measured hydrogen concentrations and temperature in an area where the fluids had passed through a *B. puteoserpentis* mussel bed. The slope of the regression line calculated for hydrogen concentration versus temperature was significantly lower in the mussel bed compared to the source fluid ($P < 0.0001$), indicating that fluids in the mussel bed are hydrogen-depleted compared to the source fluid (Fig. 3). This difference is probably due to hydrogen consumption by the sulphur-oxidizing symbionts of *B. puteoserpentis*. We observed the same effect with measurements of methane and temperature ($P < 0.0001$; Supplementary Fig. 6). These measurements confirm that significant amounts of hydrogen are being consumed in Logatchev mussel beds.

The terrestrial hydrogen biogeochemical cycle has been the topic of numerous studies (summarized in ref. 32), and interest in the global hydrogen cycle has recently grown with the prospect of a hydrogen economy[33]. In contrast to the terrestrial environment, there is a paucity of data on hydrogen turnover in the oceans. Conservative calculations based on our measurements of uptake rates in Logatchev mussels show that at 50 μM dissolved hydrogen a single mussel with a gill weight of 5 g could oxidize up to 435 μmol $H_2 h^{-1}$ (Supplementary Information). On the basis of our own estimates and those of others, the mussel population at Logatchev spreads in layers over at least 200 m^2 and accounts for most of the invertebrate biomass[13]. One square meter is covered by approximately 1,100–2,500 adult individuals corresponding to a total population of 250,000 to 500,000 individuals. This population could consume between 0.5 and 1.0 mol $H_2 m^{-2} h^{-1}$, that is, in total up to 200 mol $H_2 h^{-1}$. Previous studies have reported hydrogen uptake rates in the range of several nmol $ml^{-1} h^{-1}$ in hydrothermal fluids[34], and several nmol $m^{-3} h^{-1}$ in coastal waters[35]. On the basis of the limited data available for comparison, the symbionts of *Bathymodiolus* mussels from the MAR probably have a role as a significant hydrogen sink at these hydrothermal vents.

We do not know if the chemoautotrophic symbionts of vent mussels from other mid-ocean ridges are actively consuming hydrogen, although we show here that *B.* aff. *thermophilus* from the Pacific–Antarctic Ridge has the key gene necessary. Hydrogen is clearly present in the fluids at many basalt-hosted vents[36,37], and could well be powering biomass production at these sites as well. In addition, use of hydrogen as an energy source may also have a role in other chemoautotrophic

symbioses; the episymbionts of the hydrothermal vent shrimp *Rimicaris exoculata* from MAR vent fields have *hupL* genes[38] (Supplementary Fig. 3), as does the endosymbiont of the giant tubeworm *Riftia pachyptila* from basalt-hosted vents in the East Pacific, which it expresses *in situ* (S. Markert, personal communication). This indicates that hydrogen use could be widespread in chemosynthetic symbioses.

METHODS SUMMARY

A detailed description of the sampling sites (Supplementary Tables 1 and 2) and all methods used in this study can be found in the Supplementary Information.

For amplification of the *hupL* gene, we extracted DNA from gill samples[39] and used published primers[40]. Cloning and sequencing were done as described elsewhere[41]. Maximum likelihood and parsimony HupL phylogenies were calculated in ARB[42] using a MAFFT[43] alignment. For incubation experiments, *Bathymodiolus* mussels were collected from hydrothermal vent fields on the MAR by remotely operated vehicles and incubated immediately after recovery on board the research vessel. Mussel gill and foot tissues were incubated in sterile-filtered seawater in glass serum vials (Supplementary Table 4). Hydrogen was added to the vials, and the change in headspace concentration was measured over time by gas chromatography. CO_2 fixation was measured by incubation of gill tissues with ^{14}C-bicarbonate after adding H_2, Na_2S (for H_2S/HS^-), or no electron donor. The incorporation of labelled ^{14}C uptake over time was measured by liquid scintillation counting in separate tissue pieces from the same individual that were incubated for 0, 30, 60 and 120 min. Mussels for genome sequencing were collected from the Lilliput vent site on the MAR. Gill tissue was immediately homogenized and fixed on board. We enriched for symbiont cells by filtering sequentially through 8, 5, 3 and 2 μm GTTP filters. We extracted DNA[39] and constructed a 6 kb paired-end library, which was sequenced by 454-Titanium and Illumina. Titanium reads were assembled using Newbler (v.2.3-PreRelease-10/19/2009–454; Life Sciences Corporation). One 726 kb scaffold was identified that contained the hydrogenase operon, and this scaffold was annotated by RAST[44]. Polynucleotide probe design and geneFISH were done as previously described[31,45], with modifications listed in Supplementary Information (Supplementary Tables 5 and 6). Immunohistochemistry was done by microwaving gill sections of *B. puteoserpentis*, blocking in western blocking reagent (Roche), incubating in primary antibody, then in HRP-conjugated secondary antibody. Signal amplification was done by CARD[46]. The ISMS was deployed as previously described[47] with modifications listed in Supplementary Information. Statistical analysis was done with the software JMP5.

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Figure 3 | Hydrogen is consumed in mussel beds of *B. puteoserpentis*. Plot of hydrogen concentrations versus temperature, measured by *in situ* mass spectrometry in the source fluid and in a *B. puteoserpentis* mussel bed. The unbroken line shows linear regression analysis for the source fluid ($y = 83.308x - 240$, $R^2 = 0.63$), the broken line shows linear regression analysis for the mussel bed fluid ($y = 43.576x - 120$, $R^2 = 0.56$). The difference in the slopes of the regression lines between the source fluid and in the mussel bed is due to the consumption of hydrogen in the mussel bed.

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