Unique H₂-utilizing lithotrophy in serpentinite-hosted systems

- Supplementary Information -

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Supplementary Results

H₂ and formate metabolism
Assuming that the hydrogenases and formate dehydrogenases in situ use NADP(H) or NAD(H)+ferredoxin (i.e., electron-bifurcating) (an assumption confirmed based on analysis of the metagenome-assembled genomes we recover; see below), H₂ and formate are likely reductants. In Hakuba, we estimate ΔG of +8.64 and +4.78 kJ per mol H₂ for H₂ generation through the respective pathways, assuming (i) literature cytosolic electron carrier redox potentials (-370 mV for NADPH, -320 mV NADH, and -450 mV Fd), (ii) cytosolic pH of 8.8 (two units lower than extracellular milieu (Krulwich et al 2011)), and (iii) intracellular H₂ concentrations similar to surrounding environment. As for formate metabolism, formate dehydrogenases are predicted to run in the oxidative direction because CO₂ reduction to formate is also endergonic in situ (ΔG of +30.28 and +24.92 kJ per mol formate depending on the electron carrier, with identical assumptions). H₂ and formate generation can only become exergonic (ΔG > 0) if cytosolic H₂ and formate reach below 266 nM and 0.115 nM respectively. Similarly, in The Cedars (estimated cytosolic pH of 9.9), H₂ and formate must be less than 20.6 nM and 5.18 nM respectively.

For H₂ metabolism, we identify putative NADP-reducing hydrogenases (HoxEFUHY in Actinobacteria and “Ca. Lithacetigenota” and HndABCD in Firmicutes) and NAD/Fd-dependent electron-confusing hydrogenases (HydABC in Firmicutes) (Burgdorf et al 2005, de Bok et al 2003, de Luca et al 1998, Hidalgo-Ahumada et al 2018, Morandi et al 2000, Schneider and Schlegel 1976, Schut and Adams 2009, Yamamoto et al 1983). HoxEFUHY typically uses NAD(H) as an electron carrier, but the Hox-related hydrogenases of HKB210 and BS525 consistently associate with a sixth subunit containing a putative NADPH-binding GltD domain (tentatively HoxP), suggesting that these hydrogenases may employ NADP(H) as an electron carrier rather than NAD(H), a phenomenon that has also been reported for the Ralstonia eutropha HoxEFUHYI. For formate metabolism, we predict NADP-dependent formate dehydrogenases in one Syntrophomonadaceae population and putative electron-confusing formate dehydrogenases in Actinobacteria, NPL-UPA2, and a Syntrophomonadaceae population.

As many putative H₂-utilizers in situ depend on the NADP-reducing hydrogenase above, a redox protein for transferring electrons from NADPH to NAD⁺ and ferredoxin is necessary for driving the Wood-Ljungadhl pathway (catabolism), Rnf (energy via vectorial cation extrusion), and/or biosynthesis. Several genomes encode the NADH-dependent ferredoxin:NADP⁺ reductase (NfnAB) for interconversion of these electron carriers, which allows for energy recovery from H₂-/formate-driven homoacetogenesis by Actinobacteria and Syntrophomonadaceae members (Fig. S5). Ca. Lithacetigenota and NPL-UPA2 encode homoacetogenesis from H₂ and/or formate but lack NfnAB. Analysis of potential redox complexes encoded in these genomes revealed a gene cassette of a HydB homolog, a HydC homolog, and a putative NADPH-binding redox protein with a GltD domain highly similar to HoxP (>45% amino acid sequence identity) (Table S5). Given that the function of HydBC is to hand off electrons from an electron-donating protein (typically FeFe hydrogenase HydA) to NAD⁺ and ferredoxin, we speculate that the putative NADPH-binding redox protein likely oxidizes NADPH and transfers electrons to NAD⁺ and ferredoxin, much like NfnAB. Identification of a flavin-binding site that would support the possibility of flavin-mediated electron bifurcation would be valuable, but, no such flavin-binding site could be identified at this point (as has also been reported for the well-known electron-bifurcating hydrogenase HydABC).
Etymology of candidate taxa

Description of Candidatus Lithacetigenota phy. nov.

