RESEARCH ARTICLE

Geochemical constraints on the diversity and activity of H_2-oxidizing microorganisms in diffuse hydrothermal fluids from a basalt- and an ultramafic-hosted vent

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Abstract

Mixing processes of reduced hydrothermal fluids with oxygenated seawater and fluid–rock reactions contribute to the chemical signatures of diffuse venting and likely determine the geochemical constraints on microbial life. We examined the influence of fluid chemistry on microbial diversity and activity by sampling diffuse fluids emanating through mussel beds at two contrasting hydrothermal vents. The H_2 concentration was very low at the basalt-hosted Clueless site, and mixing models suggest O_2 availability throughout much of the habitat. In contrast, effluents from the ultramafic-hosted Quest site were considerably enriched in H_2, while O_2 is likely limited to the mussel layer. Only two different hydrogenase genes were identified in clone libraries from the H_2-poor Clueless fluids, but these fluids exhibited the highest H_2 uptake rates in H_2-spiked incubations (oxic conditions, at 18 °C). In contrast, a phylogenetically diverse H_2-oxidizing potential was associated with distinct thermal conditions in the H_2-rich Quest fluids, but under oxic conditions, H_2 uptake rates were extremely low. Significant stimulation of CO_2 fixation rates by H_2 addition was solely illustrated in Quest incubations (P-value < 0.02), but only in conjunction with anoxic conditions (at 18 °C). We conclude that the factors contributing toward differences in the diversity and activity of H_2 oxidizers at these sites include H_2 and O_2 availability.

Introduction

Hydrothermal fluids provide the grounds for life in deep-sea vent habitats. They transport reduced inorganic compounds such as H_2 and sulfide, which can be oxidized by microorganisms to yield energy for autotrophic CO_2 fixation. In these habitats, the type of host rock and the dilution of high-temperature endmember fluids with seawater influence the availability of inorganic compounds, which can vary considerably (cf. Butterfield *et al.*, 2004). As a consequence, different chemical reactions are thermodynamically favored, which are expected to govern abiotic constraints on microorganisms affecting their diversity and activity (McCollom & Shock, 1997; McCollom, 2007). At ultramafic-hosted vent sites, H_2 concentrations are high and H_2-oxidizing metabolisms are suggested to dominate, whereas in basalt-hosted settings, H_2 is substantially lower and microbial sulfide oxidation is considered to prevail (Wetzel & Shock, 2000; McCollom, 2007).

Microbial consumption and production of molecular H_2 is catalyzed by hydrogenase enzymes. Three phylogenetically unrelated types are known: [NiFe]-hydrogenases, [FeFe]-hydrogenases and [Fe]-hydrogenases. The [NiFe]-hydrogenases include, among others, the H_2-consuming membrane-bound uptake hydrogenases (group 1) and the cytoplasmic bidirectional cofactor-reducing (group 3) hydrogenases (Vignais & Billoud, 2007). Uptake hydrogenases are respiratory enzymes, which provide energy by oxidizing H_2. The electrons are fed into the respiratory chain and energy is yielded by coupling electron transport to ATP synthesis.

FEMS MICROBIOLOGY ECOLOGY

Genes encoding the membrane-bound uptake hydrogenases can be found among phylogenetically diverse bacteria and archaea (Takai *et al.*, 2005; Vignais & Billoud, 2007). Bidirectional cofactor-reducing hydrogenases are able to bind soluble cofactors, such as cofactor 420 (F_{420}), NAD or NADP. They function reversibly, i.e. they may consume or produce H_2, and can thus reoxidize these cofactors under anaerobic conditions using protons of water as electron acceptors. Members of this group are mainly of archaeal origin. For example, *Methanococcales* frequently encountered at deep-sea vents (e.g. Huber *et al.*, 2002; Perner *et al.*, 2007a) are able to use H_2 and CO_2 as substrates for methanogenesis (cf. Thauer *et al.*, 2008). These methanogens gain energy for growth by chemiosmotic mechanisms (Blaut & Gottschalk, 1985) and using F_{420}-reducing hydrogenases to oxidize H_2 (Baron & Ferry, 1989).

The autotrophic CO_2-fixing potential has been illustrated among phylogenetically diverse *Alpha-*, *Gamma-* and *Epsilonproteobacteria* in multiple hydrothermal habitats by analyzing genes encoding key enzymes of the Calvin cycle and of the reverse tricarboxylic acid cycle (Campbell *et al.*, 2003; Campbell & Cary, 2004; Perner *et al.*, 2007b; Voordeckers *et al.*, 2008; Wang *et al.*, 2009). Radioactively labeled inorganic carbon incorporation experiments with hydrothermal fluids have estimated microbial CO_2 fixation rates for these habitats (Mandernack & Tebo, 1999 and references therein). They demonstrated that CO_2 fixation can be stimulated by supplementing vent emissions with reduced sulfur compounds (e.g. Tuttle *et al.*, 1983). However, the role of H_2 in stimulating microbial CO_2 fixation in vent fluids has, to our knowledge, not been evaluated. Given the large number of H_2 oxidizers isolated from deep-sea vent biotopes (cf. Campbell *et al.*, 2006; Miroshnichenko & Bonch-Osmolovskaya, 2006) and the rich energy source provided through its oxidation (Shock & Holland, 2004), H_2 is likely of importance for chemolithoautotrophic growth in these habitats.

In this study, we chose two chemically contrasting hydrothermal vent sites to investigate the impact of chemistry on the diversity and activity of bacterial and archaeal H_2 oxidizers. These were the Clueless site within the basalt-hosted Comfortless Cove field [4°48′S Mid-Atlantic Ridge (MAR)] with H_2-poor emissions (Perner *et al.*, 2009) and the Quest site within the ultramafic-hosted Logatchev field (14°45′N MAR) with H_2-rich effluents (Schmidt *et al.*, 2007). At both localities, low-temperature diffuse fluids emanate from densely inhabited mussel patches (genus *Bathymodiolus*). We combined vertical temperature profiles, fluid chemistry and microbial data to infer local mixing models and to allocate identified microorganisms to distinct thermal layers. We further investigated the H_2-oxidizing and CO_2-fixing potential by analyzing associated functional genes. The H_2-oxidizing activity of Clueless and Quest microorganisms was assessed by supplementing the fluids with H_2 and incubating them under selected conditions. The role of H_2 as a primary inorganic electron donor for fueling CO_2 fixation was evaluated by incubating hydrothermal fluids with and without artificially added H_2 and by measuring the incorporation of radioactively labeled inorganic carbon. Additionally, the relevance of O_2 availability and thus mixing processes for the microorganisms was assessed by performing the same incubations under oxic or anoxic conditions. To date, hydrogenase gene diversity has not yet been explored in different vent habitats. Additionally, no experiments with vent microorganisms are available that can be compared with the primary reactions proposed for powering autotrophy in basalt-hosted vs. ultramafic-hosted deep-sea vent systems (McCollom & Shock, 1997; McCollom, 2007).

Materials and methods

Sampling sites, sample collection and temperature measurements

The diffuse hydrothermal fluids emanated from mussel patches at Clueless (within the Comfortless Cove hydrothermal field), and at Quest (within the Logatchev hydrothermal field), are located at 4°48′S and 14°45′N, respectively, on the MAR at water depths of ~3000 m (Schmidt *et al.*, 2007; Perner *et al.*, 2009). Hydrothermal fluids were retrieved during dives with the remotely operated vehicle Kiel ROV 6000 (IFM-GEOMAR, Kiel) during the cruises HYDROMAR V (December 2007) and MAR-SUED IV (January 2008) with the research vessel *L'Atalante*. For the sampling of hydrothermal fluids, a pumped flow-through system with perfluoralkoxy sampling bottles (Garbe-Schönberg *et al.*, 2006) was used (for detailed sampling procedure, see Perner *et al.*, 2009). Fluid chemical and microbial community analyses were conducted using aliquots of identical samples. Vertical temperature profiles were measured using a temperature lance with eight sensors placed equidistantly at 4-cm intervals inside a steel tube. This instrument was handled by the ROV manipulator and vertically inserted between the mussels into the subsurface under visual control by the ROV camera. Data were transmitted simultaneously for real-time operation control on board and also stored in the submersible temperature recorder attached to the lance.

Fluid chemistry

Immediately after sample recovery, reactive gases were quantified. Dissolved O_2 was determined using Winkler titration (Carpenter, 1965). Dissolved H_2 was quantified on board by a gas chromatograph-equipped pulsed discharge detector as described elsewhere (Perner *et al.*, 2007a). Light dissolved hydrocarbons were analyzed on board applying a

purge and trap technique (Seifert *et al.*, 1999) and treated as described (Perner *et al.*, 2007a). The pH and Mg^{2+} are by courtesy of D. Garbe-Schönberg. The pH was measured (Mettler electrodes with Ag/AgCl reference electrode) at 25 °C in unfiltered sample aliquots. The Mg^{2+} concentrations in hydrothermal fluids were determined by inductively coupled plasma-optical emission spectrometry using a CIROS SOP instrument (Spectro, Germany). Before analysis, samples were 10-fold diluted and spiked to 5 mg L^{-1} yttrium for internal standardization. Calibration standards were seawater matrix-matched, accuracy and precision were controlled with IAPSO seawater and replicate measurements and found to be better than 3% and 1% RSD, respectively. The sulfide concentrations are the courtesy of H. Strauss and were measured photometrically following the methylene blue method (Cline, 1969).

