An essential role for tungsten in the ecology and evolution of a previously uncultivated lineage of anaerobic, thermophilic Archaea

Steffen Buessecker1,14, Marike Palmer2,14, Dengxun Lai2, Joshua Dimapilis3, Xavier Mayali4, Damon Mosier3,5, Jian-Yu Jiao6, Daniel R. Colman7, Lisa M. Keller7, Emily St. John8, Michelle Miranda3, Cristina Gonzalez3, Lizett Gonzalez3, Christian Sam3, Christopher Villa3, Madeline Zhuo3, Nicholas Bodman3, Fernando Robles3, Eric S. Boyd7, Alysia D. Cox9, Brian St. Clair9, Zheng-Shuang Hua10, Wen-Jun Li6,11, Anna-Louise Reysenbach8, Matthew B. Stott12, Peter K. Weber4, Jennifer Pett-Ridge4,13, Anne E. Dekas1, Brian P. Hedlund2 & Jeremy A. Dodsworth3

Trace metals have been an important ingredient for life throughout Earth’s history. Here, we describe the genome-guided cultivation of a member of the elusive archaeal lineage Caldarchaeales (syn. Aigarchaeota), Wolframiiraptor gerlachensis, and its growth dependence on tungsten. A metagenome-assembled genome (MAG) of W. gerlachensis encodes putative tungsten membrane transport systems, as well as pathways for anaerobic oxidation of sugars probably mediated by tungsten-dependent ferredoxin oxidoreductases that are expressed during growth. Catalyzed reporter deposition-fluorescence in-situ hybridization (CARD-FISH) and nanoscale secondary ion mass spectrometry (nanoSIMS) show that W. gerlachensis preferentially assimilates xylose. Phylogenetic analyses of 78 high-quality Wolframiiraptor-aceae MAGs from terrestrial and marine hydrothermal systems suggest that tungsten-associated enzymes were present in the last common ancestor of extant Wolframiiraptor-aceae. Our observations imply a crucial role for tungsten-dependent metabolism in the origin and evolution of this lineage, and hint at a relic metabolic dependence on this trace metal in early anaerobic thermophiles.
vidence is mounting that some of the most ancient life forms inhabited terrestrial hot springs present on the anoxic early Earth. Such habitats could have mobilized and concentrated nutrients including trace metals essential to thermophiles, but that differ from the metals essential to extant aerobic mesophiles. Contemporary terrestrial hot springs are reasonable analogs for those on early Earth, often exhibiting low reduction potentials and sulfur-rich anoxic conditions. In the presence of sulfate, several trace metals, including molybdenum (Mo), copper (Cu), nickel (Ni), and cobalt (Co) react with sulfate and precipitate as solid phases, rendering them inaccessible to microbial cells. This, in turn, could have represented a strong selective pressure to evolve metalloenzymes that were dependent on less thiphilic trace metals, such as manganese (Mn) and tungsten (W).

In modern hot springs, most microbial lineages lack a cultured representative, making the assessment of nutrient requirements difficult. Although cultivation-independent "omics" techniques hold tremendous promise in assessing the functional potential and activity of communities in their natural state, results from such studies are limited by what is known of characterized proteins from cultured organisms. Yet, for uncultivated lineages, many of the proteins are poorly annotated, often leading to an incomplete picture of their ecology and physiology. As such, there is a great need to bring these organisms into the culture so that the traits that permit the habitation of terrestrial hot springs, both today and in the geological past, can be examined.

Candidatus Caldarchaeales (syn. Aigarchaeota) is a yet-uncultivated lineage detected in high-temperature environments that was first described based on a genomic assembly from a thermal stream in a subsurface gold mine in Japan. Although originally designated as candidate phylum Aigarchaeota, this lineage has been reassigned to the order Ca. Caldarchaeales within the phylum Thermoproteota and class Nitrospiraeota. For simplicity, we use this latter taxonomy without the Candidatus designation for uncultivated taxa. Caldarchaeales are commonly found in hydrothermal and geothermal ecosystems, where they can be abundant. Metagenomics and 16S rRNA gene amplicon surveys have previously shown a high abundance of Caldarchaeales in sediments of Great Boiling Spring (GBS, Nevada, USA). In particular, members of the putative genus "Aigarchaeota Group 4" (AigG4) were abundant in in-situ GBS enrichments cultivated on lignoncellulosic substrates such as corn stover. AigG4 species from GBS and from hot springs in Tengchong, China (GMQ_bin_10 and JZ_bin_10 in ref. 12) have been predicted to be anaerobic heterotrophs that degrade polysaccharides and peptides. However, these predictions have yet to be experimentally validated and other physiological traits of AigG4 remain unknown.

Here, we report the enrichment and stable laboratory growth of an AigG4 species from GBS we designate Wolfpamiraptor gerlachensis. After initial unsuccessful attempts to grow the species without amending growth media with spring water, analysis of a metagenome-assembled genome (MAG) of W. gerlachensis suggested possible dependence on the biologically rare trace metal tungsten (W), as evidenced by six annotated W-dependent ferredoxin oxidoreductases that transporters and tungstoenzymes were ancestral in this lineage, here designated the family Wolfpamiraptoraceae, and trace their evolution among 78 high-quality MAGs representing four genera and 11 species. While it is known that W is important for several archaeal groups, including methanogens, representatives of the Thermococcales and Thermoproteales are the only known archaea that require W for growth.

Our investigation is unique in showing long-term W-dependent growth dynamics in a mixed culture, identifying W-requiring archaea outside of the Methanobacteriota (formerly Euryarcheota), and demonstrating large-scale expansions of W-dependent enzymes in a microbial lineage.

Results

A stable enrichment culture of an anaerobic member of the Caldarchaeales. Because W. gerlachensis was previously enriched on corn stover incubated in GBS, we deployed new corn stover in-situ enrichments and incubated them for 6 months (Supplementary Fig. 1). The water above the incubation site was 86.5°C, with a pH of 7.31 at the time the enrichment was deployed. After incubation, W. gerlachensis was present in the in-situ enrichment at ~3% of the total community based on 16S rRNA gene tag analysis (Fig. 1a), similar to previous results, along with other taxa previously observed in enrichments or MAGs sediments. Based on the reproducible enrichment of W. gerlachensis on corn stover, and the genome-predicted anaerobic lifestyle, we used the in-situ enrichment to inoculate 13 different anaerobic media that contained corn stover and/or xyloglucan and keratin as carbon sources. Some enrichments also contained previously sampled GBS spring water that was filtered (0.2 µm) and auto-claved at a 1:1 ratio with synthetic medium (Supplementary Table 1) to test if unknown components of the spring water were required for growth. The abundance of W. gerlachensis under culture conditions without added spring water dropped below the level of detection. However, W. gerlachensis was maintained after multiple transfers in two cultures with added spring water (A8 and A10) at concentrations of ~0.25–1.0 x 10^6 16S rRNA gene copies mL^-1 culture, with a relative abundance of ~0.5–7% (Fig. 1b).

The two culture conditions that maintained stable populations of W. gerlachensis contained GBS spring water but were otherwise identical to synthetic media enrichments where W. gerlachensis was not maintained (compare A10 with A1, and A8 with A3; Supplementary Table 1). Additional experiments determined that nitrate (0.1 mM) was inhibitory to W. gerlachensis (explaining the lack of growth in culture A9) and that robust growth was dependent on anoxic conditions and on the presence of corn stover rather than other substrates (Supplementary Figs. 1 and 2 and Supplementary Table 1). As a result, most subsequent experiments were performed using a variation of the A10 medium (A10X2) with corn stover as the main carbon source under anoxia. Under these conditions, W. gerlachensis has been maintained at an abundance of 10^6–10^7 16S rRNA gene copies mL^-1 culture for over 4 years. To our knowledge, these represent only the second reported laboratory cultures containing members of the Caldarchaeales.