Lithacetigenota (Lith.a.ce.ti.ge.no’ta. N.L. fem. n. Lithacetigena, a candidatus genus name; -ota, ending to denote a phylum; N.L. neut. pl. n. Lithacetigenota, the Candidatus Lithacetigena phylum).

The Candidatus Lithacetigenota is defined by five metagenome bins (HKB111, HKB210, BS525, BS5B28, and GPS1B18) recovered using culture-independent metagenomics from two serpentinite-hosted systems (Hakuba Happo hot springs in Hakuba, Japan, and The Cedars springs in California, USA). The metagenome bins are deposited in the National Center for Biotechnology Information (NCBI) under BioProject number PRJNA453100 and WGS numbers QLUP00000000, QLUQ00000000, QLTW00000000, QLTX00000000, and QLTY00000000. See also Table S3 for the quality and completeness of each bin-genome. Genomic analysis predicts that Ca. Lithacetigenota bacteria have the capacity to produce acetate from inorganic substrates.

Description of Candidatus Lithacetigena gen. nov.

Lithacetigena (Lith.a.ce.ti’ge.na. Gr. masc. n. lithos, stone; N.L. neut. n. acido.m aceticum, acetic acid; L. suff. -genus -a -um (from L. v. gigno) producing; N.L. fem. n. Lithacetigena, producing acetate from inorganic substrate).

The type species is Candidatus Lithacetigena glycireducens with two metagenome bins (HKB111 and HKB210) recovered using metagenomics from Hakuba Happo hot springs in Hakuba, Japan.

Description of Candidatus Lithacetigena glycireducens sp. nov.

Lithacetigena glycireducens (gly.ci.ni.re.duc’ens. N.L. neut. n. glycium, glycine; L. pres. part. reducens, bringing back, leading back; N.L. part. adj. glycireducens, glycine-reducing).

The genome of this candidatus species was discovered in Hakuba Happo hot springs in Hakuba, Japan.

Description of Candidatus Psychracetigena formicireducens gen. nov.

Psychracetigena (Psychr.a.ce.ti’ge.na. Gr. masc. adj. psychros, cold; N.L. neut. n. acido.m aceticum, acetic acid; L. suff. -genus -a -um (from L. v. gigno) producing; N.L. fem. n. Psychracetigena producing acetate under psychrophilic conditions).

The type species is Candidatus Psychracetigena formicireducens with three metagenome bins (BS525, BS5B28, and GPS1B18) recovered using metagenomics from The Cedars springs in California, USA.

Description of Candidatus Psychracetigena formicireducens sp. nov.

Psychracetigena formicireducens (for.mi.ci.re.duc’ens. N.L. neut. n. formicum, formic acid; L. pres. part. reducens, bringing back, leading back; N.L. part. adj. formicireducens, formate-reducing).