DNA extraction, gene amplification, cloning and sequencing

For bacterial community analyses, 400 mL of hydrothermal fluids were concentrated on polycarbonate filters (type: GTTP, pore size 0.1 μm, Millipore, Eschborn, Germany) and kept at − 20 °C. DNA was extracted from filters using the UltraClean Soil DNA Isolation Kit (MoBio, Solana, CA) according to the manufacturer's instructions. Bacterial and archaeal 16S rRNA genes were PCR amplified using the primer sets 27F/1492R (bacteria) and 21F/958R (archaea) as described before (Lane, 1991; DeLong, 1992). The large subunit of the uptake [NiFe]-hydrogenase (group 1) (*hynL*), the β subunit of the ATP citrate lyase (*aclB*) (key enzyme of the reverse tricarboxylic acid cycle) and the RubisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) form I (*cbbL*) and form II (*cbbM*) (key enzymes of the Calvin cycle) were PCR amplified using the primers *hynL*110F/*hynL*410R (*hynL*), 892F/1204R (*aclB*), cbbLF/cbbLR (*cbbL*) and cbbMF/cbbMR (*cbbM*) according to the conditions described previously (Campbell *et al.*, 2003; Campbell & Cary, 2004; Takai *et al.*, 2005). Degenerate primers were designed for the α subunit of the F_{420}-reducing hydrogenase (*frhA*) of *Methanococcales*, namely *frhA*323F (5′-TGCAYTCVCACC CAYTAC-3′) and *frhA*1039R (5′-CTCTWGSWGCTTCRT GWAC-3′) (annealing 48 °C), and tested successfully for *Methanocaldococcus janaschii*, *Methanococcus maripaludis* S2, *Methanococcus voltae* and *Methanococcus aeolicus* Nankai-3. All amplifications were performed in triplicate, and products were pooled and separated by gel electrophoresis. The procedures for purifying the amplification products, cloning and sequencing have been described (Perner *et al.*, 2009). A total of 162 (bacterial 16S rRNA genes from Quest), 64 and 55 (archaeal 16S rRNA genes), 41 and 69 (*hynL*), 0 and 59 (*frhA*), 32 and 48 (*cbbL*), 45 and 43 (*cbbM*) and 64 and 62 (*aclB*) gene sequences were analyzed from Clueless and Quest fluids, respectively.

Clone and phylogenetic analyses

Procedures for editing sequences, translating functional genes into amino acids (Hall, 1999), comparing sequences with those in the public domain through BLAST searches (Altschul *et al.*, 1997), compiling and aligning them using the ARB FastAligner utility (Ludwig *et al.*, 2004) or performing alignments through the software tools TCOFFEE (http://www.tcoffee.org/) (Notredame *et al.*, 2000) and CLUSTALW (Larkin *et al.*, 2007) have been described before (Perner *et al.*, 2007b, 2009). Maximum-likelihood-based trees and 100 bootstrap replicates were constructed using PHYML (Guindon & Gascuel, 2003) and imported into ARB. Phylogenetic trees with *hynL*, *frhA*, *cbbL, cbbM* and *aclB* genes were determined from partial amino acid sequences (240, 220, 110, 220 and 90, respectively). 16S rRNA genes and amino acid sequences were clustered into operational taxonomic units (OTUs) with a cut-off of 97% and 93% identity, respectively. Phylogenetic trees were exported from ARB and imported into the UNIFRAC web application (http://bmf2.colorado.edu/unifrac) to perform a significance test (Lozupone *et al.*, 2006).

Catalyzed reporter deposition (CARD)-FISH and total cell counts

Hydrothermal fluids (10–90 mL) were immediately fixed upon sample recovery with formaldehyde (2% v/v) for 4–12 h and concentrated on white polycarbonate filters (type: GTTP, 0.1 μm pore size) (Millipore), left to air-dry and stored at − 20 °C. For the detection of probe-specific signals, the standard CARD-FISH procedure was performed (Pernthaler *et al.*, 2002). Horseradish peroxidase-labeled oligonucleotide probes used were EUB338 I–III, Alf968, Gam42a (along with its competitor probe cGam42a), Epsy549, Delta495a (along with its competitor probe cDelta495a), CF319a, Arch915 and NON338 (for references, see http://www.microbial-ecology.net/probebase/) (Loy *et al.*, 2003) and were purchased from Biomers.net (Ulm, Germany). All probe signal counts were corrected with the counts performed for the NON338 probe signals. Counterstaining of CARD-FISH preparations was conducted using 4′,6′-diamidino-2-phenylindole (DAPI) (5 μg mL^{-1}). A minimum of 500 DAPI-stained cells and the corresponding probe signals in different microscopic fields of view were counted. From the counts, the mean values were calculated. Microbial cell numbers were determined by counting DAPI signals and calculated for 1-mL sample. All filter sections were inspected using a Leica TCS-4D confocal microscope (Leica, Heidelberg, Germany).

Incubation experiments

Procedures

Fluid incubation experiments for determining CO_2 fixation rates and H_2 consumption rates were set up within 2 h of sample recovery. Three sets of experiments were performed, each in triplicate, for Clueless and Quest fluid samples. Two sets of fluids were spiked with H_2 and kept under oxic (oxic H_2-spiked) or anoxic (anoxic H_2-spiked) conditions. To the third set of fluids, no H_2 was added (unamended). O_2 concentrations were monitored in parallels for each set of incubations by Winkler titration (Carpenter, 1965). To establish anoxic conditions in selected H_2 incubations, vials were sparged with N_2 (4.0, Air Liquide, Germany) before and during addition of the fluids (see Alonso & Pernthaler, 2005). Headspaces of oxic and anoxic H_2-spiked incubations were flushed with H_2 (2% H_2 : 98% He, purity 3.0 and 4.5, Air Liquide) for approximately 2 min. Radioactively labeled ^{14}C-bicarbonate (Perkin Elmer) was added to a final activity of 60 kBq mL^{-1} to the unamended and H_2-spiked (oxic and anoxic) incubations. Immediately, a subsample was taken from the headspace and from the fluids for estimating the initial H_2 concentration and the activity of the radioactively labeled bicarbonate, respectively. After 30 h of incubation in the dark at 18 °C, formaldehyde was added to the samples (final concentration, 2%) and left for a further 12 h at 4 °C to stop microbial activity. Subsamples were taken from the headspace to determine the final H_2 concentrations. Excess CO_2 was removed by acidification and aeration. Incubation liquids (5 mL) were concentrated on filters to determine biologically incorporated inorganic carbon. Subsamples were taken for later CARD-FISH analyses. To exclude leakage of H_2 or O_2 from the vials, control experiments were performed, confirming the air tightness of the tubes. To exclude abiogenic reactions of fluids with the added H_2, control experiments were conducted with filtered fluids (filters: 0.1 μm pore size) and fluids treated with formaldehyde.

Estimating H_2 consumption and CO_2 fixation

The concentrations of dissolved H_2 were calculated from Crozier & Yamamoto (1974) under the assumption of Henry's law, i.e. the concentration of a dissolved gas in a solution is directly proportional to the partial pressure of that gas above the solution. The molar volume of an ideal gas (22.414 L mol^{-1}) was used to convert between the partial pressure of a gas (%) and the amount of that gas (moles) in the headspace. Based on these calculations, the initial dissolved H_2 concentrations were between 12 and 14 μM. H_2 uptake rates, i.e. H_2 removal from the headspace, were calculated for the incubation time of 30 h.

The initial activity from the subsample taken at the start of the experiment, the amount of radioactively labeled carbon incorporated into the cells after 30 h as well as blanks (filters) were measured using a scintillation counter (Perkin Elmer). CO_2 fixation rates [R_{CO_2} (mmol L^{-1} h^{-1})] were calculated from the equation:

$$R_{CO_2} = DIC \times 1.05 \times \frac{DPM_{inc}}{DPM_0} \times \frac{1}{t_{inc}}$$

where DIC is the measured dissolved inorganic carbon, i.e. 2.1 mmol L^{-1} for Clueless and 1.8 mmol L^{-1} for Quest fluids (H. Strauss, pers. commun.), 1.05 is the isotopic effect, DPM_{inc} and DPM_0 are the decays min^{-1} measured for the biologically incorporated carbon after the incubation and the initial $^{14}CO_2$ activity at the start of the experiment, respectively (after subtraction from blank DPM values of filters); t_{inc} refers to the incubation time (h). CO_2 fixation rates from formaldehyde-treated and filtered fluids were in the range of blank filters.

Nucleotide sequence accession numbers

The sequence data have been submitted to DDBJ/EMBL/GenBank databases under accession numbers FN870074–FN870139, FN870149–FN870186 (16S rRNA gene), FN908926, FN908927, FN908969-FN908979 (*hynL*), FN908937-FN908939 (*frhA*), FN908920-FN908925 (*aclB*), FN908928-FN908936 (*cbbL*) and FN908940-FN908968 (*cbbM*).