W is essential to maintain the growth of W. gerlachensis. Since GBS spring water was necessary for the maintenance of W. gerlachensis cultures (Fig. 1b and Supplementary Table 1), we compared the composition of the synthetic medium to GBS spring water using...
inductively coupled plasma mass spectrometry (ICP-MS) (Supplementary Data 1). Biologically relevant trace elements in the GBS spring water that were absent in the synthetic medium trace metal solution (Supplementary Data 2) included Ni (2.2 nM), which was however in the complete corn stover medium (19–26 nM), and W (263 nM)\(^{23}\). Only W was present in the spring water and not elsewhere, such as in the substrate (≤0.11 nM) or media components (<0.05 nM, Supplementary Data 2). Furthermore, preliminary analysis of a 16S rRNA gene-based composition of W. gerlachensis inferred from 16S rRNA gene tag sequencing. Boiling Spring indicated the presence of multiple W-associated enzymes and WO\(_4^{2-}\) ABC transporters. We therefore hypothesized that W was the key component in the GBS spring water allowing maintenance of W. gerlachensis.

The utilization of W was conceivable given its previous recognition as an essential element for diverse ferredoxin oxidoreductases in the marine heterotrophic hyperthermophile Pyrococcus furiosus (Thermococcales)\(^{24-28}\). Nevertheless, W biochemistry is rare in microorganisms, although the low redox potential of W cofactors makes them especially suitable for anaerobes\(^{29}\). For instance, the low redox potential of the \(\text{W}^6+/\text{W}^{4+}\) couple makes it preferable to Mo, which is more bioavailable in oxic environments and serves equivalent cellular functions in formaldehyde oxidoreductases and benzoyl CoA reductases\(^{30,31}\). In acetylene hydratase, W enables sharing of electrons of the outer orbital shell with the substrate and provides electrostatic stabilization to transition states\(^{32}\). In GBS, W is likely derived from minerals of the Scheelite (CaWO\(_4\)) and Wolframiiraptor groups ([Fe, Mn]WO\(_4\)), within W-rich deposits overlaying Triassic clays that can be extensive in the Great Basin\(^{33}\).

We confirmed that W. gerlachensis is dependent on W using a synthetic medium with discrete additions of WO\(_4^{2-}\) (Fig. 2). After two transfers in a synthetic medium without W amendment, W. gerlachensis fell to very low or undetectable levels based on amplicon sequence variant (ASV) counts, whereas addition of 0.1 or 2 \(\mu\)M WO\(_4^{2-}\) alleviated this effect (Fig. 2a). Importantly, the addition of W to the synthetic medium led to W. gerlachensis abundances indistinguishable from those in the original spring water-containing medium (ANOVA, \(p > 0.05\)). These results were also reflected in W. gerlachensis absolute abundance as determined by qPCR. The omission of W induced a drop of W. gerlachensis 16S rRNA genes by 2–3 orders of magnitude (Fig. 2b). Tungsten starvation induced loss of W. gerlachensis earlier (after the second transfer) in corn stover relative to sugar mix cultures (Fig. 2c and Supplementary Fig. 3), with orders-of-magnitude differences in W. gerlachensis 16S rRNA genes with varying W concentrations. Concentrations of 20 nM WO\(_4^{2-}\) yielded higher W. gerlachensis 16S rRNA gene numbers than 5 nM WO\(_4^{2-}\) (corn stover) and 1 nM WO\(_4^{2-}\) (sugar mix, ANOVA, \(p < 0.05\) for both), suggesting 20 nM WO\(_4^{2-}\) was the critical threshold to maintain W. gerlachensis over at least three transfers in synthetic medium (Fig. 2c).

Because the abundance of Caldimicrobium was also affected by W limitation (Fig. 2a and Supplementary Fig. 4), we were unable to exclude the possibility that W. gerlachensis was affected indirectly via Caldimicrobium, potentially due to a mutualistic relationship between the two. Nevertheless, the presence of multiple W-associated genes in the W. gerlachensis MAG, discussed below, suggested that the requirement for W is direct. To probe the genomic potential for W. gerlachensis anaerobic growth on carbohydrates, we annotated a high-quality MAG with 98.06% completeness and 0.49% contamination, containing both 16S and 23S rRNA genes. This MAG encoded six W-dependent ferredoxin oxidoreductases as well as a complete WO\(_4^{2-}\)-transmembrane ABC transport system of the Tup family, which corroborated our initial hypothesis that W is required for growth in synthetic medium.

![Fig. 1 16S rRNA gene-based composition of Caldarchaeales-containing cultures.](image-url)
W-dependent ferredoxin oxidoreductases are extremely oxygen-sensitive cytoplasmic tungsten enzymes that are involved in sugar and peptide catabolism in a variety of thermophiles. They oxidize various aldehydes, derived from amino acids or sugars, to corresponding carboxylic acids with ferredoxin as the electron acceptor. All characterized enzymes in this family require incorporation of W, which is coordinated by a pyranopterin cofactor, similar to molybdoenzymes. Dissolved W is mainly available as the tetrahedral oxyanion \( \text{WO}_4^{2-} \) in reduced spring waters. Tungstate is taken up by ABC-type transporters containing highly selective binding proteins (TupA, WtpA). Molybdate is transported by other distinct transmembrane proteins (ModABC). Although we found several annotated \( \text{MoO}_4^{2-} \) cofactor biosynthesis protein-encoding genes within the \( W. \) gerlachensis MAG, no Mod genes were present, supporting that multiple cellular processes in \( W. \) gerlachensis are dependent on W and not Mo.

To test if the identified genes encoding tungstoenzymes and W transporters were expressed, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) assays specific for these \( W. \) gerlachensis transcripts in community RNA. While several of the oxidoreductase genes were transcribed, \( \text{WO}_4^{2-} \) ABC-type transporters were only weakly transcribed (Supplementary Fig. 5).

---

**Fig. 2 Effects of W on Wolframiraptor (W. gerlachensis) growth in culture A10.**

a. ASV abundances based on 16S rRNA gene tags from synthetic medium with and without added spring water or W. The 12 most abundant taxa are shown, which consistently made up >98% of the total ASVs. *Caldimicrobium* and *W. gerlachensis* ASV counts from triplicate cultures were statistically different between synthetic medium with and without spring water (without W addition) as denoted by the asterisk (ANOVA, *p = 0.07, **p = 0.03, n = 3). ASV counts are reported as raw reads; see Supplementary Data 9 including normalized ASV counts that show identical trends.

b. Absolute abundance of *W. gerlachensis* based on 16S rRNA gene qPCR on corn stover incubations. Central marks in boxes indicate the median, with the bottom and top edges as the 25th and 75th percentiles. Error bars denote SD of biological replicates (n = 3).

c. Abundance of *W. gerlachensis* 16S RNA genes measured after each transfer and the resulting growth. Cultures were grown in corn stover and sugar mix in synthetic medium amended with discrete W concentrations (indicated by black arrows). 16S rRNA gene tags retained at the third transfer indicated no ASVs affiliated with *W. gerlachensis* in all replicates without W (Supplementary Fig. 4). Source data are provided as a Source Data file.
Escherichia coli transporters in other subunits have previously been observed for ABC-type transporters, including MoO₄²⁻ transporters, in *Escherichia coli*, including MoO₄²⁻ transporters, where the synthesis rate of the periplasmic binding protein exceeded that of the transmembrane portion by at least 100-fold. For the putative W-dependent ferredoxin oxidoreductases, synthesis rates correlated with increased sensitivity to W starvation and transcription of the gene encoding protein MCF3653435.1 was transcribed in corn stover cultures (Supplementary Fig. 5). The apparent higher expression of tungstoenzymes in corn stover over sugar mix cultures, whereas those encoding enzymes varied in enrichments with different ¹³C-labeled substrates (amino acids, starch, glucose, xylose, and ribose) to track carbohydrate metabolism, and with ¹⁵N-labeled ammonium to track overall assimilatory activity. Cells from non-FISH-labeled incubations collected after 24 h were significantly enriched in both ¹⁵N and ¹³C compared to unlabeled controls (Kruskal–Wallis test, p < 0.001).

Transcripts for the WO₄²⁻-binding portion of the ABC complex (MCF3653660.1) were detected in the sugar mix culture (irrespective of reverse transcriptase), and the transmembrane subunit transcripts were detected in the corn stover culture. Increased synthesis rates of the periplasmic binding proteins over other subunits have previously been observed for ABC-type transporters in *Escherichia coli*, including MoO₄²⁻ transporters, where the synthesis rate of the periplasmic binding protein exceeded that of the transmembrane portion by at least 100-fold. For the putative W-dependent ferredoxin oxidoreductases, the gene encoding protein MCF3653435.1 was transcribed in corn stover and sugar mix cultures, whereas those encoding enzymes were only transcribed in corn stover cultures (Supplementary Fig. 5). The apparent higher expression of tungstoenzymes in corn stover over sugar mix cultures is consistent with higher sensitivities to W starvation (Fig. 2c). Thus, the expression of W-dependent enzymes varied in enrichments provided with different primary carbon substrates, pointing to the direct involvement of tungstoenzymes in the catabolism of complex carbohydrates in corn stover.