The genome of this candidatus species was discovered in The Cedars springs in California, USA.
Figure S1. Thermodynamics of H₂-oxidizing reduction of CO₂ (black), formate (pink), and glycine (blue), and formate disproportionation (orange) with changing H₂ and total inorganic carbon (TIC) concentrations. The maximum observed H₂ concentration in Hakuba Happo hot springs (664 µM) is marked (triangles) in the H₂-based plots and the TIC concentrations observed in Hakuba (<0.1 µM) and The Cedars GPS1 (35 µM) are indicated correspondingly. See also Tables S1 and S2 and Supplementary Equations.
Figure S2. Thermodynamics of H₂-oxidizing CO₂-reducing homoacetogenesis under differing pH, temperature, H₂, and total inorganic carbon (TIC) concentrations. (A) For temperatures 18 (blue), 45 (green), 85 (orange), and 115 (red) °C, the H₂ and TIC concentrations at which H₂/CO₂ homoacetogenesis has a Gibbs free energy yield (∆G) of -10 kJ mol⁻¹ is shown for pH of 9 (dotted line) and 11 (solid line) (atmospheric pressure of 1 atm). For reference, the same is shown for 25 °C at pH 7 (gray solid line). (B) The ∆G of H₂/CO₂ homoacetogenesis in various serpentinite-hosted systems (Hakuba Happo hot springs - HS; The Cedars springs - CS; Lost City - LC; Voltri Massif - VM; Coast Range Ophiolite Microbiological Observatory - CR; Santa Elena ophiolite - SEO; Table Lands - TLE) are shown based on reported environmental conditions for individual sampling locations. Each data point is colored based on temperature: psychrophilic (blue), mesophilic (purple), thermophilic (green), and hyperthermophilic (orange). Samples with associated acetate measurements are labelled black, and those that have >2 µM acetate are circled. For samples with no reported acetate concentrations (gray), the average of reported concentrations was used (8.57 µM Acetate). For The Cedars spring sample, no H₂ concentration has been reported, so the highest on-land serpentinite-hosted system H₂ concentration was used (664 µM H₂ from Hakuba Happo #1). Thermodynamic calculations were performed using ∆G° and ∆H° values at 298 K values and temperature adjustment through the Gibbs-Helmholtz equation. The effect of pressure was approximated as described by Wang et al. (Wang et al 2010). See also Tables S1 and S2 and Supplementary Equations.
Figure S3. Ribosomal protein tree for high-quality MAGs. Universally conserved ribosomal proteins were collected from each genome, aligned with MAFFT v7.394 (Katoh et al 2005), trimmed with trimAl 1.2rev59 (-gt 0.70) (Capella-Gutiérrez et al 2009), and concatenated. A maximum likelihood tree was calculated using phyML 3.3.20190321 with the LG model and 100 bootstrap iterations (Guindon and Gascuel 2003). GTDBtk-based phylogeny is shown.
Figure S4. Ribosomal protein tree including high-quality MAGs from 74 GTDB-defined phylum-level lineages. See Fig. 1 for details. GTDBtk-based phylogenetic nomenclature is shown.
Figure S5. Schemes for energy metabolism. Arrow colors indicate oxidative (pink), reductive (blue), ATP-yielding (orange), and ATP-consuming (green) steps. The corresponding electron carrier (reduced form) is shown for each redox step. Redox reactions shown in gray are necessary for generating reducing equivalents for biosynthesis (e.g., if the putative catabolic pathway does not produce NADPH, an NADPH-generating pathway is shown in gray). Abbreviations: NAD – nicotinamide adenine dinucleotide, NADP – nicotinamide adenine dinucleotide phosphate, Fd – ferredoxin, THF – tetrahydrofolate, CoA – Coenzyme A, Rnf – ion-translocating NADH:ferredoxin oxidoreductase, Nfn – NADH-dependent ferredoxin:NADP+ oxidoreductase, *OR – putative NADH-dependent NADPH:ferredoxin oxidoreductase (see supplemental results). †For the 5,10-methylene-tetrahydrofolate dehydrogenase reaction, NAD+/NADH is shown as the electron carrier as has been observed for Acetobacterium woodii.
Figure S6. Complete phylogenetic tree of glycine reductase subunits GdB and homologs from Hakuba Happo hot spring*, The Cedars springs†, and other serpentinite-hosted system metagenomes‡. See Fig. 3 for details. Phylogenetic nomenclature indicated with an asterisk are GTDB-defined phylum-level nomenclature. For those with underscores, the name is abbreviated to the last capital letter (e.g., Firmicutes_A is shown as (A) and Desulfobacterota_E is shown as (E)).