Results

Physicochemical parameters in Clueless and Quest habitats

Thermal conditions according to vertical temperature profiles

The vertical temperature profiles were measured up to a depth of 33 and 38 cm below the mussel patch surface at Clueless and at Quest, respectively, and differed considerably between the two habitats (Fig. 1a and b). Two thermal layers are illustrated for the Clueless habitat (Fig. 1c): a 14-cm thick layer (1) with a downward decreasing temperature gradient (2–8.5 °C) merges into a second layer (2) characterized by constant temperatures (~8.5 °C). In contrast, the vertical temperature profile through the Quest habitat (Fig. 1d) indicates three layers: a surficial layer (1) with almost homogenous temperatures (< 20 °C) overlays a layer (2) of roughly 8 cm with a steep temperature gradient. This layer is underlain by a third layer (3) with constant temperatures above 190 °C.

Chemical conditions according to discrete sampling

The chemical data are summarized in Table 1. Diffuse hydrothermal effluents from Clueless and Quest had similar O_2

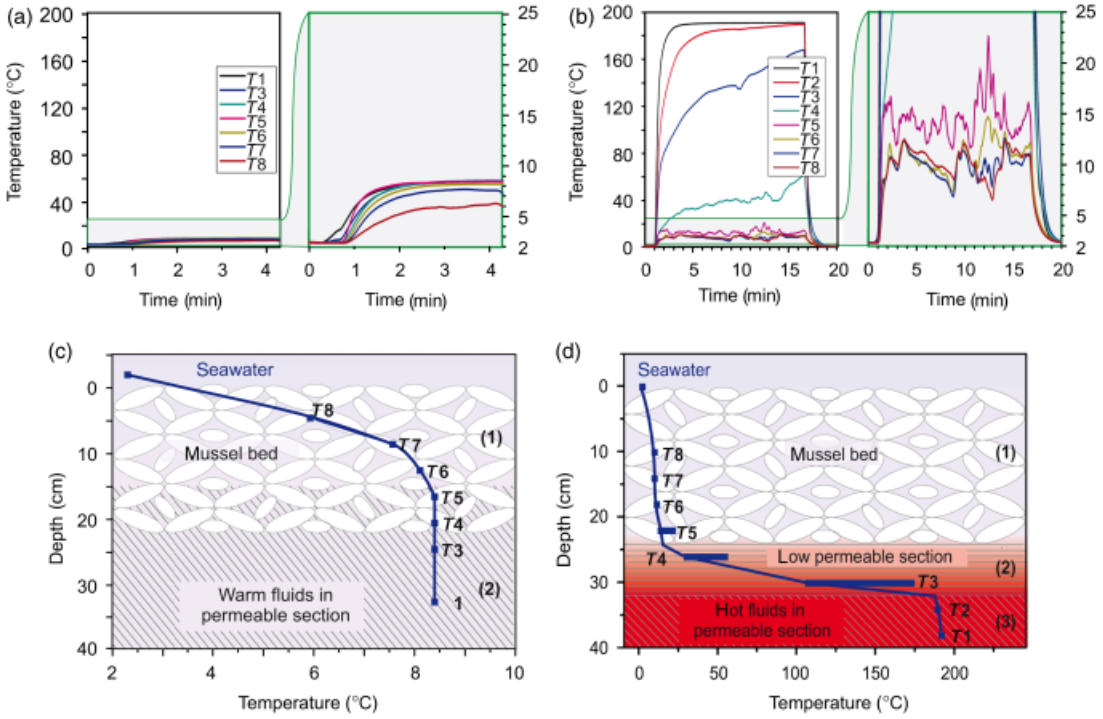

Fig. 1. Time series temperature data and temperature profiles from mussel patches associated with hydrothermal diffuse flow at the Clueless (a, c) and Quest (b, d) hydrothermal sites, respectively. Temperatures were measured simultaneously along a vertical profile at eight equidistant points, i.e. *T*1, *T*2, etc. Sensor depths [i.e. the distance from the upper mussel layer (= 0 cm)] are indicated by *T*1 = − 33 cm, *T*2 = − 29 cm, *T*3 = − 25 cm, *T*4 = − 21 cm, *T*5 = − 17 cm, *T*6 = − 13 cm, *T*7 = − 9 cm, *T*8 = − 5 cm for Clueless, and *T*1 = − 38 cm, *T*2 = − 34 cm, *T*3 = − 30 cm, *T*4 = − 26 cm, *T*5 = − 22 cm, *T*6 = − 18 cm, *T*7 = − 14 cm, *T*8 = − 10 cm for Quest. In (c, d), (1), (2) and (3) denote the different layers. The horizontal extension of the blue bars (d) indicates the range of measured temperatures while the lance was placed inside the substrate.

Table 1. Physicochemical factors from Clueless and Quest fluids

Physicochemical parameters	Ambient seawater	Clueless	Quest (LHF)
T (°C)*	2	6–9	8–190
Mg^{2+} (mM)	55	54.5 ± 0.7	43.1 ± 0.03
Fraction of endmember fluid (%)	0	< 1	22
pH (25 °C)	8.1	7.2 ± 0.3	7.6 ± 0.1
Sulfide (μM)	< 1†	21 ± 17	< 1†
H_2 (μM)	0.0005	< 0.005‡	21
CH_4 (μM)	0.001	0.9	12
O_2 (μM)	254 ± 17	224 ± 19	250 ± 21

The physicochemical factors from Clueless fluids – except for temperature – originate from Perner *et al*. (2009). Mean values and SDs were calculated whenever possible. The pH and Mg^{2+} data are by courtesy of D. Garbe-Schönberg. Sulfide concentrations are by courtesy of H. Strauss.

*Temperature is measured up to a depth of roughly 38 cm.

†Sulfide concentrations of < 1 μM denote the detection limit of the method.

‡The H_2 concentration of the sample was below the detection limit, which was 5 nM during the measurement of this sample.

concentrations (224 ± 19 and 250 ± 21 μM, respectively), but fluids contained differing amounts of Mg^{2+} (54.5 ± 0.7 and 43.1 ± 0.03 mM, respectively) and thus different fractions of hydrothermal fluid (< 1% and 22%, respectively). The availability of inorganic electron donors differed substantially between the two locations: at Clueless, H_2 was very low (< 5 nM) and sulfide was high (21 ± 17 μM). Inversely, in the Quest effluents H_2 (21 μM) was considerably enriched over sulfide (< 1 μM). In Clueless fluids CH_4 was 0.9 μM and in Quest emissions CH_4 was 12 μM.

Phylogenetic analyses from Clueless and Quest fluids

Bacterial and archaeal 16S rRNA genes

The microbial communities are overall significantly different from each other (*P*-value < 0.01). Members of *Alpha*-, *Gamma*-, *Epsilon*- and *Deltaproteobacteria*, *Bacteroidetes* and *Verrucomicrobia* are present in both fluids (see Supporting Information, Fig. S1a–c), but subordinate taxa such as genera or species are hardly shared between the two sites.

For example, *Gammaproteobacteria* unique to Clueless (95%) include sulfide-oxidizing *Thiomicrospira* spp. and *Thialkalimicrobium* spp. (Sorokin *et al.*, 2001; Takai *et al.*, 2004). *Gammaproteobacteria* unique to Quest (87%) include species related to methylotrophic *Methylophaga* (Janvier & Grimont, 1995), methanotrophic symbionts of *Bathymodiolus* mussels (Duperron *et al.*, 2006) and sulfur-oxidizing endosymbionts of an oligochaete (Dubilier *et al.*, 2001) (Fig. S1a). Some of the *Alphaproteobacteria* were shared between both habitats (Fig. S1a). These sequences were affiliated to an isolate enriched from the Sargasso Sea (Lanoil *et al.*, 2000) and a sequence from the Black Sea. *Alphaproteobacteria* unique to Quest were related to sequences from sediments of the Nankai Trough (Li *et al.*, 1999) and from the Antarctic continental shelf (Bowman *et al.*, 2003). *Deltaproteobacteria* from both sites were exclusively associated with *Desulfobacterales* (Fig. S1a). *Epsilonproteobacteria* from Clueless and Quest were related to *Sulfurimonas* spp. of group B, and *Sulfurovum* spp. of group F (classification according to Corre *et al.*, 2001) and to affiliates of the *Arcobacter* genera within the *Campylobacterales* (Fig. S1b). Members of the candidate division RE could only be identified at Clueless (Fig. S1c). *Dehalococcoidales*, *Clostridiales* and *Deinococcus* spp. were found at Quest, but not at Clueless (Fig. S1c). Many archaea were shared between Clueless (37%) and Quest (13%) (Fig. S1d and e). These were uncultured species from nonhydrothermal marine habitats (Sørensen & Teske, 2006; Gillan & Danis, 2007) likely entrained with seawater. *Thermococcales*, *Desulfurococcales* and *Methanococcales* were only identified at Quest.