**Xylose is the preferred carbohydrate monomer for *W. gerlachensis***. To investigate carbohydrate metabolism in *W. gerlachensis* in the absence of an axenic culture, we used a combined CARD-FISH-nanoSIMS approach that allowed us to directly interrogate the metabolism of single cells in the complex community of the enrichment. We amended GBS in situ corn stover enrichments with different ¹³C-labeled substrates (amino acids, starch, glucose, xylose, and ribose) to track carbohydrate metabolism, and with ¹⁵N-labeled ammonium to track overall assimilatory activity. Cells from non-FISH-labeled incubations collected after 24 h were significantly enriched in both ¹⁵N and ¹³C compared to unlabeled controls (Kruskal–Wallis test, p < 0.001; Fig. 3 and Supplementary Fig. 6), indicating active metabolism. Carbon and nitrogen enrichment were positively correlated (p = 0.003, Supplementary Fig. 6), but the ¹³C/¹⁵N enrichment varied, suggesting that some cells were metabolically active (labeled with ¹⁵N ammonium) but did not incorporate any of the ¹³C-labeled substrates.

The *W. gerlachensis* population identified with CARD-FISH and subsequently analyzed by nanoSIMS was significantly enriched with ¹³C and ¹⁵N in all except the ribose-amended incubations (Fig. 3 and Supplementary Fig. 6; Kruskal–Wallis test, p < 0.001). *W. gerlachensis* cells were the most active (¹⁵N enriched) in the xylose treatment. Xylose incubations also contained *W. gerlachensis* cells most enriched in ¹³C (median of 15 atom% excess and 100% of cells enriched), consistent with direct assimilation of xylose and a preference for it over the other substrates tested. Median ¹³C enrichment of *W. gerlachensis* cells incubated with labeled glucose, amino acids or starch was less than 1 atom% excess (potentially due to cross-feeding on metabolites derived from these substrates), and no isotope enrichment was detected in ¹³C ribose incubations.

A hot spring sediment community composed of 4% *Caldarchaeales* degraded the xylose polymer xylan and was used to purify a thermostable xylanase enzyme (>70% activity at 70 °C), indicating the participation of this lineage in polysaccharide-degrading consortia. We also found xylose isomerase (EC 5.3.1.5) and multiple xylooligosaccharide degradation enzymes (EC 2.7.1.17) encoding genes in the *W. gerlachensis* MAG. In light of our NanoSIMS results, these data suggest *W. gerlachensis* is an important contributor to the anaerobic degradation of lignocellulose by the microbial community at GBS, likely as members of a cellulolytic consortium. The precursor of xylose is likely hemicellulose in corn stover, but other sources might be present in natural hot spring environments, including both allochthonous (e.g., nearby plants) and autochthonous (e.g., biofilm) materials.
W-dependent ferrodoxin oxidoreductases putatively drive aldehyde metabolism. Next, we identified the potential substrates of the six W-requiring ferrodoxin oxidoreductases encoded by the W. gerlachensis MAG, and likely to be compromised by W limitation, employing primary sequence and structural analyses. Over 2600 sequences of known or annotated AOR, FOR, GAPOR, and WOR homologs served as reference sequences to interrogate the W-associated ferrodoxin oxidoreductase homologs encoded in the W. gerlachensis MAG. Based on phylogenetic analysis, three homologs were distantly related to well-characterized AORs, herein referred to as AOR-like 1, AOR-like 2, and AOR-like 3; one homolog was distantly related to characterized FORs, referred to herein as FOR-like; and one homolog was distantly related to characterized GAPORs, here termed GAPOR-like (Fig. 4 and Supplementary Figs. 7 and 8). To gain insight into the putative functions of these oxidoreductases, we modeled the protein structure of MAG-encoded oxidoreductase sequences belonging to the genus Wolframiraptor, along with selected reference sequences, and applied multidimensional scaling on the predicted structures (Fig. 4b). Structural similarities to a characterized AOR from P. furiosus, as quantified based on a sum-of-pairs score using a protein database reference, emerged in the three AOR-like lineages (Cluster I and II, Fig. 4b), supporting the phylogenetic analysis. The other oxidoreductases were structurally related to the GAPOR from Caldicellulosiruptor bescii (Clostridia)37 (Cluster III, Fig. 4b) and diverse FORs (Cluster IV, Fig. 4b), again congruent with the phylogenetic analysis. With the genomic assessments, coupled with the protein phylogenetic-structural analyses, we further looked for evidence of physiological dependence on W.

W-requiring ferrodoxin oxidoreductases are found at the heart of sugar and peptide metabolisms in thermophilic archaeobacteria44,45. While glycolysis in thermophilic bacteria is catalyzed by the conventional glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, glucose catabolism in several hyperthermophilic archaea differs from that canonical pathway and appears to proceed via a modified Embden–Meyerhof (EM) pathway that involves novel W-dependent enzymes46. On one hand, phylogenetic analysis and structural modeling of the homologs represented by MCF3653440.1 in W. gerlachensis predicts an encoded GAPOR (Fig. 4, GAPOR-like lineage). On the other hand, the W. gerlachensis MAG also encodes an annotated glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and a phosphoglycerate kinase (EC 2.7.2.3) and we thus cannot determine with certainty if pyruvate is originating via the canonical or modified (archaea-typical) EM pathway. Similar to the amino acid catabolism in P. furiosus, W. gerlachensis likely decarboxylates 2-keto acids, such as pyruvate, to aldehydes that are then oxidized by W-dependent ferrodoxin oxidoreductases to form carboxylic acids (Fig. 4c)47–49. The pivotal step of the metabolic inhibition occurs when W-requiring ferrodoxin oxidoreductases become insufficiently supplied with the W-cofactor tungstopterin and ferrodoxin reduction halts. A ferrodoxin-dependent, membrane-bound proton-translocating [NiFe]-hydrogenase (Mbh) encoded by the W. gerlachensis genome would then increasingly lack reduced ferrodoxin to trigger tunneling of $\mathrm{H}^+$ ions across the cell membrane, thereby leading to a decreased transmembrane $\mathrm{H}^+$ gradient and retarded ATP formation (Fig. 4).

Inactivation of W-dependent ferrodoxin oxidoreductases would be highly effective at depriving the cell of energy because (i) the encoded ATPase is predicted to be proton-dependent, based on alignment with V-type ATPases50, inducing a proton gradient of 3 ion equivalents across the membrane and (ii) these oxidoreductases can make up a significant proportion of cell biomass. For instance, in Thermococcus strain ES1, these oxidoreductases constitute 1% of the total protein mass51 and the intracellular W-dependent ferrodoxin oxidoreductase concentration in P. furiosus reached 110 $\mu$M44. Interestingly, the W. gerlachensis MAG revealed a putative ferrodoxin-dependent, membrane-bound, and proton-translocating [NiFe] hydrogenase complex of 10 subunits (Mbh-Mrp) including conserved cysteine pairs in the N- and C-terminus of the catalytic subunit MbhL (Supplementary Fig. 9) that ligate the [NiFe] metallocluster52, along with several Hyp-type [NiFe] metallocluster assembly proteins. We were not able to find $\text{rnf}$ genes53 in the W. gerlachensis MAG and we hence suspect ferrodoxin (as the reducing equivalents produced by AOR) would predominantly be recycled by the Mbh-Mrp. A similar Mbh-Mrp complex has previously been described for P. furiosus54,55 and is a type-4 [NiFe] energy-conserving hydrogenase56 that was also detected in other Caldarachaeales genomes57,58. W addition into culture media of the marine thermophile Thermotoga maritima resulted in stimulation of hydrogenase activity59. The carbon and energy metabolism in that bacterium are similar to that of W. gerlachensis60. Formation of pyruvate via the EM pathway, production of organic acids and $\mathrm{H}_2$ and therefore coupling of W enzymatic activity to a hydrogenase and alternative reduction of $\mathrm{S}^0$ to $\mathrm{H}_2\mathrm{~S}$ by $\mathrm{H}_2$. Across domains and comprising terrestrial as well as marine habitats, W-associated anaerobic heterotrophic processes are therefore important for thermophilic life.

