An ecophysiological explanation for manganese enrichment in rock varnish

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Desert varnish is a dark rock coating that forms in arid environments worldwide. It is highly and selectively enriched in manganese, the mechanism for which has been a long-standing geological mystery. We collected varnish samples from diverse sites across the western United States, examined them in petrographic thin section using microscale chemical imaging techniques, and investigated the associated microbial communities using 16S amplicon and shotgun metagenomic DNA sequencing. Our analyses described a material governed by sunlight, water, and manganese redox cycling that hosts an unusually aerobic microbial ecosystem characterized by a remarkable abundance of photosynthetic Cyanobacteria in the genus Chroococcidiopsis as the major autotrophic constituent. We then showed that diverse Cyanobacteria, including the relevant Chroococcidiopsis taxon, accumulate extraordinary amounts of intracellular manganese—over two orders of magnitude higher manganese content than other cells. The speciation of this manganese determined by advanced paramagnetic resonance techniques suggested that the Cyanobacteria use it as a catalytic antioxidant—a valuable adaptation for coping with the substantial oxidative stress present in this environment. Taken together, these results indicated that the manganese enrichment in varnish is related to its specific uptake and use by likely founding members of varnish microbial communities.

Varnish Is Governed by Sunlight, Water, and Manganese Redox Cycling. We collected varnish on a range of rock types from seven field areas across the western United States (SI Appendix, Fig. S1). Varnish occurs on diverse lithologies in different settings, yet there are some common developmental patterns. A close relationship between varnish microflora and their environment and developed a hypothesis linking specific manganese accumulation to environmental adaptations of major members of the varnish microbial community.

Significance

Rock varnish is a prominent feature of desert landscapes and the canvas for many prehistoric petroglyphs. How it forms—and, in particular, the basis for its extremely high manganese content—has been an enduring mystery. The work presented here establishes a biological mechanism for this manganese enrichment, underpinned by an apparent antioxidant strategy that enables microbes to survive in the harsh environments where varnish forms. The understanding that varnish is the residue of life using manganese to thrive in the desert illustrates that, even in extremely stark environments, the imprint of life is omnipresent on the landscape.

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To understand better the physical processes controlling varnish development, we examined depositional textures in petrographic thin sections using backscatter scanning electron microscopy (SEM). Varnish cross-sections revealed micrometer-scale, subhorizontal laminations that reflect its accretionary mode of growth (Fig. 1 B and SI Appendix, Fig. S2) (3). In all varnish samples examined, we observed laminae with crinkly to columnar or domal textures that mark an emergent topography similar to that of stromatolites—macroscopic sedimentary structures commonly understood as mineralized residue of ancient microbial mats (33). In certain stromatolites, these textures have been interpreted in terms of light-dependent growth, models of which come from studies of coral growth (34). On a topographically irregular surface, relative highs receive more light while relative lows get shaded; thus, the highs grow higher forming columnar features. In varnish, these microtextures supported the relationship with sunlight that has been documented with macroscale field observations and strengthened the evidence for a role for light in varnish genesis.

Textural data also supported the previously documented relationship with water by suggesting that varnish formation involves manganese redox cycling through a soluble phase. Desert dust samples that we collected at varnish sites contained manganese as both trace Mn^{2+} in igneous minerals and manganese oxide particles (SI Appendix, Fig. S2E). We observed detrital particles embedded in varnish, but high-resolution chemical imaging by energy dispersive spectroscopy (EDS) and nanoscale secondary ion mass spectrometry (NanoSIMS) showed that these particles are largely silicate minerals, while the manganese-rich oxide material that characterizes varnish is in the laminated cement itself (SI Appendix, Figs. S3 and S4). Since manganese is water soluble in its divalent form but not as higher-valent oxides, redox cycling is required to mobilize and reprecipitate the manganese oxide in dust to form the accreting oxide cements.

The manganese oxide mineral phase in varnish has been described as poorly crystalline birnessite (2)—a phase composed of manganese octahedra organized in layers (35); it is formally Mn^{3+}O_2 but can incorporate a substantial fraction of Mn^{4+} instead of Mn^{3+} when accompanied by charge balance with heteroatoms (Na^{+}, K^{+}, Ba^{2+}, etc.) between the layers (36). Using synchrotron X-ray absorption near-edge structure (XANES) spectroscopy and multiple-energy “redox” mapping at the manganese K-edge, we found that varnish birnessite is not homogenous in its redox properties. Although predominantly Mn^{4+}, it contains common discrete microscale domains with variable and considerable mixtures of Mn^{3+} (Fig. 1 C–E and SI Appendix, Fig. S2). This heterogeneity is consistent with the view that manganese redox cycling occurs within varnish, and the distribution of these domains indicates that such cycling is not restricted to surficial processes contributing to the ongoing accretion of varnish, but rather also characterizes the ecosystem that exists within well-developed varnish.