Bacterial uptake hydrogenases and archaeal F_{420}-reducing hydrogenases

Only two bacterial uptake hydrogenase OTUs were amplified from the Clueless effluents (Fig. 2a). One uptake hydrogenase OTU made up 98% of all clones sequenced, and was associated with a cluster of hydrogenases found in mesophilic *Beta-* and *Gammaproteobacteria*. The second OTU out (2% of all clones) was related to a hydrogenase from *Nitratifractor salsuginis* (Takai *et al.*, 2005), a mesophilic, microaerobic, nitrate-reducing epsilonproteobacterium (cf. Campbell & Cary, 2004). We could not detect any more uptake hydrogenases of other potential bacterial H_2 oxidizers from these fluids, even though we identified 16S rRNA genes of potential candidates including members of the *Bacteroidetes*, *Alpha-*, *Gamma-* and *Epsilonproteobacteria* (Fig. S1a–c). The absence of hydrogenases associated with these groups is unlikely to be due to primer bias, because uptake hydrogenase gene fragments of members of these groups can be successfully amplified with the primers we used (M. Perner, unpublished data). However, these primers are known to be biased against the hydrogenase sequences of some *Deltaproteobacteria*. The H_2 uptake potential within this group is therefore probably not covered to its full extent. No F_{420}-reducing hydrogenases were amplified from Clueless fluids.

A comparatively high richness of hydrogenases is illustrated in the Quest liquids (Fig. 2a). The bacterial uptake hydrogenases are represented by 11 OTUs. One OTU (1% clonal frequency) was related to alphaproteobacterial uptake hydrogenases. The other 10 OTUs were affiliated to uptake hydrogenases of *Epsilonproteobacteria* (Takai *et al.*, 2005), namely to mesophilic, microaerobic and nitrate-reducing *Sulfurovum* spp. and *Sulfurimonas* spp. (48% clonal frequency) and to thermophilic, anaerobic nitrate- or sulfur-reducing *Caminibacter mediatlanticus* (51% clonal frequency) (cf. Campbell *et al.*, 2006). Three F_{420}-reducing hydrogenase OTUs were detected in Quest fluids and were related to those of *M. janaschii* (Fig. 2b). Except for uptake hydrogenases related to those of *C. mediatlanticus*, hydrogenases followed 16S rRNA gene phylogeny.

Genes encoding key enzymes of the Calvin cycle and the reverse tricarboxylic acid cycle

Our RubisCO form I gene sequences were exclusively associated with those from vent habitats including plume, chimney structure, diffuse emissions and *Bathymodiolus azoricus* mussels (Elsaied *et al.*, 2006; Spiridonova *et al.*, 2006; Perner *et al.*, 2007b) (Fig. S2a). RubisCO form II genes were scattered among distinct *Alpha-* and *Gammaproteobacteria* (Fig. S2b). In both fluids, RubisCO form II genes were related to sulfide-oxidizing *Thiomicrospira* spp. and *Halothiobacillus* spp. Several RubisCO form II genes were affiliated with *Rhodobacteraceae* and were shared between Clueless and Quest. *AclB* genes were exclusively associated with *Epsilonproteobacteria*, namely of '*Candidatus* Arcobacter sulfidicus', *Sulfurimonas* spp. (group B) and *Sulfurovum* spp. (group F) as the closest cultured representatives (Fig. S2c).

Turnover rates in incubation experiments using Clueless and Quest fluids

H_2 uptake, CO_2 fixation rates and O_2 concentrations

The microbial H_2 uptake and CO_2 fixation rates in Clueless and Quest incubations are displayed in Fig. 3a–d, respectively. The consumption and fixation rates are estimated per volume (Fig. 3a and c) and per cell (Fig. 3b and d). The single values are listed in Table S1, where O_2 concentrations before and after the experiments are included. All measured

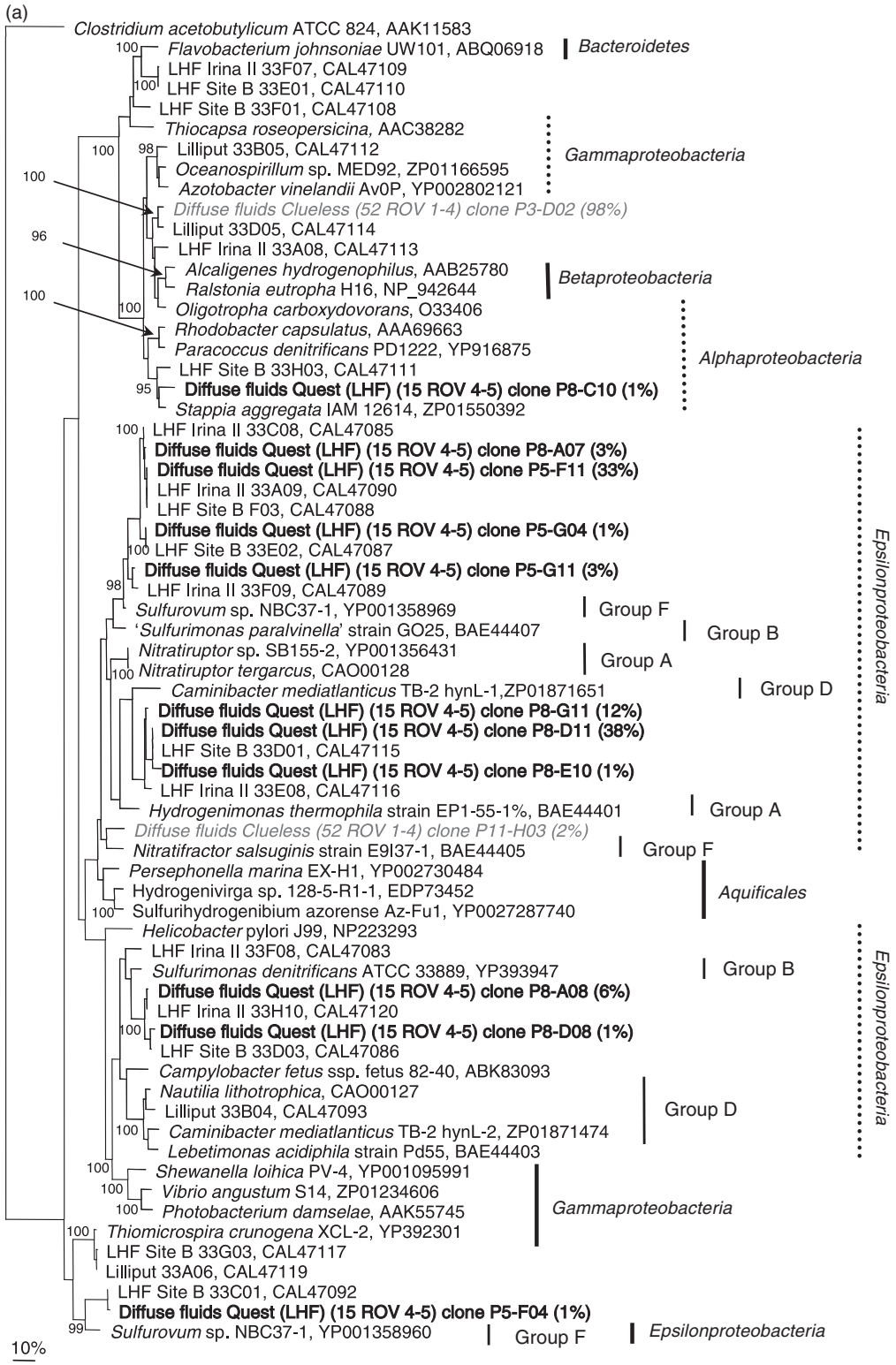

Fig. 2. Phylogenetic relationships among [NiFe]-hydrogenase gene fragments (based on amino acid sequences) of the large subunits of the bacterial uptake hydrogenase (*hynL*) (a) and of the coenzyme F_{420}-reducing hydrogenase (*frhA*) (b) are based on maximum-likelihood analysis. The percentage of bootstrap resamplings is indicated at the nodes and only bootstrap values $\geq$93 are shown. Sequences obtained from Clueless and Quest diffuse fluids are listed in gray bold/italic and black bold, respectively. Percentages in parentheses denote the clonal frequencies. The scale bar represents the expected number of changes per amino acid position. *Epsilonproteobacteria* (a) are grouped according to Corre *et al*. (2001).