Distribution of tungstoenzymes across the family Wolframiraporaceae. In evaluating the scope of tungstoenzymes in the Caldarachaeales, we recruited and annotated 77 additional high-quality MAGs related to W. gerlachensis that were extracted from metagenomes from geothermal springs in Tengchong, China; Yellowstone National Park, United States; and Taupo, New Zealand, along with a deep-sea hydrothermal mineral deposit from the Eastern Lau Spreading Center in the Southern Pacific. The assignment of these MAGs to the family Wolframiraporaceae was defined based on monophyly using conserved marker gene sets and relative evolutionary divergence values. The MAGs could be assigned to four genus-level lineages [including previously recognized AigG4 (syn. EX4484-121, herein Wolframiraptor), AigG5 (syn. JGI-000106-J15, herein Geocrenenecus), and AigG7 (syn. NZ13-MG1, herein Terraquivivens)]61, and 11 species (Fig. 5 and Supplementary Fig. 10), based on relative evolutionary divergence (Supplementary Data 3), average amino acid identity, and average nucleotide identity (Supplementary Data 4).

To assess the potential importance of W within the family Wolframiraporaceae, we interrogated the presence of tungstoenzymes within the genomes of these taxa. The three AOR-like, FOR-like, and GAPOR-like enzymes were widely distributed within the family, each present in more than half of the Wolframiraporaceae genomes (Fig. 4 and Supplementary Figs. 7 and 8), sparse in other Caldarachaeales, and absent from close relatives in the Nitrosophacia (Supplementary Fig. 11). In addition, three divergent putative tungstoenzymes were identified in the Geocrenenecus and Terraquivivens MAGs.

All protein-coding sequences annotated as the substrate-binding subunits of the Mod (K02020), Tup (K05772), or Wtp (K15495) ABC transporter systems within the family were analyzed to determine the putative substrate specificity of these transporters (Supplementary Fig. 12a, b). All species of the genera Wolframiraptor, Terraquivivens, and the sole representative of Benthortus encode homologs of the TupABC transporter. Notably, only a single genome of Geocrenenecus and members of the species T. tengchongensis encoded novel TupA- and/or WtpA-like proteins. The WtpA-like protein has been identified before as a ModA-like homolog in Pyrobaculum species14,59. The
consensus lack of a MoO$_4^{2-}$/WO$_4^{2-}$ transport system in the *Geocrenenecus* species is not unique, as several completely sequenced genomes from organisms known to utilize MoO$_4^{2-}$/WO$_4^{2-}$ lack any of these annotated ABC transporters.$^{59}$

Scaling up the distribution analysis of MoO$_4^{2-}$/WO$_4^{2-}$ transport proteins across entire thermophilic community metagenomes indicated unequal $modA$, $tupA$, and $wtpA$ gene frequencies among microbial communities inhabiting springs that span a spectrum of geochemical conditions (Supplementary Figs. 7 and Supplementary Data 7). The scale bar indicates the number of amino acid changes per site. Data points with the most similar structural neighborhoods are positioned near each other. Protein models are based on the amino acid sequences aligned in a. Reference sequences are color-coded. K-means clustering was applied to identify significant data clusters (colored ellipses) based on minimum cluster $r^2$. Clusters included from both references and those from representative members of the genus *Wolframiiraptor*. One sequence (MCF363595.1) was excluded from the analysis (<300 amino acids). Dotted lines are used to indicate putative groups identified in a and b. All AOR-like lineages grouped together in Clusters I and II, while GAPOR-like sequences grouped in Cluster III and FOR-like sequences grouped in Cluster IV.

Consensus lack of a MoO$_4^{2-}$/WO$_4^{2-}$ transport system in the *Geocrenenecus* species is not unique, as several completely sequenced genomes from organisms known to utilize MoO$_4^{2-}$/WO$_4^{2-}$ lack any of these annotated ABC transporters.$^{59}$

Scaling up the distribution analysis of MoO$_4^{2-}$/WO$_4^{2-}$ transport proteins across entire thermophilic community metagenomes indicated unequal *modA*, *tupA*, and *wtpA* gene frequencies among microbial communities inhabiting springs that span a spectrum of geochemical conditions (Supplementary Figs. 13 and 14). While the WO$_4^{2-}$ transporters show a high presence (~70%) in spring waters with temperatures >60 °C, *wtpA* is generally more abundant than *tupA* and is the most abundant MoO$_4^{2-}$/WO$_4^{2-}$ transporter among microbial communities of low-pH (<4) springs.

To better understand how *Wolframiiraptoraceae* acquired W-dependent enzymes, we inferred associated evolutionary events through phylogenetic analyses, ancestral character state reconstructions (Supplementary File 2), and gene tree/species tree
reconciliation analyses based on the W-dependent ferredoxin oxidoreductases and MoO$_4^{2-}$/WO$_4^{2-}$ transporters (Fig. 5c). These analyses revealed a complex evolutionary history characterized by rampant lateral transfer from other *Thermoproteota* to various *Wolframiiraptoraceae* lineages. Although it remains difficult to distinguish between phylogenetic noise and true signal for lateral acquisitions in single-gene trees, the presence of one tungsten-enzyme (AOR-like 1) in the ancestral node to the *Wolframiiraptoraceae* was well supported by all analyses. This ancestral enzyme, combined with extensive capture of W-associated enzymes from *Thermoproteota* and other sources (Supplementary Figs. 7, 8, and 10 and Supplementary Dataset 2), and multiple gene family expansions and losses throughout the evolution of the *Wolframiiraptoraceae*, implies an important role for W in the diversification of the family. Given the close phylogenetic grouping of the *Wolframiiraptoraceae* TupA transporter subunit, AOR-like 3, FOR-like, and GAPOR-like sequences with those from diverse *Thermoproteota* (Supplementary Figs. 7, 8, and 10 and Supplementary File 3), these enzymes may have been inherited through separate horizontal acquisitions. However, the
notable progressive increases in the number and diversity of W-dependent enzymes within Caldarchaeales, and in particular \textit{Wolframiraptoraceae}, suggest an early emergence of W utilization within the \textit{Thermoproteota} and a critical role for W metabolism in the evolution of the \textit{Wolframiraptoraceae}.

Discussion

In the present study, we demonstrate that W is required to culture the anaerobic species \textit{W. gerlachensis}, the first physiologically characterized cultured member of the Caldarchaeales. The basis of this requirement, as evidenced by poor biomass yields in cultures in synthetic medium without amended W, is most likely W-dependent ferredoxin oxidoreductases involved in the degradation of xylene-derived pyruvate, which becomes non-functional without W. This dependence on W is seemingly a characteristic of several close relatives of \textit{W. gerlachensis}, with W utilization feasibly representing an ancestral trait within the family and important for the diversification of the \textit{Wolframiraptoraceae}.

The levels of W required by \textit{W. gerlachensis} (≥20 nM) are below those that are typically inhibitory to the growth of other microorganisms where W inhibition has been demonstrated\(^\text{18}\). The consensus presence or absence profiles for species with multiple genome sequences available were determined by data as indicated for the majority of genomes in the species. Where genes encoding these proteins are present in some genomes within a taxon, colored dots are partially filled, while fully filled dots in the dot matrix indicate the presence in the majority of the genomes belonging to that taxon. For full A/B-type transporter phylogeny, including KOs K02020, K05772, and K15495, see Supplementary Figs. 10 and Fig. 5, and Supplementary Fig. 7 for phylogenies and structural modeling of W-dependent ferredoxin oxidoreductases.

