Applied and Environmental Microbiology

Community Microrespirometry and Molecular Analyses Reveal a Diverse Energy Economy in Great Boiling Spring and Sandy's Spring West in the U.S. Great Basin

Caitlin N. Murphy, Jeremy A. Dodsworth, Aaron B. Babbitt and Brian P. Hedlund

Appl. Environ. Microbiol. **2013, 79(10):3306. DOI: 10.1128/AEM.00139-13.**
Published Ahead of Print 8 March 2013.

AEM
Journals.ASM.org

Community Microrespirometry and Molecular Analyses Reveal a Diverse Energy Economy in Great Boiling Spring and Sandy's Spring West in the U.S. Great Basin

Caitlin N. Murphy,* Jeremy A. Dodsworth, Aaron B. Babbitt,* Brian P. Hedlund

School of Life Sciences, University of Nevada, Las Vegas, Nevada, USA

Microrespirometry showed that several organic and inorganic electron donors stimulated oxygen consumption in two ~80°C springs. Sediment and planktonic communities were structurally and functionally distinct, and quantitative PCR revealed catabolically distinct subpopulations of *Thermocrinis*. This study suggests that a variety of chemolithotrophic metabolisms operate simultaneously in these springs.

Because photosynthesis does not occur above ~73°C (1), microbial communities in high-temperature environments must obtain organic carbon from allochthonous photosynthetic carbon, organics synthesized abiotically in the subsurface under high temperature and pressure (e.g., Fischer-Tropsch synthesis) (2), diagenesis products of organic-laden sedimentary formations (thermogenesis) (3), or primary production coupled to chemolithotrophy. Collectively, known thermophiles can use a variety of simple (e.g., methane, formate, and acetate) and complex organics, as well as inorganic compounds, including hydrogen (H_2), carbon monoxide, ammonia (NH_3), hydrogen sulfide (H_2S), elemental sulfur (S^0), thiosulfate ($S_2O_3^{2-}$), and reduced metals (4, 5), as electron donors. It has been proposed that H_2 may be the most important electron donor for life at high temperature based on the physiological and phylogenetic diversity of thermophilic hydrogenotrophs (6, 7), the high energy yield of H_2 respiration (8–11), the high diffusivity of H_2, and the ubiquity of *Aquificales* in Yellowstone National Park (YNP) and other geothermal systems (8). However, many redox couples in addition to hydrogen oxidation are also highly exergonic, suggesting that a diversity of energy metabolisms may coexist without competition for electron donors (9–11).

Few studies have experimentally addressed the importance of H_2 and other electron donors in a natural geothermal setting. D'Imperio et al. (12) described microbial mats in Dragon Spring, YNP (73°C, pH 3.1), that consumed H_2S at a rate of about three orders of magnitude greater than H_2 and showed that *Hydrogenobaculum* isolates from the spring either oxidized only H_2S or cooxidized H_2S and H_2 concomitantly, but with a much higher rate of H_2S consumption. Boyd et al. (13) showed that CO_2 fixation at the same site was stimulated by addition of O_2, suggesting electron acceptor limitation. In contrast, neither H_2S nor H_2 addition stimulated $^{14}CO_2$ uptake.