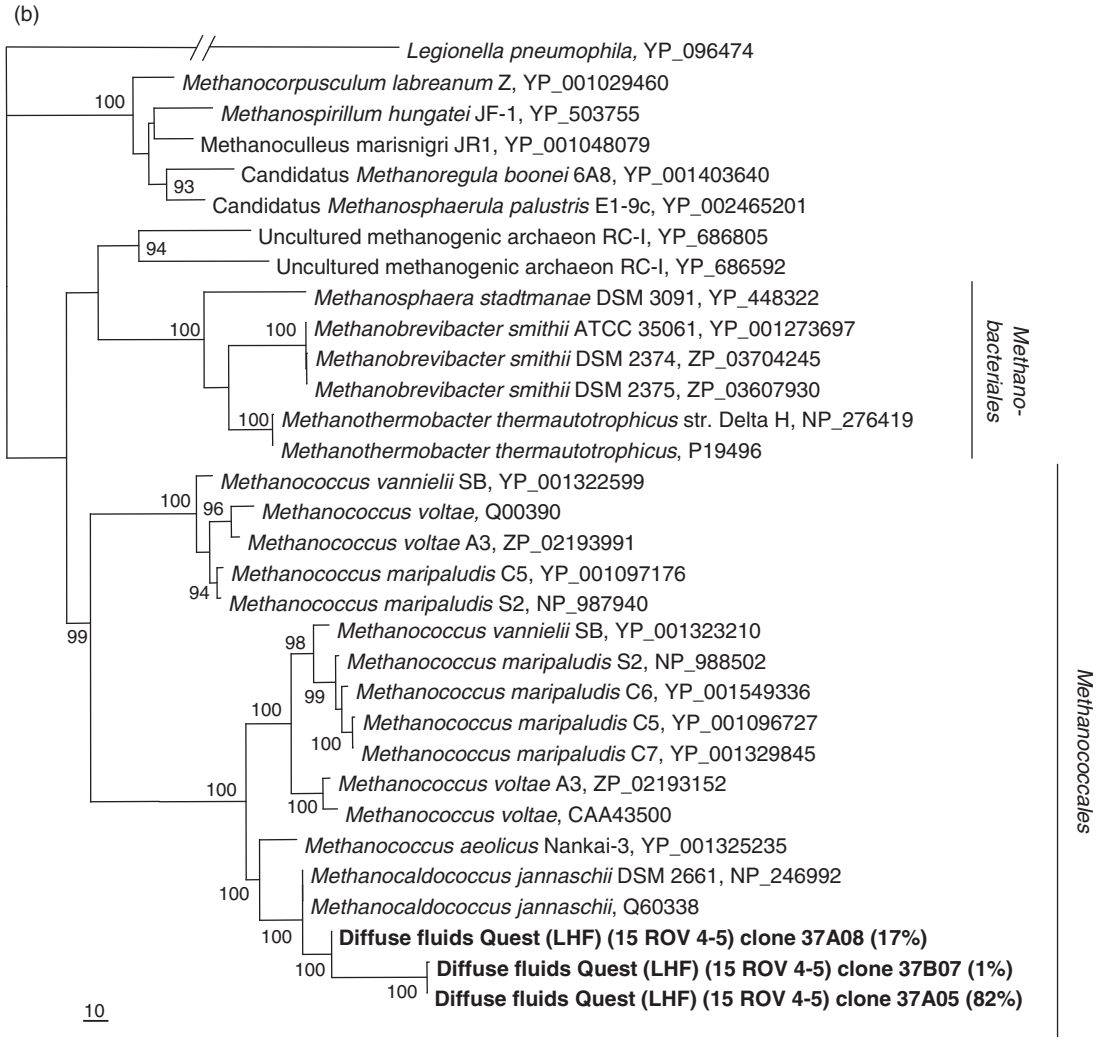

Fig. 2. Continued.

unamended and oxic Clueless and Quest incubations remained oxic throughout the experiments.

The highest H_2 consumption rates and CO_2 fixation rates were in the oxic H_2-spiked Clueless fluids. H_2 was also consumed in the anoxic H_2-spiked Clueless fluids, but no CO_2 was fixed. In the Quest H_2-supplemented incubations, the highest H_2 uptake rates and CO_2 fixation rates were in the oxic H_2-spiked incubation, but only on a per volume estimation (Fig. 3a and c). When calculating the rates per cell, the highest rates are found in the anoxic H_2-spiked incubations (Fig. 3b and d). Estimated ratios of H_2 oxidized to CO_2 fixed are displayed in Table S1 and were between 11 : 1 and 22 : 1 for oxic H_2-amended Clueless fluids. For oxic H_2-amended Quest fluids, they ranged between 5 : 1 and 9 : 1 and for anoxic H_2-amended Quest fluids they were between 3 : 1 and 12 : 1.

Abundance of microbial groups in Clueless and Quest fluids

Environmental fluids before incubation experiments

The total cell numbers in the Clueless fluid samples were higher than those from Quest ($0.8 \pm 0.1 \times 10^6$ and $0.3 \pm 0.2 \times 10^6$ cells mL^{-1}, respectively) (Fig. 4a) and are comparable to the cell numbers reported from other hydrothermal habitats (e.g. Tuttle *et al.*, 1983; Nakagawa *et al.*, 2005b). The relative abundance of the different microbial groups based on CARD-FISH with general probes was similar between the Clueless (Perner *et al.*, 2009) and the Quest fluid samples. These were $32 \pm 9\%$ and $28 \pm 6\%$ *Gamma*-, $9 \pm 5\%$ and $3 \pm 2\%$ *Alpha*-, $3 \pm 2\%$ and

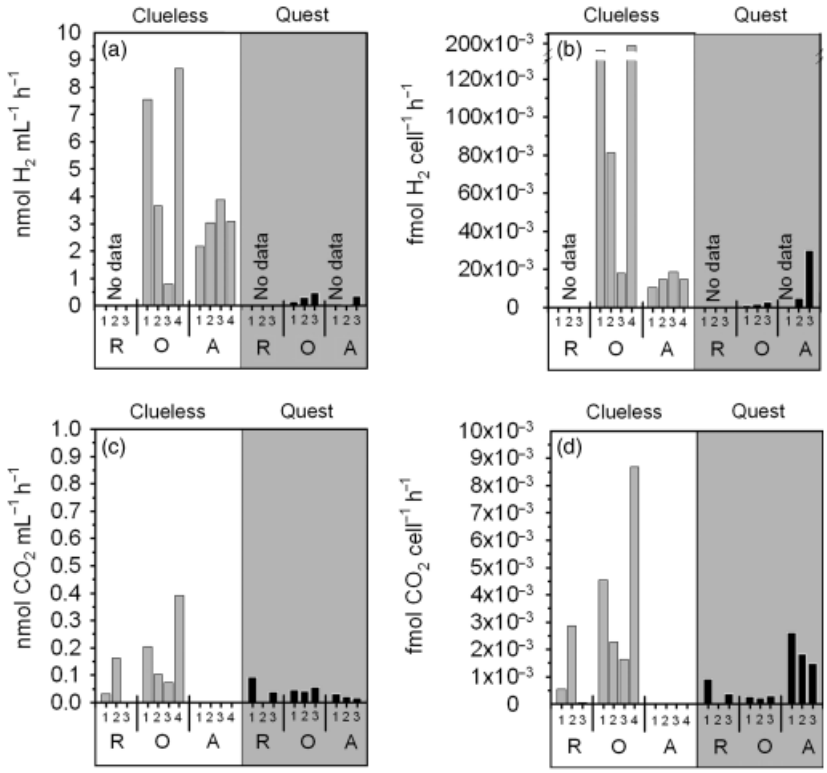

Fig. 3. H_2 consumption rates (a, b) and CO_2 fixation rates (c, d) from incubation experiments with fluids from Clueless (white background) and Quest (gray background). Rates in (a, c) are estimated per volume and those in (b, d) are calculated per cell. Consumptions on single cells are based on the assumption that all organisms are utilizing the substrate, and thus denote conservative estimates. The unamended fluids are labeled by 'R' and the H_2-supplemented fluids by 'O' for oxic conditions (the O_2 concentrations are listed in Table S1) and 'A' for anoxic conditions. The different replicates are denoted 1, 2, 3 and 4.

$5 \pm 3\%$ *Delta*-, $37 \pm 3\%$ and $44 \pm 5\%$ *Epsilonproteobacteria*, $12 \pm 6\%$ and $13 \pm 4\%$ *Cytophaga–Flavobacteria* group and $< 2\%$ and $8 \pm 1\%$ archaea, respectively. For absolute numbers of microbial groups, see Fig. 4a.

Fluids after incubation experiments

In all cases, an increase in the microbial abundance after the incubation period (Fig. 4b) compared with the environmental fluids was observed (see also Table S1). The highest increase in cell numbers was associated with the anoxic H_2-spiked Clueless (ninefold), with the unamended Quest (11-fold) and with the oxic H_2-spiked Quest (19-fold) fluids. The community compositions changed more drastically in Clueless fluid incubations than in Quest incubations (Fig. 4b). In all Clueless incubations, *Gammaproteobacteria* became the dominant group after incubation. Particularly in the anoxic H_2-amended fluids, *Gammaproteobacteria* increased 23-fold over the environmental sample. *Epsilonproteobacteria* could not be detected following oxic and anoxic H_2-spiked Clueless incubations. *Deltaproteobacteria* only proliferated to a significant level in the anoxic H_2-spiked Clueless incubations (14-fold). The Quest fluid incubations maintained a larger number of different microbial subclasses/phyla than the Clueless fluids after the incubation periods (Fig. 4b). *Gamma*-, *Alphaproteobacteria* and affiliates targeted by the *Cytophaga–Flavobacteria* probe were below the detection limit in the unamended fluids, but proliferated in the oxic H_2-spiked incubations 11-, 77- and 3-fold, and in the anoxic H_2-spiked incubations 2-, 3- and 0.4-fold, respectively. A considerable increase of *Deltaproteobacteria* was found in various Quest incubations: 66 times in unamended, 108 times in oxic H_2-spiked and nine times in anoxic H_2-spiked fluids. *Epsilonproteobacteria* proliferated in the unamended (12-fold) and in the oxic H_2-spiked (fourfold), but decreased sevenfold in the anoxic incubation.