Methods

\textbf{In-situ enrichments and sampling.} GBS water used in the preparation of cultivation medium was sampled in 2015, 2016, 2017, and 2018, passed through a 0.2 µm filter, and stored in 20 L plastic containers in the laboratory without temperature control. In situ enrichments on ammonia-methionine explosion-treated corn stover (kindly provided by Bruce Dale, Michigan State University) in GBS were performed as described in Peacock et al.\(^\text{72}\). Samples were taken after approximately six months of incubation on March 29, 2016. At that time, the temperature of the water was 86.0 °C and the pH was 7.15, typical for the main source pool of GBS.\(^\text{18}\) For sampling, a nylon bag containing corn stover was removed from the conical tube, the bag was cut open with a sterile scissors, and approximately half of the contents was immediately transferred to 10 mL of 0.2 µm filtered GBS water (previously sampled, sparged with N\(_2\), reduced by the addition of cysteine to 0.05 g/L and sterilized by autoclaving) in a 25 mL Balch tube under a stream of N\(_2\) gas applied through a 0.2 µm filter. The tube was sealed with a butyl rubber stopper, the headspace was flushed for 3 min with N\(_2\) gas, and the tube was then shaken to disperse the corn stover. Previously prepared anaerobic media (50 mL in 160 mL serum vials sealed with butyl rubber stoppers; see below) were inoculated with 0.2 mL of the corn stover slurrying a sterile, N\(_2\)-flushed needle and syringe. The bottles were transported back to the laboratory at ambient temperature and were transferred to high-temperature incubators within 24 h of sample collection. The remaining in-situ corn stover enrichment was placed in 1.5 mL centrifuge tubes, frozen immediately, and transported back to the laboratory on dry ice, and then stored at ~80 °C for DNA extraction.

\textbf{Laboratory culturing.} The 13 different media and incubation conditions used are shown in Supplementary Table 1. All media were based on GBS salts synthetic medium, which contained per liter of 18.2 M\(_2\)O water: 3 g NaCl, 0.3 g Na\(_2\)SO\(_4\), 0.15 g KCl, 0.015 g CaCl\(_2\), 0.123 g MgSO\(_4\), 0.5 mL of a trace mineral solution\(^\text{64}\). The trace mineral solution, based on ref.\(^\text{65}\), contained per liter: 3.7 g Na\(_2\)-EDTA, 1.1 g FeSO\(_4\), 0.028 g ZnSO\(_4\), 0.098 g MnCl\(_2\), 0.031 g NaMoO\(_4\). The "spring water medium" consisted of a 1:1 mix of the above synthetic medium and water collected from GBS as described above. All media contained 0.107 g/L NH\(_4\)Cl. The medium was then sparged with N\(_2\) gas and transferred into serum vials in an anaerobic chamber. The vials were sealed with butyl rubber stoppers and aluminum crimps, the headspace was exchanged with N\(_2\) by three cycles of vacuuming to 385 mm Hg and pressurizing to 15 psi with N\(_2\), and the vials were sterilized by autoclaving\(^\text{66}\). Prior to inoculation, the following were added to all media at the indicated final concentrations from concentrated, anoxic stocks: 0.025% keratin, 0.002% xylolgen, 0.02% Na\(_2\)S, 0.02% cysteine, 0.002% yeast extract, 0.014% NaH\(_2\)PO\(_4\), and 1.6 mL/L of a vitamin solution\(^\text{66}\). All stocks were sterilized by autoclaving except yeast extract and vitamins, which were passed through a 0.2 µm filter. Additions for different media conditions in the initial enrichments were made from sterile, anoxic stocks to final concentrations shown in Supplementary Table 1, except the corn stover (0.02% m/v) was added to individual serum vials in the anaerobic chamber prior to sealing. Follow-up experiments used the same base synthetic or spring water media with modifications of the specific substrates that were excluded or added anaerobically. After stable laboratory cultures were obtained, the culture conditions for routine transfer and maintenance used the base spring water medium with 2 mM sodium bicarbonate and either corn
stover (0.02% m/v) or a “sugar mix” (0.01% m/v each of glucose, xylose, mannose, and D- and L-arabinose) for major carbon sources. Laboratory cultures were kept at 80°C and initially using 5 g packets of corn stover, and then after 2 weeks for the first two transfers after inoculation with in situ enrichments and then every 3 weeks thereafter.

**Quantification of W in water and media by ICP-MS and ICP-OES.** W levels in laboratory media and filtered GBS spring water were measured using ICP-MS after the addition of nitric acid (OPTIMA grade, Fisher Chemical, Fair Lawn, NJ, USA) in a fume hood at Huffman Laboratory (Golden, CO, USA). W levels in filtered GBS spring water were also measured using inductively coupled plasma optical emission spectroscopy (ICP-OES) with an Agilent 7100 ICP-OES (Santa Clara, CA, USA) and dilutions of a W calibration standard (PLW-2Y, SPEX CertiPrep, Metuchen, NJ, USA). Nitric acid was added to samples and standards to a final concentration of 1% before analysis, and optical emission at 207.91 nm was used for W quantification. Broad trace element analysis by ICP-MS on GBS spring water and media was performed on samples prepared as described above at the Analytical Chemistry Laboratory of New Mexico Bureau of Geology and Mineral Resources, New Mexico Institute of Mining and Technology.

**16S rRNA gene tag sequencing.** 16S rRNA gene tag amplification and sequencing on internal enrichment cultures was performed essentially as described111. A modified forward primer 5’ (GGGCGACCCGCGCGGTAAA) with a Y instead of a C at the fourth position from the 5’ end was used to increase coverage of Archaea, and a corresponding change was made to the SeqRead1 primer. Sequencing (2 x 250 bp) was performed on an Illumina MiSeq sequencer using the V2 reagent kit. Additional 16S rRNA gene sequencing on laboratory cultures tested with different levels of W was performed as described12 at Argonne National Laboratories.

**Genomic DNA and RNA extraction.** For the laboratory culture, biomass (from 10 to 40 mL culture) was harvested by centrifugation at 10 min at 9300 g and 4°C in 50 mL conical tubes. Resulting pellets were resuspended in 1 mL of supernatant, transferred to 1.5 mL tubes, and centrifuged at 16,100 g for 5 min at 4°C. The supernatant was removed and the pellets were stored at −80°C until nucleic acid extraction. DNA was extracted using the Fast DNA Spin Kit for Soil (MP Biomedicals) as described following the manufacturer’s recommendations, except that homogenization by bead-beating was performed twice for 30 s at a speed setting of 4.5 m/s. DNA was eluted in 75–100 µL of DEPC water and further diluted 10-fold in 10 mM Tris buffer pH 8. DNA was isolated long fragments of DNA for Oxford Nanopore sequencing. DNA was extracted using the cetyltrimethyl ammonium bromide protocol of the Joint Genome Institute66. RNA was extracted using the FastRNA Spin Kit for Soil (MP Biomedicals) and the High Pure RNA Isolation Kit (Roche) as described in Hamilton et al.20, followed by an additional DNase treatment using the TURBO DNase kit (Ambion) according to the manufacturer’s instructions. For all other information regarding metagenomic DNA extraction, please refer to Supplementary Note 1.

**Quantitative PCR.** Assays were performed in 96-well plates (Applied Biosystems) in a StepOnePlus Quantitative Real-Time PCR machine (Applied Biosystems) using PowerUp SYBR Green Master Mix (Applied Biosystems). Cycling conditions for AigG4-specific primers (Supplementary Table 2) were as follows: initial denaturation for 15 min at 95°C, followed by 45 cycles of denaturation (20 s at 95°C), annealing (25 s at 64°C), and extension (30 s at 72°C). Specificity of qPCR primers was verified by melt curve analysis. Using a plasmid with a fragment of the AigG4 16S SSU rDNA sequence as a standard (SSW_L4_C03; ref.23), an additional DNase treatment using the TURBO DNase kit (Ambion) according to the manufacturer’s instructions. For all other information regarding metagenomic DNA extraction, please refer to Supplementary Note 1.

**Reverse transcriptase PCR.** RNA was converted to cDNA with random hexamer primers using the Verso cDNA kit (ThermoScientific), including control reactions without added reverse transcriptase. cDNA was amplified with primers targeting AigG4 predicted W-associated genes and AigG4 16S rRNA (Supplementary Table 3) in the Axxygen® Maxygene™ II Thermal Cyclers as follows: at 95°C for 3 min followed by 45 cycles of denaturation (20 s at 95°C), annealing (30 s at annealing temperature appropriate for specific primer set), and extension (72°C for 30 s), then 72°C for 5 min. Primers targeting AigG4 predicted W-associated enzymes were designed using Primer3.25 DNA was quantified using the Qubit 4.0 with HS DNA reagent kit.