Numerous manganese redox cycling processes occur in Earth surface environments. Manganese oxidation with atmospheric O_2...
is thermodynamically favorable and can be catalyzed by metal oxide surfaces or bacterial and fungal enzymes (37–40). Manganese reduction can also be catalyzed by microbial processes, notably anaerobic respiration (41), and with an appropriate electron donor present (e.g., organic carbon) photochemical reduction of manganese oxides occurs readily even in aerobic settings (42–44). While any of these processes might contribute to varnish development and the manganese redox heterogeneity we observed, the relationship between varnish and sunlight raised the hypothesis that photochemistry—-and perhaps photobiology—might play particularly important roles.

To visualize organic matter in varnish, we used NanoSIMS imaging of sulfur. $^{32}$S appeared throughout varnish, notably concentrated in micrometer-scale particles that sit along varnish laminations and are distinct from detrital grains observed in the $^{28}$Si and $^{57}$Fe mass channels (SI Appendix, Fig. S4A). To confirm that this sulfur reflects organics rather than just sulfur-bearing mineral phases, we used XANES spectroscopy to assess electronic structure at the sulfur K-edge. The sulfur in varnish displayed complex speciation; sulfur is present in organic forms observed in biological material (variable mixtures of thiols with disulfides, sulfides, and sulfonates) as well as sulfate salts (SI Appendix, Fig. S4B). The fact that varnish is rich in organics and exposed to light—conditions that promote manganese photoreduction—and yet the manganese is maintained largely as $\text{Mn}^{4+}$ oxides supports the view that dynamic redox cycling occurs within varnish.

The Varnish Microbial Community Is Characterized by Cyanobacteria.

To investigate the microbial diversity in varnish, we extracted DNA from varnish samples along with samples of surrounding surface soils for comparison. 16S rRNA gene amplicon sequencing revealed a varnish-specific microbial community that is distinct from surrounding soils, but common among varnishes from different rock types and locations (SI Appendix, Figs. S5–S7). The taxa we recovered are consistent with previous studies of varnish microbiology (45-48), with the bacterial families Xenooccaceae, Rubrobacteraceae, Acetobacteraceae, Sporichthyaceae, and Gemmatimonadaceae distinguishing the varnish community.

The most striking observation from our community analyses was the high abundance of Cyanobacteria associated with varnish, specifically members of Chroococcidiopsidaceae—a genus of the family Xenooccaceae noted for its ability to live in extreme environments, with high tolerance for radiation and desiccation (49–52) (Fig. 2). 16S sequences assigned to the Xenooccaceae (either Chroococcidiopsis or unassigned below the family level) were recovered from 48 out of 49 varnish samples and accounted for 25.9% of all sequence reads and 98.7% of cyanobacterial reads recovered from varnish. In contrast, the Xenooccaceae represented only 0.06% of all reads and 1.4% of cyanobacterial reads from soil. The most abundant cyanobacteria Chroococcidiopsis sequence variant accounted for 8% of all reads from varnish and was completely absent from soil samples. Top BLAST hits for this sequence in the National Center for Biotechnology Information (NCBI) nr database included a remarkable representation of hits from previous varnish studies, and 99.6% identity with isolate Ryu 1–3 from the University of the Ryukyus in Okinawa, Japan (53), which we obtained for further study. Additionally, epifluorescence microscopy on varnish flakes revealed sarcinoid clusters of cells that are morphologically characteristic of baeocytes, Chroococcidiopsis cells (Fig. 2D).