Discussion

Mixing models for Clueless and Quest habitats

Mixing models for Clueless (Fig. 5a) and Quest (Fig. 5b) are inferred from vertical temperature profiles (Fig. 1), fluid chemistry (Table 1) and the microorganisms identified in the hydrothermal fluids (Fig. 2 and S1). These parameters suggest distinct mixing properties in the two sampled habitats.

At Clueless, a high dilution of reduced liquids with fresh seawater is indicated by the Mg^{2+} concentration, which is close to that of seawater. According to the vertical temperature profiles, two thermal layers are suggested passing over into each other (Fig. 5a). The upward decreasing temperatures (2–8.5 °C) in the upper section (layer 1) appear to be caused by an increasing amount of fresh seawater diluting

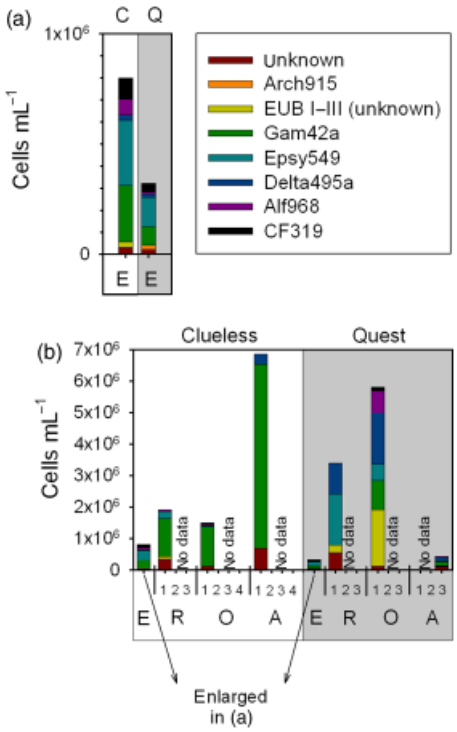

Fig. 4. Microbial community composition of fluid samples from Clueless (background shaded in white) and Quest (background shaded in gray), before (a) and after incubation experiments (b). Cell numbers result from cell counts after DAPI staining and CARD-FISH with group-specific probes targeting different microbial groups (indicated by different colors; non-hybridized DAPI counts are labeled 'unknown'). The community compositions of fluid samples before experimental incubations are indicated by 'E'. Community compositions after incubation (30 h) are designated 'R' (unamended fluids), 'O' and 'A', i.e. hydrothermal fluids spiked with H_2 and kept under oxic and anoxic conditions, respectively. In (a), 'C' and 'Q' indicate Clueless and Quest, respectively. For clarity, community compositions of nonincubated environmental fluid samples (a) are shown at scales different from those from the experiments (b). The different replicate samples are denoted 1, 2, 3 and 4 (b). Cell counts and relative abundance of the Clueless sample were from Perner *et al.* (2009) and were estimated as absolute cell counts of distinct microbial lineages.

and cooling the ascending warm hydrothermal fluids on their way through the mussel bed. In the subsurface (layer 2), the absence of a vertical thermal gradient and the very low amplitude of temperature variations (Fig. 1a and c) with a SD of $< 0.1\,^{\circ}C$ suggest that the temperature differences between hot hydrothermal fluids and cool seawater are already well adjusted. This means mixing of hot hydrothermal fluids with fresh seawater is taking place at some distance and the homogenous warm fluids are moving through the sections. In agreement with this mixing model, no genes were found in the Clueless effluents, which were associated with those of thermophiles (Fig. 2 and S1) (Perner *et al.*, 2009). This provides a further indication for the absence of an immediate hot subground at Clueless.

A very different mixing model is suggested for the Quest habitat. Mg^{2+} values indicate a greater contribution of reduced fluids to the emanating liquids. According to the vertical temperature profile, a distinction between three thermal depth intervals is evident (Fig. 5b). Compared with Clueless, a 15-fold higher temperature variability in time layer 1 with an SD of $1.5\,^{\circ}C$ and upward decreasing temperatures and temperature variability is observed. This lack of small-scale temperature homogenization suggests that minor amounts of very hot vent fluids are locally admixed to the fresh seawater that is passing through the 23-cm thick mussel bed. The approximately 8-cm thick layer 2 below is characterized by a very steep temperature gradient ($> 20\,^{\circ}C\,cm^{-1}$). The low thermal conductivity of layer 2 as indicated by the strongly increased thermal gradient in comparison with layers 1 and 3 indicates a nearly blocked advective heat transport and thus a low permeability for layer 2. This low permeable section at the bottom of the mussel bed appears to be the lower boundary of fresh seawater circulation and allows only a minor vertical outflow of the hot hydrothermal fluids. The continuous increase of temperature with time in layer 2 is probably caused by penetration of the inserted temperature probe, allowing some hot extra fluids to rise along this newly created pathway. This indicates a – generally blocked – tendency to upward flow from layer 3 instead of downward intrusion of cold seawater into layers 2 and 3. The smooth shape of the temperature vs. time curve in the bottom section layer 3 with an SD of only $0.3\,^{\circ}C$ at the lowest sensor *T*1 demonstrates the absence of sudden and local mixing with cold seawater (Fig. 1d). The Quest mixing model is supported by the microorganisms identified in the fluids (Fig. 2 and S1). They included microorganisms associated with mesophilic and (hyper)thermophilic lifestyles, which are constrained by the thermal regimes and can thus be allocated to the different layers. Typical mesophiles included *Epsilonproteobacteria* of groups B and F (cf. Corre *et al.*, 2001; Campbell *et al.*, 2006) (Fig. S1b), which were likely entrained from the mussel layer (layer 1, $< 20\,^{\circ}C$) and the upper part of layer 2 ($20–50\,^{\circ}C$) (Fig. 5b). An area supportive of thermophilic growth is limited to the immediate shallow subsurface (layer 2), probably characterized by strongly reduced chemical features. Indications for bacterial and archaeal thermophiles stem from hydrogenases affiliated with *C. mediatlanticus* (temperature optimum, $55\,^{\circ}C$) (cf. Campbell *et al.*, 2006) (Fig. 2a) and from the presence of (hyper)thermophilic *Methanococcales*, *Thermococcales* and *Desulfurococcales* (cf. Miroshnichenko & Bonch-Osmolovskaya, 2006) (Fig. 2b, S1d and e). At depths below layer 2, life is highly unlikely because of temperatures $> 190\,^{\circ}C$, and thus exceeding the currently accepted maximum temperatures supporting microbial growth (Kashefi & Lovley, 2003; Takai *et al.*, 2008a).

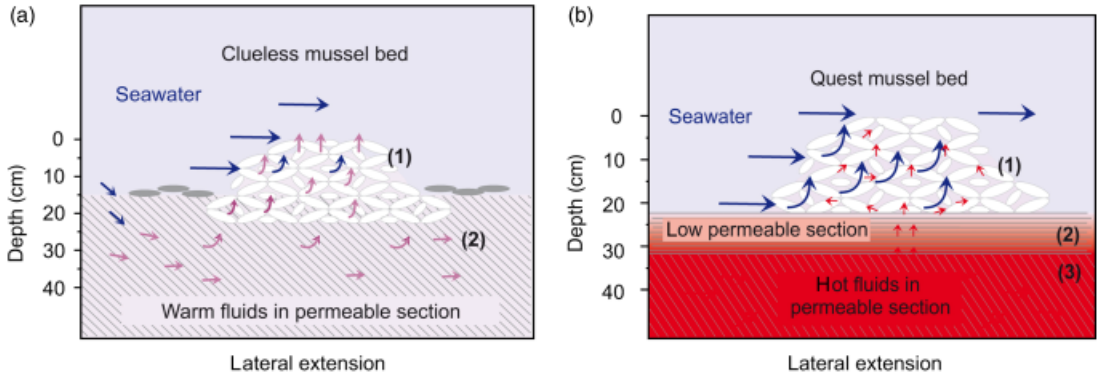

Fig. 5. Mixing models for Clueless (a) and Quest (b) habitats. The models are inferred from the vertical temperature profiles (Fig. 1), from the fluid chemistry (Table 1) and from the microorganisms identified in the hydrothermal fluids (Fig. 2 and S1). (1), (2), and (3) denote the different thermal layers.

Geochemical constraints on the microbial diversity from Clueless and Quest

H_2 availability and diversity of H_2 oxidizers

Owing to the basalt rocks hosting the Clueless environment, H_2 is considerably lower than in the fluids from the ultramafic-hosted Quest location (Logatchev) (Schmidt *et al.*, 2007; Perner *et al.*, 2009) (Table 1). According to catabolic energy calculations performed for three fluid samples from Clueless, the extremely low H_2 concentration (< 5 nM) is also attributed to microbial H_2 consumption (Perner *et al.*, 2009). Consequently, if microbial H_2 consumption occurs along the fluid pathways, remnants of H_2 oxidizers should be found in the H_2-poor emanated fluids. Based on 16S rRNA genes, no typical H_2 oxidizers were identified in the effluents (Fig. S1). However, phylogenetically diverse H_2 oxidizers could be too rare to be detected among more abundant metabolically versatile microorganisms such as *Alpha-*, *Gamma-*, *Delta-* and *Epsilonproteobacteria* (Campbell *et al.*, 2006; Vignais & Billoud, 2007). Therefore, genes encoding H_2-utilizing enzymes were analyzed. The hydrogenase genes exhibited a low richness in the Clueless effluents (Fig. 2a), thus reflecting the limited phylogenetic diversity among the targeted H_2 oxidizers at this site. If the low H_2 concentration in the Clueless habitat is indeed partially a consequence of microbially mediated H_2 oxidation (Perner *et al.*, 2009), then the responsible H_2 oxidizers likely belong to a few phylogenetically divergent lineages.