Here, we used microrespirometry to determine whether various electron donors could stimulate aerobic respiration in two springs in the U.S. Great Basin and complemented the activity measurements with quantification of key catabolic genes and comparisons to previously published 16S rRNA gene pyrotag data sets derived from samples collected at the same time and location (within 0.5 m; all sediment samples were at the sediment/water interface, top ~1 cm) (14). Experiments at Great Boiling Spring (GBS) were carried out on ~82°C samples collected from a shallow shelf at site B, as described previously (15, 16). Experiments at Sandy's Spring West (SSW) were carried out using ~79°C samples collected from the source pool (9, 14). A fast-responding Clark-type O_2 microsensor modified with an organic electrolyte for temperature tolerance up to 100°C was used for microrespirometry (Unisense AS, Århus, Denmark), and data were logged using MicOx Software (Unisense AS, Århus, Denmark) (see Fig. S1 in the supplemental material) (17, 18). Samples were mixed continuously with a magnetic stir bar to prevent formation of an O_2 gradient. Electrodes were polarized for >15 min to −0.5 V to remove O_2 from the electrolyte, and then, a linear calibration was performed using aerated spring water (100% saturation) and water reduced using 0.1 M sodium ascorbate and 0.1 M NaOH (0% saturation) (17, 18). Fresh water or dilute sediment slurries were collected without the introduction of O_2 and without cooling, and experiments were initiated within 30 min of collection in a water bath maintained at *in situ* temperature. After the intrinsic respiration rate was measured for 3 min, potential electron donors were added via syringe, the sensor was replaced, and measurements were recorded every 6 s for 20 min. To determine whether O_2 consumption in samples was due to abiotic activity, controls consisted of either filtered spring water (0.2 μm) or aliquots of the sediment slurries used for respiration that were frozen within 30 min of collection and transported to the laboratory, where they were thawed, autoclaved, and tested. Manipulations of controls led to a ~10% decrease in O_2 concentration. The following framework of axioms was used to guide interpretation of microrespirometry experi-

Received 18 January 2013 **Accepted** 4 March 2013

Published ahead of print 8 March 2013

Address correspondence to Brian P. Hedlund, brian.hedlund@unlv.edu.

* Present address: Caitlin N. Murphy, Department of Microbiology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA; Aaron B. Babbitt, Coe College, Cedar Rapids, Iowa, USA.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00139-13.