**Stable isotope labeling.** Stable isotope labeling was performed on-site at GBS in July 2017 using in situ corn stover enrichments prepared and incubated as described10. Two packets were placed, and cDNA was isolated from samples collected at the GBS site (outflow)66 for 34 days. Two packets (approximately 10 g) of freshly harvested corn stover enrichment were added to 180 mL of anaerobic, autoclaved GBS water in a sealed 500 mL bottle with an N2 headspace and shaken to form a slurry. Samples of this slurry (2 mL) were then added via syringe to sealed 60 mL amber serum vials containing 40 mL of filtered GBS water, generating 10 mL microcosms. Various individual 13C-labeled substrates (Cambridge Isotope Laboratories) were then added from concentrated, anaerobic stocks to the microcosms at 0.001% (mass/volume) final concentration: algal amino acids, algal starch, glucose, xylose, ribose, or an equal-mass mixture of acetate, propionate, and butyrate. Incubations were controlled with no labeled carbon source as a positive control for enriching cells. In addition, 0.5 mM 15NH4Cl was added to all microcosms. Preparation of microcosms was done in a hot water bath at 75°C to prevent excessive cooling. Unlabeled controls were processed as described above immediately after inoculation. Sealed microcosms with labeled substrates were incubated at 75°C for 4 or 24 h. After incubation, microcosm vials were immersed in ice water to cool, shaken vigorously for 30 s, opened, and decanted into 15 mL conical tubes. Tubes were centrifuged for 30 s at 11 g in a clinical centrifuge to pellet large fragments of corn stover. Supernatant (4.5 mL) was then split between three 1.5 mL tubes and centrifuged for 5 min at 16,100 g to pellet cells. Pellets from the three microcosms were pooled in 0.25 mL of 1% PBS, and 0.5 mL of 3% paraformaldehyde (PFA) was added and mixed. Samples were fixed for 1 h on ice. After fixation, tubes were centrifuged for 5 min at 16,100 g. Resulting pellets were washed twice with 1× PBS, resuspended in 200 µL of 50% ethanol, and stored on ice (in the field) and then at −20°C in the laboratory. Prior to performing FISH, Nycodenz density cushion centrifugation25 was used to separate cells from higher density particles in the samples. Cells harvested from the aqueous-Nycodenz interface were pelleted by centrifugation, washed twice in water, and resuspended in 50% ethanol.

**CARD-FISH.** Detection of W. gerlachii (AigG4) cells was performed using CARD-FISH using a horseradish peroxidase-labeled probe 5’ CRGAAAAGGCTTCAAACGTGT (Biomers.net, Germany). This probe is specific to Aigarchaeota Group 4 and Group 5, and was designed based on multiple sequence alignment of 16S rRNA genes of Aigarchaeota and other Archaea and checked using the Probe Match Database of the Ribosomal Database Project.15 Optimization of dNTP concentrations used during hybridization was performed using the Clone-FISH technique70 with the AigG4 16S rRNA gene sequence SSW_L4_C03 (EU635908)23 expressed from the T7 promoter on plasmid pET23(+) in E. coli strain JM109(DE3). Formamide concentrations from 0 to 50% were tested in 10% increments. The highest formamide concentration that gave strong signal (20%) was then used to test for signal in the E. coli strain without induction of expression of the AigG4 16S rRNA as well as Staphylococcus aureus and Bacillus subtilis as negative controls. CARD-FISH was performed as previously described70 with minor modifications. Briefly, cells were fixed using a 1% PFA solution for 1 h, followed by storage at −20°C until use. The protocol was performed on fixed cells spotted and air dried on custom synthesized 14-well tin oxide (ITO)–coated slides rather than using filters. Cells were permeabilized by treatment with 10 mg/mL lysozyme (for Clone-FISH in E. coli and negative controls) or 1 µg/mL proteinase K (for samples with AigG4) in 50 mM EDTA and 100 mM Tris (pH 8) for 60 min at 37°C, endogenous peroxidases were inactivated by treatment with 0.01 M HCl for 15 min at ambient temperature (~25°C), and dehydrated using 96% EtOH. Hybridization was performed at 20% formamide for 16 h at 46°C, followed by washing at 48°C, CARD using tyramide labeled with AlexaFluor 555 (Invitrogen), and counterstaining by incubation in ice-cold 1 µg/mL DAPI for 10 min. Cells were visualized with a Carl Zeiss LSM510MBRII microscope in laser confocal dry immersion objective. Fluorescence and brightfield images were collected at diverse locations with FISH-positive cells and the X-Y location of those images was noted, as well as fiducial locations to enable navigation in the nanoSIMS.

**NanoSIMS.** ITO-coated slides were cut with a diamond saw in order to fit on metal sample holders and analyzed on a CAMECA nanoSIMS 50 at Lawrence Livermore National Laboratory. The charge-coupled device camera was used to find fiducial locations and locations of the FISH-positive cells using X-Y coordinates and the real-time imaging unit. The primary 13C-2 μm ion beam was set to 1.3 pA, corresponding to an approximately 100 nm diameter beam diameter at 16 kV. Sputtering was performed over 20 × 20 µm analysis areas with a dwell time of 1 ms per pixel14 for 19–30 scans (cycles) and generated images containing 256 × 256 pixels. Sputtering equilibrium at each analysis area was achieved with an initial beam current of 90 pA to a depth of <~10 nm, thus ensuring analysis of intracellular isotopic material but without sputtering the ITO coating. After tuning the secondary ion spectrometer for mass resolving power of ~7000, secondary electron images and quantitative secondary ion images were simultaneously collected for 12C2/13C2, 14N/15N, and 12C14N/12C15N on individual electron multipliers in pulse counting mode. All nanoSIMS datasets from two independent experiments using initially prepared 1H2O microcosms were collected at Lawrence Livermore National Laboratory (August 2021) to perform deadtime and image shift correction of ion image data.
Metagenome sequencing, assembly, and binning. For the AigQ4 cultures established from the in-situ enrichment at GBs, extracted DNA were sequenced using the Illumina MiSeq platform (2 x 250 bp), along with Oxford Nanopore sequencing using a MinION device. All extracted metagenomic DNA from geo-thermal springs from Tengchong, China, and Yellowstone National Park, USA, and the marine hydrothermal vent in the Western Pacific were sequenced on the Illumina NovaSeq, HiSeq3000, or HiSeq4000 platforms, in a paired-end or single-read configuration (Supplementary Note 2). All MAGs were subjected to quality checks with CheckM v. 1.0.18-1.1.373 and classification with GTDB Toolkit v. 1.1.1.374. This was followed by gene calling using Prodigal v. 2.6.275 and annotation of all MAGs using eggNOG-maps v. 2.76, and gene calling and annotation with Rapid Annotation using Subsystem Technology77 in the case of the hybrid assembly for W. gerlachensis.

Phylogenomic analyses. All MAGs classified as belonging to the family NZ23-MG1 in the order Caldarchaeales were used for the construction of a species tree. As reference sequences, genomes for members of the Nitrososphaerales (syn. Thaumarchaeota), along with publicly available genomes for the Caldarchaeales (syn. Aigarchaeota) were included in the taxon set. The 122 conserved archaeal marker sequences in the ar122 dataset were identified from the genomes of these taxa using the GTDB Toolkit v. 1.4.174. Aligned markers were subjected to testing for appropriate evolutionary models using ProtTest v. 3.4.78, followed by concatenation and partitioning with FASconCAT-G v. 1.0.479. This partitioned, concatenated matrix was subjected to maximum-likelihood tree reconstruction with RAxML v. 8.2.1280, with the appropriate evolutionary model applied to each partition, and branch support inferred from 1000 bootstrap pseudoreplicates. For description on the phylogenomic analysis for the domain Archaea, please see Supplementary Notes 3-6.

Phylogenetic analyses of genes. For phylogenetic confirmation of function of the predicted genes related to physiology, and specifically tungsten enzymes, all Wolframitriarctiacceae sequences annotated as belonging to the KEGG Orthology (KO) numbers of interest were used to infer phylogenetic trees to place the identified sequences into evolutionary context within the protein families. A detailed description of these analyses is provided in the Supplementary notes (see also Supplementary Data 7 and 8).