Glycine reductase GdB

Firmicutes_A

Phylum_Rhodothermota

Chloroflexi

Ca. Lithiactigenota

Ca. G. hatharoa OCA_01039905

GdB

D-proline reductase PrdAB
Figure S7. Genome streamlining of publically available genomes (Joint Genome Institute Integrated Microbial Genome) and selected high completeness bins from Hakuba Hppo hot springs (red) and The Cedars springs (blue) (inset on left). Genomes with known streamlining are marked (black): *Aurantimicrobium minutum*, *Ca. Pelagibacter ubique*, *Polynucleobacter asymbioticus*, *Rhodoluna lacicola*, *Ca. Serpentinomonas raichei*, and *Ca. S. mccroryi*. 
Figure S8. Hakuba-derived enrichment culture of *Actinobacteria* UBA1414. (A) Culture medium based on Widdel medium (pH 10) with an N₂-CO₂ (80:20, vol/vol) headspace was supplemented with 0.01 g l⁻¹ yeast extract and 25 g l⁻¹ elemental iron granules. Hakuba hot spring water 100 mL was passed through a membrane filter and the filter was submerged in the culture medium. After a 4 month incubation at 40 °C, 1 mL of the culture was used for DNA extraction, PCR amplification, and clone library construction (about 600 bp of 16S rRNA gene). UBA1414-derived 16S rRNA gene fragments, which shared high sequence identity (>99%) to bin HKB206, comprised 7 out of 20 clones. The remaining 13 clones consisted of obligately aerobic *Methylobacterium*- and *Pseudomonas*-related sequences that may be contaminants, but further investigation is required. (B) Micrographs: phase-contrast (left) and SYBR-Green-I-stained microbial cells (green, right); scale bar, 2 μm.
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Gibbs-Helmholtz equation:

$$\Delta G_1 = R \times T_1 \times \left( \frac{\Delta G_2}{R \times T_2} - \ln \left( \frac{K_{eq1}}{K_{eq2}} \right) \right)$$

van’t Hoff equation:

$$\ln \left( \frac{K_{eq2}}{K_{eq1}} \right) = \frac{\Delta H}{R} \times \left( \frac{1}{T_1} - \frac{1}{T_2} \right)$$

Pressure adjustment equation (optional):

$$\Delta G_1 = R \times T_1 \times \left( \frac{\Delta G_2 + \Delta V \times (P_1 - P_2)}{R \times T_2} - \ln \left( \frac{K_{eq1}}{K_{eq2}} \right) \right)$$

$\Delta G_1$ : Gibbs free energy of the reaction at in situ temperature

$\Delta G_2$ : Gibbs free energy of the reaction at the reference temperature

$R$ : Universal gas constant

$T_1$ : In situ temperature (Kelvin)

$T_2$ : Reference temperature (Kelvin)

$\Delta H$ : Molar enthalpy change of the reaction at the reference temperature

$K_{eq1}$ : Equilibrium constant at in situ temperature

$K_{eq2}$ : Equilibrium constant at the reference temperature

$\Delta V$ : Molar volume change in the reaction at the reference temperature and pressure

$P_1$ : In situ pressure (atm)

$P_2$ : Reference pressure (atm)
Table S1. Gibbs free energy ($G^\circ_f$) and enthalpy ($H^\circ_f$) of formation of compounds of interest.

| Compound name | Formula | State       | $G^\circ_f$ [kJ mol$^{-1}$] | $H^\circ_f$ [kJ mol$^{-1}$] | $V_m$ (cm$^3$ mol$^{-1}$) |
|---------------|---------|-------------|-----------------------------|-----------------------------|---------------------------|
| Hydrogen      | H$_2$   | g           | 0                           | 0                           | 26.7                      |
| Carbon dioxide| CO$_2$  | g           | -394.4                      | -393.5                      | 33.9                      |
| Carbon monooxide| CO      | g           | -137.2                      | -110.5                      | 37.3                      |
| Acetate       | CH$_3$COOH | ionized, aq | -369.4                      | -486.0                      | 56.3                      |
| Formate       | HCOOH   | ionized, aq | -351.0                      | -425.6                      | 37.7                      |
| Glycine-†     | NH$_2$CH$_2$COO$^-$ | ionized, aq | -325.7                      | -521.17                     | 64.7                      |
| Ammonia       | NH$_3$  | aq          | -26.6                       | -80.3                       | 26.56                     |
| Water         | H$_2$O  | l           | -237.2                      | -285.8                      | 18.03                     |