doi:10.1128/AEM.00139-13

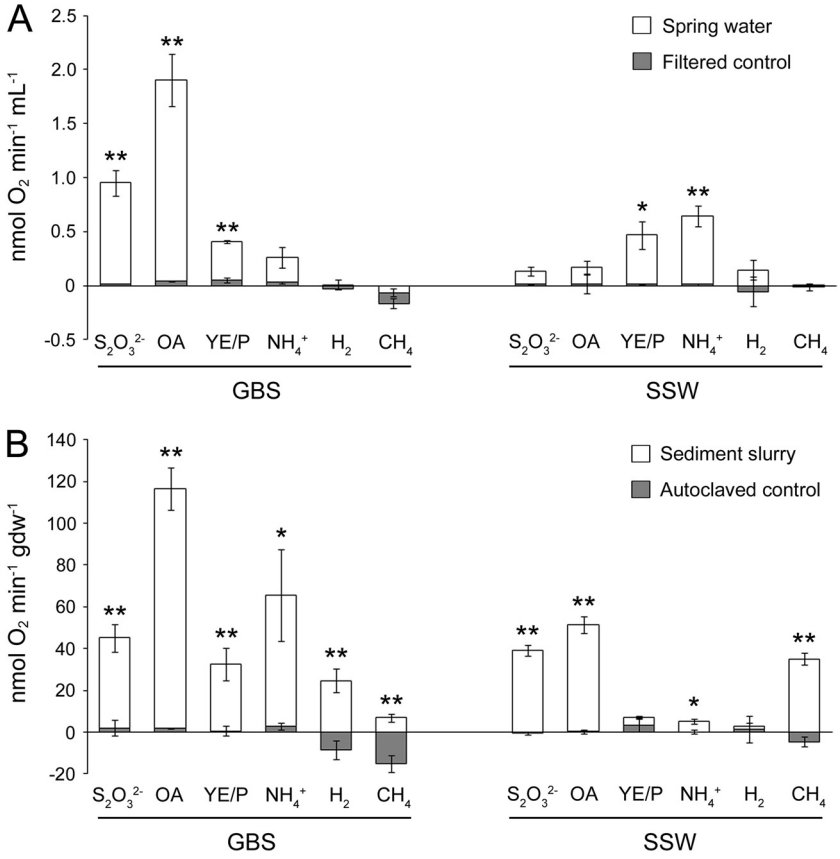

FIG 1 Stimulation of O_2 consumption in GBS and SSW water (A) and sediment slurries (B). Samples (2 ml) were amended to the indicated final concentrations with one of the following electron donors: 1 mM sodium thiosulfate ($S_2O_3^{2-}$) or ammonium chloride (NH_4^+); organic acid (OA) mixture of formate, acetate, lactate, and propionate (1 mM each); mixture of yeast extract (0.5%, wt/vol) and peptone (0.1%, wt/vol; YE/P); or H_2 or CH_4 (10 μM). Rates of O_2 consumption over 20 min in samples (white bars) and filtered or autoclaved controls (gray bars) are shown. Error bars indicate the standard errors of the means ($n = 3$). Amendments that exhibited stimulation of O_2 consumption significantly above that of the control (paired t test, $\alpha = 0.05$) before (*) or after (**) correction for multiple tests (Bonferroni correction) within a given experiment are indicated.

ments: (i) the rate of O_2 consumption prior to the addition of exogenous electron donors represented the intrinsic rate of aerobic respiration, (ii) a stimulation of O_2 consumption would be observed upon addition of excess electron donor if the necessary enzymes were present in members of the microbial community (i.e., no time was allowed for enrichment or induction of gene expression), (iii) only a reaction not already occurring at V_{max} could be stimulated, and (iv) the stimulated respiration rates are considered potential rates, which are likely to be higher than rates occurring *in situ* because the bulk water and sediment slurries used in these experiments are electron donor limited for most reactions (9, 10).

A variety of electron donors stimulated O_2 consumption, and their effects varied both between the two springs and between water and sediments. In all samples, the intrinsic rate of O_2 consumption was low (0.01 to 0.02 nmol O_2 min^{-1} ml^{-1} in bulk water samples and 2.8 to 4.5 nmol O_2 min^{-1} g^{-1} in sediment slurries), although this might be an artifact of electron donor consumption during brief delays as experiments were set up. In GBS bulk water, $S_2O_3^{2-}$, yeast extract and peptone (YE/P), and a mixture of organic acids (OA) stimulated O_2 consumption by 7- to 49-fold without lag, with the highest rates in response to addition of $S_2O_3^{2-}$ or OA (Fig. 1A). In GBS sediment slurries, all electron donors tested stimulated O_2 consumption rates (Fig. 1B). In SSW bulk water, YE/P and NH_4^+ stimulated O_2 consumption by 6- to 52-fold, with the highest rates in response to NH_4^+ (Fig. 1A). In SSW sediment slurries, $S_2O_3^{2-}$, OA, NH_4^+, and CH_4 stimulated O_2 consumption (Fig. 1B). Thus, the potential catabolic capacity of sediment microbial communities was both distinct from and more diverse than those of the planktonic communities. This is consistent with differences in microbial communities observed between the sediment and planktonic communities in both springs (14, 15) and, in the case of GBS, a higher richness and evenness observed in sediments compared to planktonic communities (14, 15). In general, the stimulation of O_2 consumption suggests that communities in GBS and SSW are limited by electron donor supply, in contrast to the electron acceptor (O_2) limitation of CO_2 fixation observed in Dragon Spring (13). Although only a limited chemistry data set was collected at the time and location of the respirometry experiments (14), extensive geochemistry measurements made at GBS on several different dates document a much higher concentration of O_2 (19 to 56 μM) than of many of the electron donors used here, with the exception of NH_4^+/NH_3, (15 to 93 μM) (9, 10, 14, 15). The lower stimulated O_2 consumption rates in SSW were consistent with