In contrast, a phylogenetically diverse H_2-oxidizing potential in the H_2-rich Quest effluents is illustrated by a high richness of hydrogenase genes (Fig. 2), which has been detected in other H_2-rich Logatchev fluids before (M. Perner, unpublished data). The affiliation of the hydrogenases to mesophilic and (hyper)thermophilic microorganisms reflects the broad thermal range at which H_2 oxidation in the Quest habitat could be performed. The identification of *Dehalococcoidales*, *Methanococcales* or *Desulfurococcales* (Fig. S1c–e), which have been described as H_2 oxidizers (Löffler *et al.*, 1999; Huber & Stetter, 2006; Thauer *et al.*, 2008), further supports the high H_2-oxidizing diversity in this fluid sample.

Chemistry and CO_2-fixing metabolisms

The concentrations of erupted gases in hydrothermal plumes have been correlated with the diversity of genes encoding CO_2-fixing enzymes (Elsaied *et al.*, 2006). However, the RubisCO form I, RubisCO form II and *aclB* gene diversities did not significantly differ between the Clueless and the Quest emissions. As the RubisCO OTU richness exceeded that of the *aclB* genes (Fig. S2), autotrophic CO_2 fixation might be predominantly carried out by organisms operating the Calvin cycle rather than the rTCA cycle as shown for other vent habitats (Wang *et al.*, 2009). Yet, the limited number of *aclB* OTUs might simply reflect the limited phylogenetic resolution (Voordeckers *et al.*, 2008) of the comparatively short and conserved region of the amplified *aclB* gene fragments. Alternative autotrophic pathways might considerably contribute to biomass synthesis, but genes encoding these key enzymes were not sought.

Geochemical constraints on the microbial activities from Clueless and Quest

Microbial H_2 uptake rates based on incubation experiments

Under the provided incubation conditions (i.e. 18 °C; after H_2 addition at the start of the experiment 12–14 μM H_2), species from Clueless consumed more H_2 than those from Quest (Fig. 3a and b; Table S1). Generally, very little data are available for comparing our H_2 consumption rates with

those from natural environments (H_2 uptake per unit volume per unit time) or enrichments (H_2 uptake per cell per unit time). The microbial H_2 consumption rates for an uncontaminated aerobic aquifer were within the range of those from the Clueless incubations, but exceeded those from the Quest incubations (Fig. 3a) (Harris *et al.*, 2007). In contrast, H_2 uptake from a contaminated aquifer site, where SO_4^{2-} reduction prevailed, considerably exceeded the rates from Clueless and Quest incubations (Harris *et al.*, 2007). H_2 uptake rates between 420×10^{-3} and 7140×10^{-3} fmol H_2 per cell h^{-1} have been determined for different isolates (Häring & Conrad, 1991; Klüber & Conrad, 1993; Olsen & Maier, 2002). Based on our measurements, comparable H_2 uptake is only found in the oxic H_2-spiked Clueless incubations ($115 \pm 81 \times 10^{-3}$ fmol H_2 per cell h^{-1}) (Fig. 3b). Consequently, this indicates the ability of specific Clueless microorganisms to respond to H_2 addition – under the provided conditions, i.e. 18 °C, and 172 ± 27 μM O_2, 12–14 μM H_2 at the start of the incubation – by rapidly consuming the H_2 offered. Indeed, fluctuations in H_2 concentrations have been observed in the Clueless effluents (< 5–36 nM) (Perner *et al.*, 2009). Under these conditions, microorganisms able to exploit sudden inputs of H_2 would have a considerable advantage.

The H_2 uptake rates in all the other incubations were considerably lower (Fig. 3b). Thus, H_2 oxidizers likely comprise a small component of these incubation communities and/or exhibit very low H_2 uptake rates. Temperature issues can be of major concern when evaluating our H_2 consumption rates. This particularly applies to Quest incubations, as incubation temperatures only reflect thermal conditions of the Quest mussel layer (< 20 °C). Our incubation conditions therefore favor the microorganisms from this layer. To assess the H_2-oxidizing potential from the hotter Quest subsurface (> 20 °C), fluids would have to be incubated at higher temperatures. Roughly half of the identified hydrogenase OTUs were associated with (hyper)thermophiles, putatively contributing to the total H_2 turnover in Quest fluids. Hence, it appears that increased incubation temperatures could cause elevated H_2 uptake rates.

Microbial CO_2 fixation rates based on incubation experiments

Overall, the CO_2 fixation rates in Clueless and Quest fluid incubations exceed those of heterotrophic dark fixation (Fig. 3c; Table S1) (e.g. Labrenz *et al.*, 2005) and can thus be associated with autotrophic CO_2 fixation. The high variability of Clueless and Quest CO_2 fixation rates among replicate samples of the same site and the corresponding incubation treatments (Fig. 3c and d) is common for vent fluids and can be likely attributed to the uneven distribution of particles (Tuttle *et al.*, 1983). Our *ex situ* rates (Fig. 3c) exceed those from deep-sea vent *in situ* incubations (Mandernack & Tebo, 1999 and references therein) and likely deviate from the actual *in situ* rates, because of differences in hydrostatic pressure (Tuttle *et al.*, 1983; Fisher *et al.*, 1989). In any case, the rates provide a rough estimate of the microbial CO_2-fixing ability and allow a comparison between the rates of the equally treated incubations with fluids from the two sites.

The highest CO_2 fixation rates were exhibited in the Clueless fluid incubations (Fig. 3d; Table S1) and are in the same range of the lowest rates determined for marine nitrifying bacteria (Glover, 1983) and vent *Bathymodiolus* mussel symbionts (Belkin *et al.*, 1986). This illustrates the huge autotrophic potential for Clueless microorganisms under oxic incubation conditions. The highest CO_2 fixation rates from Quest incubations (Fig. 3d) are also within the magnitude of those determined for the above-mentioned bacteria, displaying the autotrophic potential of Quest microorganisms. However, the highest Quest CO_2 fixation rates are associated with anoxic incubation conditions and not with oxic conditions as observed for Clueless incubations. The generally lower rates from Quest could be related to the incubation temperature as has been discussed above within the context of H_2 uptake. The absence of CO_2 fixation in the anoxic Clueless incubation and the comparably low inorganic CO_2 fixation rates in the unamended and oxic H_2-spiked Quest incubations, but the considerable cell proliferation, are suggestive of experimental conditions supporting heterotrophs that feed syntrophically or on residual organic matter, commonly found in vent fluids (Brault *et al.*, 1988; Konn *et al.*, 2009).

Putative residual inorganic electron donors fueling CO_2 fixation in incubations

In most of the unamended Clueless and unamended Quest incubations, CO_2 fixation takes place and is indicative of residual inorganic electron donors fueling autotrophy. Putative inorganic electron donors enriched in vent fluids include H_2, H_2S, reduced metals for example Fe^{2+}, Mn^{2+} (e.g. McCollom, 2007; Schmidt *et al.*, 2007) and NH_4^+ (Lam *et al.*, 2004). In the unamended Clueless sample, H_2 (< 5 nM) is close to or below the H_2 threshold values of most microorganisms (e.g. Karadagli & Rittmann, 2007) and is therefore unlikely to be the residual inorganic electron donor powering autotrophy. In these effluents, sulfide accounts for 21 ± 17 μM and can therefore be considered a putative inorganic electron source for energy-yielding oxidation processes in the unamended Clueless incubations. Sulfide would also be available in the oxic H_2-spiked fluid incubations and even in the anoxic H_2-spiked incubation. In the latter, despite vigorous sparging with N_2 for establishing

anoxic conditions and thus expulsion of gaseous H_2S, dissociated anionic sulfide (HS^-) would still be available at the pH of the fluids (Stumm & Morgan, 1981). Therefore, sulfide oxidation could contribute toward CO_2 fixation in all of the Clueless incubations. In the Quest fluids, 21 μM of H_2 was measured, whereas sulfide was below the detection limit (< 1 μM). Hence, H_2 represents a potential residual inorganic electron donor in the unamended Quest incubation, whereas sulfide likely plays a minor role due to limited availability. Besides H_2 and sulfide, other putative inorganic electron donors capable of powering CO_2 fixation were not measured in the effluents.