Gene tree/species tree reconciliation and ancestral character state reconstruction. For reconciliation analyses, a species tree was inferred from the domain-level phylogenomic tree with Bacteria specified as the outgroup. Similarly, subtrees for all W-associated homologs were inferred from the maximum-likelihood phylogenetic analyses of single-gene trees (see Supplementary materials for full description of single-gene tree construction). The inferred gene trees were reconciled with the species tree for the family ecceTERA v. 1.2.691, with default reconciliation penalties, partially ultrametric trees specified, and the median reconciliation graph pruned as syvX reconciliations. Resulting syx reconciliations were viewed in SylvX v. 4.525, and median symmetrical reconciliations were summarized and edited in Inkscape v. 0.92.4. A cladogram inferred from the species tree constructed from the genomes was used as the basis for performing reconstruction of ancestral character states for the family, with a particular focus on genes relating to physiology and W utilization. As the presence or absence of genes within single MAGs can be unreliable, a majority-rules consensus approach was employed for all genomes of the respective species. This entailed using all eggNOG annotated genomes, and summarizing the genome content based on KO numbers for each genome. The absence (0), presence (1), or presence in multiple copies (22) for each species was determined based on all genomes belonging to the species. These consensus presence/absence profiles for the species were then used to reconstruct all potential evolutionary events (gene birth, HGT, duplication, or loss) by optimizing rates and inferring the history of gene families by posterior probabilities, as implemented in COUNT15. The COUNT sessions for the full genome analysis and the tungsten-enzyme-specific analysis are available as Supplementary Data 6.

Protein structure modeling. W-dependent oxidoreductase sequences were submitted to the iterative threading assembly refinement (I-TASSER) server164 to generate models of oxidoreductase homologs. Models were selected based on C-score165. All Wolframitriarctiacceae W-dependent oxidoreductases reached at least a C-score of 1.3. Structural comparison of all-against-all was done with DALi v5 based on PDB coordinates of the models166,167. The first two eigenvectors of the output were used for the multidimensional-scaling and K-means clustering168 applied in JMP Pro (Version 13.1.0, SAS Institute Inc.).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Sequence data for nomenclatural types and some non-type genomes were deposited on GenBank under accession numbers listed in Supplementary Data 3, and all other genomes were submitted to eLMSG and/or IMG under Biotic project nos. PRJNA495050, PRJNA791658, PRJNA807863, PRJNA381623, and PRJNA780009. For nomenclatural types, raw sequence reads were submitted to the Sequence Read Archive (SRA) under the SRA run accessions listed in Supplementary Data 3. All other data are contained within the manuscript and supplements/source data files. Source Data are provided with this paper.

Received: 26 October 2021; Accepted: 17 June 2022;
Published online: 30 June 2022

References
1. Weiss, S. et al. Correlation detection strategies in microbial data sets vary widely in sensitivity and precision. ISME J. 16, 1699–1716 (2016).
2. Djokic, T., Kranendonk, M. J. V., Campbell, K. A., Walter, M. R. & Ward, C. R. Earliest signs of life on land preserved in ca. 3.5 Ga hot spring deposits. Nat. Commun. 8, 1–9 (2017).
3. Archibald, B. & Deamer, D. The Hot Spring Hypothesis for an origin of life. Astrobiology 20, 429–452 (2020).
4. Van Kranendonk, M. J. et al. Elements for the origin of life on land: a deep-time perspective from the Pilbara Craton of Western Australia. Astrobiology 21, 39–59 (2021).
5. Colman, D. R. et al. Phylogenomic analysis of novel Diaforarchaea is consistent with sulfite but not sulfate reduction in volcanic environments on early Earth. ISME J. 14, 1316–1331 (2020).
6. Anbar, A. D. & Knoll, A. H. Proterozoic ocean chemistry and evolution: a bioinorganic bridge? Science 297, 1137–1142 (2002).
7. Lloyd, K. G. et al. Phylogenetically novel uncultured microbial cells dominate Earth microbomes. mSystems 3, 431 (2018).
8. Hedlund, B. P. et al. Uncultivated thermophiles: current status and spotlight on ‘Aigarchaeota’. Curr. Opin. Microbiol. 25, 136–145 (2015).
9. Nounoua, T. et al. Genetic and functional properties of uncultivated thermophilic crenarchaeotes from a subsurface gold mine as revealed by analysis of genome fragments. Environ. Microbiol. 7, 1967–1984 (2005).
10. Nounoua, T. et al. Insights into the evolution of Archaea and eukaryotic protein modifier systems revealed by the genome of a novel archaeal group. Nucleic Acids Res. 39, 3204–3212 (2010).
11. Rinke, C. et al. A standardized archaeal taxonomy for the Genome Taxonomy Database. Nat. Microbiol. 6, 946–959 (2021).
12. Hua, Z.-s. et al. Genomic inference of the metabolism and evolution of the archaeal phylum Aigarchaeota. Nat. Commun. 9, 1–11 (2018).
13. Takami, H., Arai, W., Takemoto, K., Uchiyama, I. & Taniguchi, T. Functional classification of uncultured ‘Candidatus Caldaraechaeum subterraneum’ using the Maple system. PLoS ONE 10, e0132994 (2015).
14. Beam, J. P. et al. Ecophysiology of an uncultivated lineage of Aigarchaeota from an oxic, hot spring filamentous ‘streamer’ community. ISME J. 10, 210–224 (2016).
15. Rinke, C. et al. Insights into the phylogeny and coding potential of microbial dark matter. Nature 499, 431–437 (2013).
16. Cole, J. K. et al. Sediment microbial communities in Great Boiling Spring are controlled by temperature and distinct from water communities. ISME J. 7, 718–729 (2013).
17. Peacock, J. P. et al. Pyrosequencing reveals high-temperature cytolysyl microbial consortia in Great Boiling Spring after in situ lignocellulose enrichment. PLoS ONE 8, e59927 (2013).
18. Kletzin, A. & Adams, M. W. W. Tungsten in biological systems. FEMS Microbiol. Rev. 18, 5–63 (1996).
19. Hagedorn, P. L. et al. Purification and characterization of the tungsten enzyme aldheyde:ferredoxin oxidoreductase from the hyperthermophilic denitrifier Pyrobaculum aerophilum. J. Biol. Inorg. Chem. 10, 259–269 (2005).
20. de Vries, S. et al. Adaptation to a high-tungsten environment: Pyrobaculum aerophilum contains an active tungsten nitrate reductase. Biochemistry 49, 9911–9921 (2010).
21. Bräsen, C., Eiser, D., Rauch, B. & Siebers, B. Carbohydrate metabolism in Archaea: current insights into unusual enzymes and pathways and their regulation. Microbiol. Mol. Biol. Rev. 78, 89–175 (2014).
22. Kato, S. et al. Long-term cultivation and metagenomics reveal ecophysiology of previously uncultivated thermostoichi anaerobes. *Microbes Environ.* 33, 107–110 (2018).

23. Costa, K. C. et al. Microbiology and geochemistry of great boiling and mud hot springs in the United States Great Basin. *Extremophiles* 13, 447–459 (2009).

24. Mukund, S. & Adams, M. W. The novel tungsten-iron-sulfur protein of the hyperthermophilic archaeon, *Pyrococcus furiosus*, is an aldehyde ferredoxin oxidoreductase. Evidence for its participation in a unique glycolytic pathway. *J. Biol. Chem.* 266, 14208–14216 (1991).

25. Mukund, S. & Adams, M. W. W. Glyceraldehyde-3-phosphate ferredoxin oxidoreductase, a novel tungsten-containing enzyme with a potential glycolytic role in the hyperthermophilic archaeon *Pyrococcus furiosus*. *J. Biol. Chem.* 270, 8389–8392 (1995).

26. Roy, R. et al. Purification and molecular characterization of the tungsten-containing formaldehyde ferredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*: the third of a putative five-member tungstenoxygen family. *J. Bacteriol.* 181, 1171–1180 (1999).

27. Roy, R. & Adams, M. W. W. Characterization of a fourth tungsten-containing enzyme from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J. Bacteriol.* 184, 6952–6956 (2002).

28. Bevers, L. E., Bol, E., Hagedoorn, P.-L. & Hagen, W. R. WORS, a novel tungsten-containing aldehyde oxidoreductase from *Pyrococcus furiosus* with a broad substrate specificity. *J. Bacteriol.* 187, 7056–7061 (2005).

29. Cabib, U. & Hofmann, M. Effect of molybdenum and tungsten on the reduction of nitrate in nitrate reductase, a DFT study. *Chem. Cent. J.* 11, 1–12 (2017).