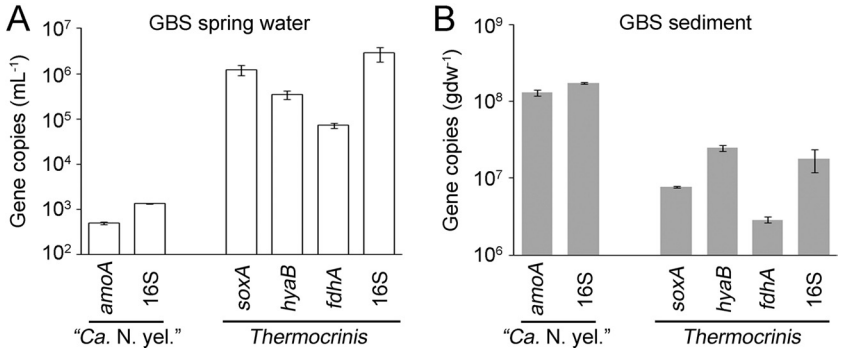

FIG 2 Quantification of 16S rRNA genes (16S) and various putative catabolic genes in *Thermocrinis* and "*Ca.* Nitrosocaldus yellowstonii" ("*Ca.* N. yel.") in DNA isolated from spring water (A) and sediment (B) of GBS. Bars represent the averages and standard errors of three replicates. Primers and qPCR methods are described in the supplemental material and Tables S1 to S3.

the lower redox status, lower O_2 concentration, and, in the case of the planktonic community, lower cell counts in SSW than in GBS (9, 14).

Possible links could be made between catabolic capacities inferred by respirometry and prominent members of the resident microbial communities in both springs based on previously reported 16S rRNA gene pyrotag surveys. For GBS sediment and SSW experiments, pyrotag data sets derived from samples collected at the same time and location as samples used for respirometry are summarized in Fig. S2 in the supplemental material (14). Although community surveys directly associated with the GBS water respirometry samples are lacking, GBS planktonic communities are relatively stable and dominated by *Thermocrinis*, making up 91.5% ± 6.7% of pyrotags, and *Pyrobaculum*, ranging from <0.1% to 13.9% pyrotags (see Fig. S2) (14, 15). Most metabolisms inferred by the respirometry data are consistent with catabolic activities of well-characterized organisms with close relatives in the communities. For example, GBS and SSW contain close relatives of known hydrogenotrophs and $S_2O_3^{2-}$ oxidizers (*Thermocrinis*, *Sulfurihydrogenibium*, and *Pyrobaculum*), organic acid oxidizers (*Pyrobaculum* and *Thermocrinis*), ammonia oxidizers ("*Candidatus* Nitrosocaldus yellowstonii"), and aerobic, proteolytic heterotrophs (*Hydrogeniphilus*, *Anoxybacillus*, and *Thermus*) (19–21). NH_4^+ stimulation of O_2 consumption in sediments of both springs is consistent with relatively high rates of ammonia oxidation calculated using a $^{15}NO_2^-/NO_3^-$ pool dilution and with extremely high abundance of "*Ca.* Nitrosocaldus yellowstonii" in these springs (Fig. 2) (14). In contrast, no known methane-oxidizing microorganisms or consortia are known above 72°C (22), despite the stimulatory effect of CH_4 on O_2 consumption in sediments and the large amount of energy potentially available from methane oxidation in these springs (9, 10). We failed to amplify genes for methane monooxygenase by PCR (T. Vick and B. P. Hedlund, unpublished results) and genetic markers for known methane oxidizers were not found in 16S rRNA gene pyrotag data sets (14, 15) or metagenomes (see below) from GBS water and sediment; thus, the organism(s) responsible for stimulation of O_2 consumption by CH_4 are unknown.