*Data from Hanselman K.W. Microbial energetics applied to waste repositories. Experientia, 47, 645–687, (1991). https://doi.org/10.1007/BF01958816
†For glycine- only, the $G^\circ_f$ reported in Amend & Shock (2001) was adjusted using the pKa value (9.6) of the amine group of glycine.
Amend J.P. & Shock E.L. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and bacteria. FEMS Microbiol Rev., 25, 175-243, (2001). https://10.1111/j.1574-6976.2001.tb00576.x
| Ecosystem          | Location      | Sample   | pTIC concentration below the detection limit | Reference |
|--------------------|---------------|----------|---------------------------------------------|-----------|
| Hakuba Happo       | Japan         | HS 1     | 10.8, 52                                   | 694, 0.1  |
| Hakuba Happo       | Japan         | HS 3     | 10.7, 48                                   | 201, 0.1  |
| The Cedars         | USA           | GPE1     | 11.9, 17                                   | 1644, 0.35|
| Voltri Massif      | Italy         | GOR34-3  | 11.8, 18.5                                 | 26, 28    |
| Voltri Massif      | Italy         | BR2-2012 | 12.1, 20.3                                 | 0.5, 29   |
| Voltri Massif      | Italy         | BR2-2013 | 12.3, 20.3                                 | 1.8, 7.8  |
| Voltri Massif      | Italy         | GOR34-3  | 12.3, 18.5                                 | 9.2, 17.2 |
| Voltri Massif      | Italy         | GOR34-1  | 12.3, 18.5                                 | 3.9, 14   |
| Table Lands        | Canada        | WHHC2b   | 12.3, 16.2                                 | 520, 372  |
| CROMO*             | USA           | QV1.1    | 11.5, 17.9                                 | 0.075, 96 |
| CROMO*             | USA           | CSW1.3   | 10.1, 16.9                                 | 0.283, 172|
| CROMO*             | USA           | CSW1.1   | 12.2, 17.2                                 | 0.289, 253|
| Lost City          | Mid-Atlantic  | 3871-GT15 | 9.4, 62                                   | 7900, 0.1 |
| Lost City          | Mid-Atlantic  | 3871-G9  | 9.4, 55                                   | 3330, 0.1 |
| Lost City          | Mid-Atlantic  | 3871-G7  | 9.4, 90                                   | 9220, 0.1 |
| Lost City          | Mid-Atlantic  | 3871-G7  | 9.4, 90                                   | 8970, 0.1 |
| Lost City          | Mid-Atlantic  | 3871-G7  | 9.4, 60                                   | 1270, 0.6 |
| Lost City          | Mid-Atlantic  | 3871-G7  | 9.4, 90                                   | 3690, 1    |
| Lost City          | Mid-Atlantic  | 3871-G7  | 9.4, 60                                   | 2210, 2    |
| Lost City          | Mid-Atlantic  | 3871-G7  | 9.4, 60                                   | 5444, 2    |
| Lost City          | Mid-Atlantic  | 3871-G7  | 9.4, 28                                   | 5430, 4    |
| Lost City          | Mid-Atlantic  | 3871-G7  | 9.4, 60                                   | 14190, 9   |
| Lost City          | Mid-Atlantic  | 3871-G7  | 9.4, 61                                   | 11580, 10  |
| Lost City          | Mid-Atlantic  | 3871-G7  | 9.4, 73                                   | 11940, 26  |