Shotgun metagenomic sequencing of select samples further emphasized the importance of these Cyanobacteria in the varnish community. In total, 21.9% of raw metagenome reads were assigned to Cyanobacteria, corroborating their high abundances in the 16S amplicon data (SI Appendix, Fig. S6C). We recovered six high-quality cyanobacterial metagenome-assembled genomes (MAGs), all belonging to the members of the Chroococcidiopsidaceae (SI Appendix, Fig. S8). These Cyanobacteria appeared to be the main primary producers of the varnish community—of nine MAGs containing genes for the Calvin–Benson–Bassham cycle, six were Cyanobacteria, with three others representing considerably less abundant taxa (of Armatimonadota, Rhodobacteraceae, and Beijerinckiaaceae). No MAGs appeared to encode alternative carbon fixation pathways. Furthermore, we only recovered one additional MAG with phototrophic reaction center genes (an aerobic phototroph of the Acetobacteraceae), indicating that the Cyanobacteria are the only taxa in varnish capable of using light as an energy source for autotrophic growth. Sulfur K-edge spectra of Chroococcidiopsis cells were similar to the organic content we observed within varnish (SI Appendix, Fig. S4), supporting the interpretation that these taxa are the main primary producers of the ecosystem.

By both abundance and function, we concluded that Chroococcidiopsis are extremely important taxa in varnish and are likely founding autotrophic members of the community. When considered in this context, the physical evidence that sunlight and water play important roles in varnish development can be interpreted to suggest that these Cyanobacteria, which grow with light and water, might be involved in the formation of varnish itself.

Metagenomic data also revealed that the varnish microbial communities mark a highly aerobic ecosystem, far more so than typical sediments or soils. No obligate anaerobic metabolisms were represented by the gene content of our MAGs—consistent with the known physiologies of the major taxa identified in our 16S analysis. Indeed, nearly all MAGs contained high-potential bioenergetic systems utilized in aerobic respiration as well as reactive oxygen species detoxification systems. Of the 38 MAGs...
we recovered from varnish samples, we observed heme-copper
O$_2$ reductase and/or bd O$_2$ reductase complexes in 37 of them, a
superoxide dismutase in 34 of them, and a catalase in 24 of them.
These aerobic adaptations are unsurprising considering the
proximity of this community to atmospheric O$_2$ and exposure to solar
irradiation; they are biochemical attestations to the high degrees
of oxidative stress encountered in this harsh environment.

**Cyanobacteria Accumulate Manganese Likely as a Nonenzymatic Antioxidant System.** The significance of cyanobacterial taxa as abundant keystone members of the varnish ecosystem suggested to us a previously unexplored connection between the microbial community and the manganese content of varnish. The model freshwater cyanobacterium *Synechocystis* sp. PCC 6803 has been shown to accumulate a massive pool of intracellular Mn$^{2+}$, up to 10$^7$ atoms per cell, which when averaged over cell volume is the equivalent concentration of 100 mM manganese—for four orders of magnitude higher than their growth medium (54). We hypothesized that if this hyperaccumulation of manganese occurs broadly in the Cyanobacteria—particularly those taxa that are dominant members of the varnish community—then this physiological peculiarity might underpin varnish development.

We examined two strains of *Chroococcidiopsis* (PCC 7433 and Ryu 1–3, the closest cultured relative to the varnish sequences), *Synechocystis* sp. PCC 6803, and *Gloeobacter violaceus* PCC 7421, a very deep branching member of the photosynthetic Cyanobacteria (*SI Appendix, Fig. S9*), along with noncyanobacterial model organisms *Escherichia coli* K12 and *Shewanella oneidensis* MR-1 for comparison. Using inductively coupled plasma mass spectrometry (ICP-MS) to measure total cellular manganese, we observed dramatic manganese accumulation in all cyanobacterial strains; the highest values were seen in Ryu 1–3, with over two orders of magnitude greater manganese content than that seen in *E. coli* or *S. oneidensis* (Fig. 3A).

To probe the speciation of this copious intracellular manganese, we combined electron paramagnetic resonance (EPR), electron nuclear double resonance (ENDOR), and electron spin echo envelope modulation (ESEEM) spectroscopies. The EPR spectra of all Cyanobacteria examined showed that the majority of the Mn$^{2+}$ pool exists in soluble high-symmetry complexes with low–molecular-weight ligands (denoted H–Mn$^{2+}$) and not in low-symmetry complexes with strongly chelating ligands or bound to proteins (denoted L–Mn$^{2+}$) (Fig. 3B and *SI Appendix, Fig. S10 A and B*). $^3$P, $^1$H ENDOR, and $^{11}$N ESEEM measurements on labeled cells further revealed that this manganese binds undetectably low amounts of phosphate and nitrogenous ligands, and is instead predominantly bound to carboxylato ligands (represented by Mn–HCO$_3$ standard), which was confirmed by ENDOR measurements of $^{13}$C-labeled cells (*SI Appendix, Fig. S10D*).