Pyrotag data indicated that *Thermocrinis* and "*Ca.* Nitrosocaldus" spp. comprised the two most abundant operational taxonomic units (OTUs) in GBS water and sediment about which catabolic capacity could be predicted based on their relationship to characterized thermophiles (see Fig. S2 in the supplemental material). We leveraged existing metagenomic data from GBS water (JGI-IMG taxon object identifier 2084038020; available at http://img.jgi.doe.gov/m) and sediment (taxon object identifier 2053563014) to design nondegenerate primers targeting key genes in these two organisms, which enabled us to quantify these two OTUs and to explore potential catabolic capacity of the *Thermocrinis* OTU. Characterized members of the genus *Thermocrinis* are capable of aerobically respiring H_2 and thiosulfate (20, 23, 24), and some can additionally oxidize formate (20). Primers were designed to target *Thermocrinis* genes putatively involved in chemolithotrophic oxidation of thiosulfate (sulfur oxidation enzyme complex subunit *soxA*), hydrogen (NiFe hydrogenase large subunit *hyaB*), and formate (formate dehydrogenase large subunit *fdhA*), as well as the 16S rRNA gene (see Table S1 in the supplemental material). Quantitative PCR (qPCR) with these primers was performed essentially as described previously (14) using template DNA isolated from GBS water and sediment collected at the time and location of sample collection for respirometry experiments (see the supplemental material and Tables S2 and S3 for details). All qPCR amplicons had sizes and melting curves identical to standards, confirming the primers' fidelity. *Thermocrinis* 16S rRNA genes were less abundant in GBS sediments but approximately equal to total cell counts ($\sim 2 \times 10^6$ ml^{-1}) observed in GBS water (14), consistent with this *Thermocrinis* being a dominant member of the GBS water community. *Thermocrinis soxA*, *hyaB*, and *fdhA* were all detected in both sediment and water of GBS (Fig. 2), but their relative abundances in each environment differed by as much as an order of magnitude, with *soxA* most abundant in water and *hyaB* most abundant in sediment at levels equivalent to the 16S rRNA gene. These data are consistent with the existence of several distinct populations of *Thermocrinis* with different catabolic capacities and different habitat distributions in GBS. This interpretation is consistent with the detection by PCR of *soxA*, but not *hyaB* or *fdhA*, in genomic DNA obtained from multiple *Thermocrinis* isolates from GBS water and the ability of these strains to oxidize thiosulfate but not H_2 or formate (A. B. Babbitt and B. P. Hedlund, unpublished results). Multiple attempts to isolate RNA from the samples associated with respirometry experiments were unsuccessful; thus, we could not test whether these genes were expressed at different levels in GBS. Although similar experiments could, in principle, be done at

SSW to probe the catabolic capacity of *Sulfurihydrogenibium*, the most abundant organism in SSW water (see Fig. S1 in the supplemental material), we lack both metagenomic data and laboratory cultures from which nondegenerate PCR primers could be designed. All five known *Sulfurihydrogenibium* species use oxidized sulfur compounds as electron donors and three use H_2 (19), so these are possible donors for *Sulfurihydrogenibium* in SSW; however, $S_2O_3^{2-}$ was stimulatory only in SSW sediments and H_2 was not stimulatory in SSW at all (Fig. 1).

Quantification of the ammonia monooxygenase subunit A (*amoA*) and 16S rRNA genes from "*Ca*. Nitrosocaldus yellowstonii" was done by using nondegenerate primers previously described and optimized (14, 25). Similar to previous results, "*Ca*. Nitrosocaldus yellowstonii" *amoA* and 16S rRNA genes were abundant in sediments of both springs (Fig. 2) (SSW, 1.8×10^9 *amoA* copies g dry weight [gdw]$^{-1}$ and 4.1×10^9 16S rRNA gene copies gdw^{-1}) (14), which is consistent with the stimulatory effect of NH_4^+ addition to sediment slurries from both springs. NH_4^+ addition also stimulated O_2 consumption in SSW water, even though "*Ca*. Nitrosocaldus yellowstonii" populations were not abundant in SSW water at the time and location of sampling (see Fig. S2 in the supplemental material) (4.5×10^3 *amoA* copies ml^{-1} and 7.7×10^3 16S rRNA gene copies ml^{-1}) (14).