30. Liao, R.-Z. Why is the molybdenum-substituted tungsten-dependent formyldihydroxyferredoxin oxidoreductase not active? A quantum chemical study. *J. Biol. Inorg. Chem.* 18, 175–181 (2013).

31. Qian, H.-X. & Liao, R.-Z. QM/MM study of tungsten-dependent benzoyl-coenzyme A reductase: rationalization of regioselectivity and predication of W vs Mo selectivity. *Inorg. Chem.* 57, 10667–10678 (2018).

32. Liu, Y.-F., Liao, R.-Z., Ding, W.-J., Yu, J.-G. & Liu, R.-Z. Theoretical investigation of the first-shell mechanism of acetylene hydration catalyzed by a biomimetic tungsten complex. *JBC* 16, 745–752 (2011).

33. Korr, P. P. Tungsten-bearing manganese deposit at Golconda, Nevada. *Adv. Prot. Chem.* 48, 101 (1995).

34. Mukund, S. & Adams, M. W. Molybdenum and vanadium do not replace tungsten-containing oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*: the third of a putative five-member tungstenoxygen family. *J. Bacteriol.* 181, 1171–1180 (1999).

35. Heider, J. et al. Simultaneous characterization of a fourth type of 2-keto acid oxidizing enzyme from a hyperthermophilic archaeon: 2-ketogluconate ferredoxin oxidoreductase from *Thermococcus litoralis*. *J. Bacteriol.* 178, 5890–5896 (1996).

36. Adams, M. W. & Kletzin, A. Oxidoreductase-type enzymes and redox proteins involved in fermentative metabolisms of hyperthermophilic archaea. *Adv. Prot. Chem.* 48, 101 (1995).

37. Mulik, J. A., Galperin, M. Y., Makarova, K. S., Wolf, Y. I. & Koonin, E. V. Evolutionary primacy of sodium bioenergetics. *Biol. Direct* 3, 1–19 (2008).

38. Heider, J., Ma, K. & Adams, M. W. W. Purification, characterization, and metabolic function of tungsten-containing aldehyde ferredoxin oxidoreductase from the hyperthermophilic and proteolytic archaeon *Thermotoga maritima* strain ES-1. *Bacteriol.* 177, 4757–4766 (1995).

39. Schütz, G. et al. The modular respiratory complexes involved in hydrogen and sulfur metabolism by heterotrophic hyperthermophilic archaebacteria and their evolutionary implications. *FEBS Lett.* 37, 182–203 (2013).

40. Kuhn, M., Trefunovic, D., Huber, H. & Müller, V. The Rnf complex is a Na⁺/coupling respiratory enzyme in a fermenting bacterium, *Thermotoga maritima*. *Commun. Biol.* 3, 1–10 (2020).

41. Supra, R., Verhagen, M. J. F. J. M. & Adams, M. W. W. Purification and characterization of a membrane-bound hydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J. Bacteriol.* 182, 3423–3428 (2000).

42. Supra, R., Bagramyan, K. & Adams, M. W. W. A single energy-conserving system: Proton reduction coupled to proton translocation. *Proc. Natl Acad. Sci. U.S.A.* 100, 7545–7550 (2003).

43. Schütz, G. J. et al. The role of geochemistry and energetics in the evolution of modern respiratory complexes from a proton-reducing ancestor. Biochim. Biophy. Acta Bioenerg. 1857, 958–970 (2016).

44. Luzczak, A., Aono, S. & Adams, M. W. The extremely thermophilic rubEBacterium, *Thermotoga maritima*, contains a novel iron-hydrogenase whose cellular activity is dependent upon tungsten. *J. Biol. Chem.* 266, 13834–13841 (1991).

45. Selig, M., Xavier, K. B., Santos, H. & Schönheit, P. Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium *Thermotoga*. *Arch. Microbiol.* 167, 217–232 (1997).

46. Zhang, Y. & Gladyshev, V. N. Molybdoproteins and evolution of molybdenum utilization. *J. Mol. Biol.* 379, 881–899 (2008).

47. Anbar, A. D. et al. If a whiff of oxygen before the Great Oxidation Event? *Science* 364, 1781–1784 (2019).

48. Neubert, N., Nägler, T. F. & Böttcher, M. E. Sulphur cycling in the oxygenic phototrophic bacterium *Cyanobium vulcani* and its evolutionary implications. *FEMS Microbiol. Lett.* 6956 (1996).

49. Helz, G. R. et al. Mechanism of molybdenum removal from the sea and its concentration in black shales: EXAFS evidence. *Geochim. Cosmochim. Acta* 60, 3631–3642 (1996).

50. Hanada, S., Hiraishi, A., Shimada, K. & Matsuura, K. Molecular phylogeny and characterization of microorganisms from the deep hot springs in the United States Great Basin. *Extremophiles* 13, 447–459 (2009).

51. Knapik, K., Becerra, M. & González-Siso, M.-I. Microbial diversity analysis and screening for novel xylanase enzymes from the sediment of the Lobios Hot Spring in Spain. *Sci. Rep.* 9, 11195 (2019).

52. Roy, R., Dhawan, I. K., Johnson, M. K., Rees, D. C. & Adams, M. W. Aldehyde Ferredoxin Oxidoreductase. 266 (American Cancer Society, 2011).

53. Sevenco, A.-M. et al. The tungsten metallome of *Pyrococcus furiosus*. *Metallomics* 1, 395–402 (2009).

54. Papamichail, K., Becerra, M. & González-Siso, M.-I. Microbial diversity analysis and screening for novel xylanase enzymes from the sediment of the Lobios Hot Spring in Spain. *Sci. Rep.* 9, 11195 (2019).

55. Sakuraba, H. & Ohshima, T. Novel energy metabolism in anaerobic hyperthermophilic archaea: a modified Embden-Meyerhof pathway. *Biochim. Biophys. Acta* 93, 441–448 (2002).
Acknowledgements
We thank Jonathan Covington (UNLV) for help assembling a repository of tungsten-enzymes, Jim Noblet and Andreas Beyersdorf (CSUSB) for assistance with ICP-OES, Dave and Sandy Jamieson for access to GBS, high school teachers Kathleen Diver, Heather Garcia, Juan Salgado, and Chuong Vu for assistance with fieldwork, and Prof. Aharon Oren for assistance on nomenclature. We acknowledge Tikiterre Trust for their enthusiasm for our research and assistance in access and sampling of the Hell's Gate geothermal features. We extend our gratitude to Mackenzie Lynes and Roland Hatzenpichler for permitting use of metagenome 3300028735 and providing geochemistry information on hot spring LCB-006, as well as to Donald Bryant, Vera Thied, Mircea Podor, Susannah Tringe, Robert Kelly, Brian Yu, and William Insko for sequence data use permissions. We also thank Everett Shock for discussions on W geochemistry in hot springs. Part of this work was carried out at Lawrence Livermore National Laboratory (LLNL) under Contract DE-AC52-07NA2734. Funding was provided by the U.S. National Science Foundation (DEB 1557042 and EAR-1820658), NASA (80NSSC17K0548 and 80NSSC19M0150), and the National Natural Science Foundation of China (No. 91951205).

Author contributions
J.A.D., B.P.H., A.E.D., S.B. and M.P. conceived of the study. J.D., M.M., C.G., L.G., C.S., C.V., M.Z., N.B., and F.R. conducted cultivation experiments, monitoring, and quantitative PCR. S.B. and J.A.D. analyzed qPCR and 16S data. D.M. performed CARD-FISH. X.M., P.K.W., and J.P.-R. analyzed culture samples with nanosimS and S.B. and A.E.D. joined for the nanosimS data analysis/interpretation. J.-Y.J., D.R.C., L.M.K., E.S.I., E.S.B., A.D.C., B.S.C., Z.-S.H., W.-L., A.-L.R., and M.B.S. were involved in metagenomic sampling, sequencing, assembly, annotation and binning. M.P. performed phylogenomic analyses and ACS reconstructions. S.B. carried out the protein structural modeling. S.B., M.P., and J.A.D. wrote the manuscript and all authors contributed to the final version of the paper.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-31452-8.

Correspondence and requests for materials should be addressed to Steffen Buesseker, Marike Palmer or Jeremy A. Dodsworth.

Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022