**Discussion**

Varnish develops in environments that are extremely harsh, where protective strategies against irradiation and oxidative stress are essential for life to survive. The presence of the varnish microbial community has been noted in such terms; for example, varnish provides a habitat for microbial life shielded by oxide minerals that absorb UV radiation (62). However, up until now, how and why the varnish—with its high and specific enrichment in manganese—got there in the first place was a mystery. Based on the data presented here, we propose that varnish itself is a hallmark of life prevailing in these extreme environments.

We showed that Cyanobacteria of the genus *Chroococcidiopsis* are intimately and abundantly associated with varnish. Moreover, being the main primary producers of the ecosystem, these organisms must establish prior to heterotrophic taxa that depend upon them for organic substrates, implicating them as likely founding members of the microbial community. Evidence tying sunlight and water to varnish development provided further, albeit circumstantial, evidence that photosynthetic Cyanobacteria play a fundamental role. We then demonstrated that diverse Cyanobacteria, including *Chroococcidiopsis*, accumulate substantial quantities of H–Mn$^{2+}$. This phenomenon is well established as an antioxidant strategy that enables tremendous oxidative stress resistance (58, 59, 63); indeed, there is no other known physiological purpose for such elevated manganese concentrations. The cyanobacterial H–Mn$^{2+}$ pool that we observed is comparable in magnitude to the most radiation-resistant organisms known (60).
Many previous studies have focused on a mechanism of manganese oxidation as the key to varnish formation. However, in such aerobic environments, manganese oxidation may proceed through numerous pathways with both biological (SI Appendix, Text and Fig. S8) and abiotic (including photochemical and autocatalytic) mechanisms. Rather than oxidation, we argue that it is the selective enrichment of manganese that represents a process of singular importance to the development of varnish. The manganese hyperaccumulation that we observed in *Chroococcidiopsis* provides a simple and effective, ecologically relevant, physiological explanation for this manganese enrichment.

Thus, we propose a hypothesis for varnish formation (Fig. 4). These Cyanobacteria grow on sunlit rock surfaces with intermittent access to water, sequestering high concentrations of manganese in their cells and exploiting the unique redox chemistry of manganese complexes as a catalytic antioxidant system that enables their survival in such a harsh environment. When they die, the residue from their biomass provides an enriched manganese source that is ultimately oxidized to form the oxide mineral components that comprise varnish. Since varnish forms over timescales of millennia, a well-developed varnish sample represents the time integrated manganese accumulation of many, many generations of cells, which are sparsely distributed at any given time. This was demonstrated in our analyses of varnish samples in petrographic thin section—the Mn$^{2+}$ content of any living cells is negligible next to thousands of years of accumulated Mn$^{2+}$/Mn$^{4+}$ mineral. In addition to solving the mystery of manganese enrichment, cyanobacterial exudates and necromass also supply fresh organic carbon to the varnish ecosystem. This provides a growth substrate for the heterotrophic microbes that inhabit varnish, as well as an effective electron donor for photochemical manganese reduction. Taken together, our results place the activity of extremophilic Cyanobacteria as a key driver of both the physical and biological development of varnish ecosystems.

Fig. 4. Natural history of varnish. (A) Manganese is delivered largely as oxide particles in windborne dust, reduced by either photochemical or biological processes, and taken up by *Chroococcidiopsis* cells for use as a catalytic antioxidant. (B) *Chroococcidiopsis* grows with light and water, fixing carbon and trapping accumulated manganese. (C) Dust material not adhered to the rock surface is removed by wind or precipitation. (D) When *Chroococcidiopsis* cells die, the manganese-rich residue left behind by their biomass is oxidized to generate the manganese oxides that comprise varnish. This oxidation could be biologically catalyzed and/or abiotic. (E) Products from cyanobacterial photosynthesis serve as substrates for heterotrophic community members. (F and G) Manganese reduct cycling continues in developed varnish, with abundant O$_2$ as an electron acceptor (F) and organic matter as an electron donor (G).

**Methods**

**Study Locations and Sampling.** Samples were taken from seven field locations across the southwestern United States, ranging from arid to semiarid climates, corresponding to rock varnish Type I and Type II designated by Macholdt et al. (66). Sampling included mafic, felsic, and sedimentary rocks. Varnished rocks were collected with ethanol-sterilized gloves into Whirl-Pak sample bags, and surface soil samples were collected using sterile spatulas into Falcon tubes. Ultrathin sections cut orthogonal to the varnished surface were prepared by David Mann at High Mesa Petrographics (Los Alamos, NM). The thin sections revealed varnishes ranging in thickness from ~5 to ~100 μm, likely representing hundreds to thousands of years of development (27). For DNA sampling, varnished rocks were returned to the laboratory, gently rinsed with sterile nanopure water using a 50-μL syringe and 16-gauge needle, and allowed to dry. For DNA extraction, varnish was removed from rock surfaces by scraping with flame-sterilized steel brushes, spatulas, and dental picks, and collected in a weigh boat. Each varnish sample was obtained from a separate rock. Dust analysis was conducted on a fine-grained (clay-silt sized) fraction of surface soil from the Barstow field location.