In conclusion, the stimulation of O_2 consumption by a variety of organic and inorganic compounds in samples from GBS and SSW suggest a diverse energy economy in these springs. It is noteworthy that H_2 oxidation was stimulatory only in GBS sediments, despite the dominance of *Aquificales* in the bulk water of each spring. The response of the sediment community but not the planktonic community to H_2 may be partially attributed to the differential distribution of functionally distinct *Thermocrinis* subpopulations. In addition, experiments with pure cultures of *Thermocrinis ruber* (C. N. Murphy and B. P. Hedlund, unpublished results) and *Sulfurihydrogenibium subterraneum* HGMK-1 (26, 27) show O_2-sensitive hydrogenase activity but constitutive $S_2O_3^{2-}$ oxidation activity, irrespective of H_2, O_2, or $S_2O_3^{2-}$ concentrations. Together, these data argue against the hypothesis that H_2 is a key electron donor in relatively oxidized or aerobic terrestrial geothermal springs, which are relatively common in the U.S. Great Basin, but do not rule out the possible importance of H_2 in more reduced or electron acceptor-limited geothermal settings. This study also underscores the need for caution when using 16S rRNA gene sequence to infer microbial activity, particularly for *Aquificales*, due to the presence of catabolically distinct subpopulations within 16S rRNA gene phylotypes and catabolic versatility due to gene regulation.

ACKNOWLEDGMENTS

We thank David and Sandy Jamieson for access to GBS and SSW and Anna-Louise Reysenbach for sharing *Thermocrinis ruber* pure cultures for controls. We also thank the Unisense team, particularly Lars Larsen, for training and discussions, and UNLV travel funds for support to visit Unisense. We thank members of the Hedlund lab for advice and discussion, particularly Scott Thomas and Mandy Williams.

This work was supported by NSF grants MCB-0546865 and OISE-0968421. B.P.H. acknowledges generous support from Greg Fullmer through the UNLV Foundation.