**Ecosystem**

- Hakuba Happo
- Table Lands
- CROMO
- Lost City

| Sample code in Figure S2B | h | Temp (°C) | pCO2 | pH | pO2 | Ref. |
|---------------------------|---|-----------|------|----|-----|-----|
| HS 1                      | 10.8 | 52       | 694  | 0.1 | 3.38E-07 | 6 | -20.47 | 2.18 | -10.29 | 1.23 |
| HS 3                      | 10.7 | 48       | 201  | 0.1 | 5.71E+04 | 7 | -13.89 | 8.60 | -3.71 | 1.23 |
| GPE1                      | 11.9 | 17       | 1644 | 0.35 | 2.26E+06 | 69.3 | -27.54 | -34.06 | -4.34 |
| GOR34-3 2012              | 11.8 | 18.5     | 268  | 29  | 2.82E+06 | 8.57 | -1.54 | -7.40   | 5.6  |
| BR2-2012                  | 12.1 | 20.3     | 0.5  | 29  | 6.78E+07 | 8.57 | 43.15 | 38.21    | 5.6  |
| BR2-2013                  | 12.3 | 20.3     | 1.8  | 7.8 | 7.28E+07 | 8.57 | 40.36 | 35.46    | 5.6  |
| GOR34-3 2013              | 12.3 | 18.5     | 9.2  | 17.2| 1.70E+07 | 8.57 | 20.13 | 13.79    | 5.6  |
| GOR34-3 2011              | 12.3 | 18.5     | 3.9  | 14 | 2.19E+07 | 8.57 | 7.96  | 21.45    | 5.6  |
| WHHC2b                    | 12.3 | 16.2     | 520  | 372 | 3.95E+06 | 8.57 | -35.27 | -42.22   | 7    |
| QV1.1                     | 11.5 | 17.9     | 0.075| 96  | 3.70E+05 | 10.2 | 46.19 | 38.65    | 8    |
| CSW1.3                    | 10.1 | 16.9     | 0.283| 172 | 1.97E+02 | 1.55 | 5.30  | -2.20    | 8    |
| CSW1.1                    | 12.2 | 17.2     | 0.289| 253 | 4.13E+06 | 70.79 | 44.55 | 36.31    | 8    |
| Costa Rica Spring 9       | 11.5 | 26.4     | 53.1 | 254 | 6.33E+05 | 8.57 | -22.88 | -21.72   | 9    |
| Costa Rica Spring 9       | 11.5 | 26.4     | 53.1 | 254 | 6.33E+05 | 8.57 | -22.88 | -21.72   | 9    |
| Costa Rica Spring 9       | 11.5 | 26.4     | 53.1 | 254 | 6.33E+05 | 8.57 | -22.88 | -21.72   | 9    |
| Costa Rica Spring 9       | 11.5 | 26.4     | 53.1 | 254 | 6.33E+05 | 8.57 | -22.88 | -21.72   | 9    |

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| Habitat | Sample | Bin | Prefix | Phylum (phylum) | Phylum (bowtie level) | based on GDTobin | GDTobin | RED value | Genome size (MB) | Contig (% | Complete reads (%) | Complete alien (%) |
|---------|-------|-----|--------|----------------|----------------------|-----------------|---------|----------|-----------------|-------------|-----------------|-----------------|
| Hakuba HKB702 | HKB206 | DDT19 | Firmicutes | **Actinobacteria** | **Actinobacteria** | **Actinobacteria** | 0.533909 | 1.079 | 346 | 85.5 | 5.6 |
| Hakuba HKB702 | HKB209 | DDT19 | Firmicutes | **Syntrophomadaceae** | **Syntrophomadaceae** | **Syntrophomadaceae** | 0.650559 | 2.68 | 562 | 73.8 | 6.8 |
| Hakuba HKB702 | HKB212 | DDT20 | Firmicutes | **Syntrophomadaceae** | **Ochrobacterales** | **Ochrobacterales** | 0.662550 | 2.97 | 112 | 88.4 | 9.1 |
| Hakuba HKB702 | HKB214 | DDT20 | Firmicutes | **Syntrophomadaceae** | **Syntrophomadaceae** | **Syntrophomadaceae** | 0.663900 | 3.00 | 149 | 80.5 | 8.3 |
| Hakuba HKB702 | HKB217 | DDT20 | Firmicutes | **Syntrophomadaceae** | **Syntrophomadaceae** | **Syntrophomadaceae** | 0.663900 | 3.00 | 149 | 80.5 | 8.3 |
| Hakuba HKB701 | HKB111 | DDT23 | Ca. *Lithaeroglobus* (novel) | **Chloroflexota** | **Chloroflexota** | **Chloroflexota** | 0.018102 | 1.24 | 204 | 83.8 | 0.0 |
| The Cedars GSS 2011 | BS517 | DDT35 | Chitinolytic | **Ochrobacterales** | **Ochrobacterales** | **Ochrobacterales** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |
| The Cedars BSS 2011 | BS517 | DDT35 | Dithiobacterales | **Ochrobacterales** | **Ochrobacterales** | **Ochrobacterales** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |
| The Cedars BSS 2011 | BS517 | DDT36 | Chitinolytic | **Ochrobacterales** | **Ochrobacterales** | **Ochrobacterales** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |
| The Cedars BSS 2011 | BS518 | DDT36 | Dithiobacterales | **Ochrobacterales** | **Ochrobacterales** | **Ochrobacterales** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |
| The Cedars BSS 2011 | BS519 | DDT37 | Chitinolytic | **Ochrobacterales** | **Ochrobacterales** | **Ochrobacterales** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |
| The Cedars BSS 2011 | BS520 | DDT37 | Dithiobacterales | **Ochrobacterales** | **Ochrobacterales** | **Ochrobacterales** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |
| The Cedars GSS 2011 | BS519 | DDT38 | Firmicutes | **Firmicutes** | **Firmicutes** | **Firmicutes** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |
| The Cedars GSS 2011 | BS519 | DDT38 | Firmicutes | **Firmicutes** | **Firmicutes** | **Firmicutes** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |
| The Cedars GSS 2011 | BS519 | DDT38 | Firmicutes | **Firmicutes** | **Firmicutes** | **Firmicutes** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |
| The Cedars GSS 2011 | BS519 | DDT38 | Firmicutes | **Firmicutes** | **Firmicutes** | **Firmicutes** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |
| The Cedars GSS 2011 | BS519 | DDT38 | Firmicutes | **Firmicutes** | **Firmicutes** | **Firmicutes** | 0.620393 | 0.89 | 233 | 82.0 | 0.0 |