Relevance of substrate availability for stimulating CO_2 fixation in incubations

Clueless CO_2 fixation rates were considerably elevated after H_2 addition, but only under oxic conditions (Fig. 3c and d). Estimated ratios for autotrophically growing H_2-oxidizing bacteria (Ruhland, 1924; Bongers, 1970) suggest that more H_2 is being consumed in the Clueless H_2-spiked oxic incubations (Table S1) than would be required when coupling H_2 oxidation to CO_2 fixation. In the Clueless incubations, H_2 availability alone is likely not the prime determinant for stimulating CO_2 fixation as (1) no CO_2 was fixed in the anoxic incubations despite H_2 addition and (2) CO_2 fixation was also stimulated in some unamended incubations (Fig. 3c and d), where H_2-alternative inorganic electron donors appear to be used (see the discussion above). In fact, while CO_2 fixation seems to be considerably stimulated by H_2 addition under oxic conditions, statistically, CO_2 fixation of the oxic H_2-spiked incubation was not significantly elevated over that of the unamended incubations. Because a shared chemical feature of the unamended and the oxic H_2-spiked incubations exhibiting elevated CO_2 fixation rates is the presence and consumption of O_2 (*P*-values 0.04 and 0.02, respectively), O_2 availability appears to be one vital factor for promoting autotrophy in the Clueless incubations.

The $H_2:CO_2$ ratios for the oxic H_2-spiked Quest incubations (Table S1) can be interpreted as much of the energy gained by H_2 oxidation powering CO_2 fixation. In contrast, the $H_2:CO_2$ ratios for anoxic H_2-spiked Quest incubations show that more H_2 is being consumed than is likely required for fueling autotrophy. Microorganisms in the Quest incubations responded quite differently to the experimental conditions than those in the Clueless incubations. This might be related to the fact that temperatures comparable to those of the incubations (18 °C) are only provided in the Quest mussel patch and are well below those measured in the subsurface. In the Quest incubations, microbial CO_2 incorporation was significantly stimulated by H_2 addition under anoxic conditions compared with the unamended and oxic H_2-spiked fluids (*P*-values 0.02 and < 0.01, respectively). The very little CO_2 fixed in the unamended and oxic H_2-spiked Quest fluids suggests the limited capacity of microbial H_2 oxidation to fuel autotrophy under oxic conditions at 18 °C. As all three incubation types harbor H_2 (unamended: < 21 μM and oxic or anoxic H_2-spiked: 12–14 μM at the start of the experiment), the reasons for the comparably small amount of CO_2 fixed in the unamended and in the oxic H_2-spiked incubations appear to be related to the presence of O_2 in the unamended and oxic incubations (Table S1). These experimental data from Quest incubations suggest the importance of H_2 for elevated CO_2 fixation at this temperature, but only when devoid of O_2.

Possible causes of observed compositional changes in incubation experiments

During all Clueless incubations, the microbial community compositions changed drastically to *Gammaproteobacteria* as the predominant microbial lineage (Fig. 4b). Consequently, organisms less abundant in the natural community respond to incubation conditions and start proliferating. The predominance of *Gammaproteobacteria* can be attributed to (1) H_2 addition, (2) heterotrophic feeding (3) and/or residual inorganic electron donors as a strategy for survival and proliferation in the incubations. The predominance of *Gammaproteobacteria* in the H_2-spiked Clueless fluids is accompanied by elevated H_2 uptake rates (Fig. 3b) and coincides with the high clonal frequency of an uptake hydrogenase associated with *Beta-* or *Gammaproteobacteria* in the natural fluids (Fig. 2a). Several RubisCO genes associated with those of phylogenetically diverse *Gammaproteobacteria* were identified from the environment (Fig. S2a and b) and support the dominance of *Gammaproteobacteria* associated with elevated CO_2 fixation rates in the unamended and oxic incubations (Fig. 3d). In view of the absence of fixed CO_2 in the Clueless anoxic H_2-spiked incubations, the considerable increase in *Gammaproteobacteria* can be probably attributed to the consumption of organic carbon and possibly the effects of confinement (Azam & Worden, 2004; Allers *et al.*, 2007). The viability of some Clueless *Epsilonproteobacteria* at incubation temperatures is illustrated by their presence in the unamended replicates. The absence of *Epsilonproteobacteria* from the H_2-spiked incubations either demonstrates their inability to consume H_2 under the conditions provided or the ability of H_2 oxidizers to outcompete them. Based on temperature likeness between the habitat and the incubations, it appears that the population shifts are not related to temperature, but rather to the discrepancy between the chemical properties in the incubations and the natural habitat.

Multiple microbial groups are sustained in the Quest incubations (Fig. 4b). Despite incubation temperatures only

targeting microorganisms from the Quest mussel patch and not those from the hotter subsurface, community compositions changed less drastically than in the Clueless incubations. The proliferation of *Alpha-* and *Gammaproteobacteria* and affiliates of the *Cytophaga–Flavobacteria* group is limited to the oxic and anoxic H_2-spiked fluids and is particularly successful under oxic conditions (Fig. 4b). However, the low H_2 uptake rates and the little biomass synthesized in the oxic H_2-spiked incubations (Fig. 3b and d) argue against their proliferation being largely bound to H_2 uptake and autotrophic growth. In contrast, under anoxic conditions, the elevated H_2 uptake and CO_2 fixation rates in the anoxic H_2-spiked incubations (Fig. 3b and d) make it highly likely that some members of these groups are participating in these processes. Support for the H_2 uptake potential among some of these affiliates comes from an uptake hydrogenase associated with a cluster of *Alphaproteobacteria* found in the natural fluids (Fig. 2a). The growth of *Epsilon-* and *Deltaproteobacteria* is only considerably stimulated in the unamended and oxic H_2-spiked incubations where H_2 and O_2 ($243 \pm 17\,\mu M$ at the start of the experiment) are present. While the H_2 levels in the Quest incubations and in the natural habitat are fairly comparable, the temperatures are not. Thus, increasing incubation temperatures might lead to the substantial proliferation of thermophilic microorganisms as suggested by the high clonal frequency of hydrogenases associated with thermophilic microorganisms.

Ecological models inferred for Clueless and Quest habitats

Owing to the characteristics of the hydrothermal habitats, different chemical reactions are thermodynamically favored and are expected to govern abiotic constraints on microorganisms by affecting their diversity and activity (McCollom & Shock, 1997; McCollom, 2007). Because many metabolic reactions share the same reactants (e.g. H_2 or O_2), microorganisms are likely to compete for the limiting electron donor or electron acceptor (McCollom, 2007). While models exist inferring distinct predominant sources of metabolic energy in venting from different host rocks (McCollom & Shock, 1997; McCollom, 2007), complementary experiments with vent microorganisms are largely lacking. Our analyses suggest that H_2 availability and mixing processes, dictating electron acceptor availability, can affect the diversity and activity of H_2 oxidizers from the fluid samples at Clueless and Quest.

The H_2 levels at Clueless are likely close to or below the microbial H_2 threshold values of certain terminal electron acceptor processes (cf. Karadagli & Rittmann, 2007), constraining the H_2-oxidizing versatility to organisms using electrochemically more positive electron acceptors. Because O_2 appears to be available throughout much of the Clueless habitat, microorganisms coupling H_2 oxidation to electrochemically more positive electron acceptors such as O_2 would have an advantage over H_2 oxidizers using electron acceptors yielding less energy. Consequently, they can gain more energy, can thus maintain lower H_2 concentrations and can therefore outcompete other organisms transferring electrons to acceptors with less favorable energy yields (Lovley & Goodwin, 1988; Thauer *et al.*, 2008). As a strategy for survival, they would likely be adapted to rapidly make use of H_2, once it is sufficiently available. The restricted H_2 availability would also make way for microorganisms operating metabolisms using inorganic electron donors other than H_2, for example sulfide.

H_2 enrichment in hydrothermal fluids has been suggested to be a major factor for increased hydrogenotrophic chemolithoautotrophs (Nakagawa *et al.*, 2005a; Takai *et al.*, 2008b) and in H_2-rich hydrothermal effluents especially thermophiles appear to be sustained by H_2 oxidation (Nanoura & Takai, 2009). As H_2 in Quest fluids is substantially above microbial H_2 thresholds and mixing processes appear to restrict oxygenated seawater to the mussel layer, phylogenetically diverse H_2 oxidizers can thrive in the thermal and chemically distinct layers, allowing a high microbial versatility to arise. In the less well-oxygenated areas below the Quest mussel patch, O_2 rather than H_2 would be the limiting reactant, thus allowing H_2-consuming organisms utilizing alternative electron acceptors, such as NO_3^-, SO_4^{2-}, S^0 or CO_2, to thrive. This is indeed reflected by the identification of hydrogenases from *Epsilonproteobacteria* and methanogens requiring O_2-alternative electron acceptors (Campbell *et al.*, 2006; Thauer *et al.*, 2008). Organisms from this type of habitat would not be dependent on rapidly consuming H_2 as it is plentifully available.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phylogenetic relationships of 16S rRNA genes affiliated to bacteria, *Alpha-*, *Gamma-* and *Deltaproteobacteria* (a), *Epsilonproteobacteria* (b), deeply rooted lineages (c) and archaea, of the euryarchaeota (d) and crenarchaeota (e) from Clueless and Quest diffuse hydrothermal fluids are based on maximum-likelihood analysis.

Fig. S2. Phylogenetic relationships of RubisCO form I (a), RubisCO form II genes (b) and the β subunit of the ATP citrate lyase (c) using amino acid sequences are based on maximum likelihood analysis.

Table S1. Total cell counts, O_2 concentrations, H_2 consumption and CO_2 fixation rates of experimental incubations with Clueless and Quest diffuse fluids.

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