REFERENCES

1. **Brock TD.** 1978. Thermophilic microorganisms and life at high temperatures. Springer-Verlag, New York, NY.
2. **McCollom TM, Seewald JS.** 2007. Abiotic synthesis of organic compounds in deep-sea hydrothermal environments. Chem. Rev. **107**:382–401.
3. **Shock EL.** 1988. Organic acid metastability in sedimentary basins. Geology **16**:886–890.
4. **Amend JP, Shock EL.** 2001. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. FEMS Microbiol. Rev. **25**:175–243.
5. **Reysenbach AL, Shock E.** 2002. Merging genomes with geochemistry in hydrothermal ecosystems. Science **296**:1077–1082.
6. **Stetter KO.** 1992. Life at the upper temperature border, p 195–219. *In* Tran Thanh Van J, Tran Thanh Van K, Mounolou JC, Schneider J, McKay C (ed), Frontiers of life. Editions Frontieres, Gif-sur-Yvette, France.
7. **Stetter KO.** 2006. History of discovery of the first hyperthermophiles. Extremophiles **10**:357–362.
8. **Spear JR, Walker JJ, McCollom TM, Pace NR.** 2005. Hydrogen and bioenergetics in the Yellowstone geothermal ecosystem. Proc. Natl. Acad. Sci. U. S. A. **102**:2555–2560.
9. **Costa KC, Navarro JB, Shock EL, Zhang CL, Soukup D, Hedlund BP.** 2009. Microbiology and geochemistry of Great Boiling and Mud Hot Springs in the United States Great Basin. Extremophiles **13**:447–459.
10. **Dodsworth JA, McDonald AI, Hedlund BP.** 2012. Calculation of total free energy yield as an alternative approach for predicting the importance of potential chemolithotrophic reactions in geothermal springs. FEMS Microbiol. Ecol. **81**:446–454.
11. **Inskeep WP, Ackerman GG, Taylor WP, Kozubal M, Korf S, Macur RE.** 2005. On the energetics for chemolithotrophy in nonequilibrium systems: case studies of geothermal springs in Yellowstone National Park. Geobiology **3**:297–313.
12. **D'Imperio SC, Lehr R, Oduro H, Druschel G, Kuhl M, McDermott TR.** 2008. The relative importance of H_2 and H_2S as energy sources for primary production in geothermal springs. Appl. Environ. Microbiol. **74**:5802–5808.
13. **Boyd ES, Leavitt WD, Geesey GG.** 2009. CO_2 uptake and fixation by a thermoacidophilic microbial community attached to sulfur flocs in a geothermal spring. Appl. Environ. Microbiol. **75**:4289–4296.
14. **Dodsworth JA, Hungate BA, Hedlund BP.** 2011. Ammonia oxidation, denitrification, and dissimilatory nitrate reduction to ammonium in two US Great Basin hot springs with abundant ammonia-oxidizing archaea. Env. Microbiol. **13**:2371–2386.
15. **Cole JK, Peacock JP, Dodsworth JA, Williams AJ, Thompson DB, Dong H, Wu G, Hedlund BP.** 2013. Sediment microbial communities in Great Boiling Spring are controlled by temperature and distinct from water communities. ISME J. **7**:718–729.
16. **Hedlund BP, McDonald AI, Lam J, Dodsworth JA, Brown JR, Hungate BA.** 2011. Potential role of *Thermus thermophilus* and *T. oshimai* in high rates of nitrous oxide (N_2O) production in ~80°C hot springs in the US Great Basin. Geobiology **9**:471–480.
17. **Revsbech NP.** 1989. Diffusion characteristics of microbial communities determined by use of oxygen microsensors. J. Microbiol. Methods **9**:111–122.
18. **Santegoeds CM, Schramm A, de Beer D.** 1998. Microsensors as a tool to determine chemical microgradients and bacterial activity in wastewater biofilms and flocs. Biodegradation **9**:159–167.
19. **Flores GE, Liu Y, Ferrera I, Beveridge TJ, Reysenbach AL.** 2008. *Sulfurihydrogenibium kristjanssonii* sp. nov., a hydrogen- and sulfur-oxidizing thermophile isolated from a terrestrial Icelandic hot spring. Int. J. Syst. Evol. Microbiol. **58**:1153–1158.
20. **Huber R, Eder W, Heldwein S, Wanner G, Huber H, Rachel R, Stetter KO.** 1998. *Thermocrinis ruber* gen. nov., sp. nov., a pink-filament-forming hyperthermophilic bacterium isolated from Yellowstone National Park. Appl. Environ. Microbiol. **64**:3576–3583.
21. **de la Torre JR, Walker CB, Ingalls AE, Könneke M, Stahl DA.** 2008. Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. Environ. Microbiol. **10**:810–818.
22. **Bodrossy L, Kovács KL, McDonald IR, Murrell JC.** 1999. A novel thermophilic methane-oxidising γ-Proteobacterium. FEMS Microbiol. Lett. **170**:335–341.
23. **Caldwell SL, Liu Y, Ferrera I, Beveridge T, Reysenbach AL.** 2010. *Thermocrinis minervae* sp. nov., a hydrogen- and sulfur-oxidizing, thermophilic member of the Aquificales from a Costa Rican terrestrial hot spring. Int. J. Syst. Evol. Microbiol. **60**:338–343.
24. **Eder W, Huber R.** 2002. New isolates and physiological properties of the

Aquificales and description of *Thermocrinis albus* sp. nov. Extremophiles **6**:309–318.
25. **Dodsworth JA, Hungate B, de la Torre JR, Jiang H, Hedlund BP.** 2011. Measuring nitrification, denitrification, and related biomarkers in terrestrial geothermal ecosystems. Methods Enzymol. **486**:171–203.
26. **Takai K, Kobayashi H, Nealson KH, Horikoshi K.** 2003. *Sulfurihydrogenibium subterraneum* gen. nov., sp. nov., from a subsurface hot aquifer. Int. J. Syst. Evol. Microbiol. **53**:823–827.
27. **Takai K, Hirayama H, Sakihama Y, Inagaki F, Yamamoto Y, Hoikoshi K.** 2002. Isolation and metabolic characteristics of previously uncultured members of the order Aquificales in a subsurface gold mine. Appl. Environ. Microbiol. **68**:3046–3054.