**Table S3**: Phylogeny and quality of bins from Hakuba Hachijo hot springs and The Cedars springs (GPS1 and BSS). Phylogeny was defined by GDTobin (g) or comparing GDTobin-defined phylogeny with SH-BL (e) or SH-UK (g). GDTobin annotations with RED values less than 0.5 were not considered and phylogeny was checked by constructing a concatenated ribosomal protein tree (see Supplement). Low quality bins that were only used for comparative purposes (e.g., whether a function found in a high-quality bin from Table S3 was present in Table S2 with >80% similarity).
Table S4. Distribution of acetogen-related pathways among bins recovered from Hakuba Happo hot springs and The Cedars springs. Low quality bins are grayed out. * Missing one non-substrate-binding subunit of a multi-subunit protein complex.

| Bin     | Prefix | Phylum       | Specific              | Hydrogenase | Formate dehydrogenase | THF pathway | CO dehydrogenase | CODH/ACS | Archael CODH/ACS | H-ODS | NADH CODH/ACS | NADH CODH/ACS | H-ODS | NADH CODH/ACS | CODH/ACS | Cyclo reductase | Electron transport |
|---------|--------|--------------|-----------------------|-------------|------------------------|-------------|------------------|-----------|------------------|-------|--------------|--------------|-------|--------------|----------|-----------------|-------------------|
| GPS109  | DCF25  | Firmicutes   | Syntrophomonadaceae   |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| GPS1089 | DCF250 | Firmicutes   | Syntrophomonadaceae   |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| HK5B19  | DCF219 | Firmicutes   | Syntrophomonadaceae   |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| GPS214  | DCF214 | Firmicutes   | Syntrophomonadaceae   |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| GPS1801 | DCF312 | MPL-UPA2     |                        |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| BS33    | DCF33  | MPL-UPA2     |                        |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| HK206   | DCF18  | Actinobacteria | UBA1414 (g)          |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| BS33    | DCF4   | Ca. Lithoacetigena (novel) |                 |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| GPS1089 | DCF4   | Ca. Lithoacetigena (novel) |                 |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| GPS30   | DCF4   | Chitinifera  | Dehalococcoida       |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| GPS1084 | DCF55  | Chitinifera  | Dehalococcoida       |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| BS50    | DCF27  | Chitinifera  | Dehalococcoida       |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| BS17    | DCF55  | Chitinifera  | Dehalococcoida       |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| BS291   | DCF55  | Chitinifera  | Dehalococcoida       |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| BS330   | DCF38  | Firmicutes   | SRB2 (e)             |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| GPS119  | DCF37  | Firmicutes   | SRB2 (e)             |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| HK212   | DCF37  | Firmicutes   | SRB2 (e)             |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| BS334   | DCF34  | Firmicutes   | SRB2 (e)             |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| GPS123  | DCF35  | Firmicutes   | SRB2 (e)             |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| BS34    | DCF36  | Firmicutes   | SRB2 (e)             |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| HK111   | DCF33  | Ca. Lithoacetigena (novel) |                 |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| HK210   | DCF32  | Ca. Lithoacetigena (novel) |                 |             |                        |             |                  |           |                  |       |              |              |       |              |          |                 |                   |
| HoxE (2Fe2S thioredoxin; pfam01257) | Subunit K | MnhF? | CooS | NhaD | RnfG | AcsB | FchA | RnfD | Acs1 | Ack | c |
|----------------------------------|----------|-------|------|------|------|------|------|------|------|-----|---|
| DDT22_00646                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT22_00644                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT22_00128                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT22_00283                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT22_00702                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT22_00346                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT22_00347                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT22_00351                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT22_00171                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT22_00295                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT22_00297                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT41_00874                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT41_00043                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT41_01410                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT41_00604                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT41_00871                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT18_01219                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT18_01220                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT18_01401                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT18_01669                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT18_00627                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT18_00661                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT18_00659                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT32_00101                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT32_00103                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT32_00564                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT32_00571                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT32_00098                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT32_00309                     |          |       |      |      |      |      |      |      |      |     |  |
| GPS1B11                         |          |       |      |      |      |      |      |      |      |     |  |
| Syntrophomonadaceae             |          |       |      |      |      |      |      |      |      |     |  |
| DDT21_00671                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT21_00066                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT21_00068                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT21_01163                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT21_00067                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT21_00065                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT21_00069                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT21_01162                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT21_00670                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT20_01369                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT20_00647                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT20_00646                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT20_00529                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT20_01394                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT37_00796                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT37_00801                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT37_00638                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT37_00575                     |          |       |      |      |      |      |      |      |      |     |  |
| DDT37_00574                     |          |       |      |      |      |      |      |      |      |     |  |
| GPS119                           |          |       |      |      |      |      |      |      |      |     |  |

**Table S5.** Subunits of the protonotive electron transport chain (HoxE-CooS-NhaD-RnfG-AcsB-FchA-RnfD-Acs1-Ack) for the Escherichia coli (E. coli) strain K-12 MG1655 (GenBank accession number NC_000913). The table lists the protein names and their respective accession numbers.
Table S6. Environmental parameters and chemical composition of Hakuba Happo spring water (artificially pumped from a drilling well named Happo #3) used for microbiological analysis.

|                      | July 2016 | October 2016 | October 2017 |
|----------------------|-----------|--------------|--------------|
| pH                   | 10.95     | 10.80        | 10.67        |
| Temperature (°C)     | 47.5      | 47.4         | 45.6         |
| ORP (mV)             | -435      | -432         | -453         |
| electrical conductivity (mS/m) | 47.7 | 43.6         | 51.7         |
| dissolved oxygen (mg/L) | <dl | <dl         | <dl          |
| Na⁺ (ppm)            | 32        | 30           | <dl          |
| K⁺ (ppm)             | <dl       | <dl          | <dl          |
| Ca²⁺ (ppm)           | <dl       | <dl          | <dl          |
| NO₃⁻ (ppm)           | <dl       | <dl          | <dl          |
| NH₃ (μM)             | -         | -            | 2.9          |
| Amino acids          |           |              |              |
| Aspartic Acid (nM)   | -         | -            | <dl          |
| Threonine (nM)       | -         | -            | <dl          |
| Serine (nM)          | -         | -            | <dl          |
| Glutamic Acid (nM)   | -         | -            | <dl          |
| Glycine (nM)         | -         | -            | 5.4 ± 1.6    |
| Alanine (nM)         | -         | -            | <dl          |
| Cysteine (nM)        | -         | -            | <dl          |
| Valine (nM)          | -         | -            | <dl          |
| Methionine (nM)      | -         | -            | <dl          |
| Isoleucine (nM)      | -         | -            | <dl          |
| Leucine (nM)         | -         | -            | <dl          |
| Tyrosine (nM)        | -         | -            | <dl          |
| Phenylalanine (nM)   | -         | -            | <dl          |
| Histidine (nM)       | -         | -            | <dl          |
| Lysine (nM)          | -         | -            | <dl          |
| Arginine (nM)        | -         | -            | <dl          |

<dl, below our quantification limit; -, no data.