**SEM/EDS/NanoSIMS.** SEM and EDS analyses were conducted at the Caltech Geological and Planetary Sciences Division Analytical Facility on a ZEISS 1550VP Field Emission SEM, with a Robinson-type backscatter electron detector and an Oxford X-Max SDD X-ray EDS system. Varnish was imaged at 10–20 kV with working distances of 7–10 mm and magnifications of 500–4,500×. NanoSIMS analyses were conducted in the Caltech Microanalysis Center, on a Cameca NanoSIMS 500 using 50-nm resolution, primary ion current of 1 pA, and dwell time of 3.5 ms/pixel. The masses of 12C, 12C14N, 32Ar, 31P, 30Si, 55Mn16O, and 57Fe16O were collected. Petrographic thin sections were coated with 20-nm graphite using a Turbo carbon evaporator for SEM/EDS, and with 40-nm gold using a Cressington HR metal spattering coater for NanoSIMS. EDS and NanoSIMS images were examined using ImageJ.

**Synchrotron X-ray Spectroscopy.** Synchrotron analyses were conducted at the Stanford Synchrotron Radiation Lightsource, on X-ray microprobes at beamlines 2-3 for the manganese K-edge and 14-3 for the sulfur K-edge. The 2-3 beam was energy calibrated using the pre-edge feature of KMnO$_4$ at 6,543.34 eV, and 14-3 was calibrated using the pre-edge feature of Na$_2$SO$_4$ at 2472.02 eV. Multiple energy maps for producing images of manganese redox state were collected at 6,553, 6,557, 6,559, 6,562, and 6,570 eV, with 3-μm resolution. For both spectra and maps, least-squares fitting was done using a sputter-sputter standard spectrum (65) representative of igneous Mn$^{3+}$, manganic oxide (65), and feitknechtite (66) standard spectra as two different Mn$^{3+}$/Mn$^{4+}$ bearing phases, and an internal endmember spectrum for Mn$^{4+}$O$_2$ Spectra and maps were viewed and fit using the SIXPACK (67) and SMAK (68) software packages, respectively (https://www.sams-xrays.com/). Colormaps were converted to viridis using fixthejet (https://fixthejet.ecrlife.org/).

**DNA Extraction and Sequencing.** Genomic DNA was extracted from 10 to 50 mg varnish scrapings or soil using the FastDNA SPIN kit for soil (MP Biomedicals). DNA yields were quantified using a Qubit 2.0 fluorometer with the HS dsDNA assay kit (Thermo Fisher Scientific).

To generate 16S rRNA gene libraries, the V3–V4 hypervariable region of the 16S gene was amplified using degenerate primers (341–806 pair) from ~12.5 ng of genomic DNA with KAPA HiFi HotStartReadyMix (denaturation at 95°C for 3 min, 20 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 30 s before holding at 4°C). A second round of PCR added Nextera XT v2 indexes (Illumina) (denaturation at 95°C for 3 min, 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension of 72°C for 5 min before holding at 4°C). The amplicons were cleaned up using AMPure XP beads (Beckman Coulter). A no-template control was processed and did not show a band in the amplicon region. The amplicons were pooled and sequenced on the Illumina MiSeq platform using paired end 301-bp reads using the Miseq reagent kit v3 (600 cycles) (Illumina).

Two representative varnish DNA samples (sample 24, sandstone from Babbitt Ranch, AZ, and sample 41, basalt from Mesa Prieta, NM) were selected for metagenomic sequencing. These samples were selected based on 16S amplicon data as likely candidates to recover high-quality genomes for the major taxa in the varnish community. No aspects of the communities in these two samples were outliers, and together these represent samples from two different locations and very different rock types.

Illumina shotgun libraries were prepared using the Next Ultra DNA II library preparation kit (New England Biolabs). DNA was fragmented using a Covaris E220; the ends were made blunt and adapters and indexes added.

**DNA Sequencing and Assembly.** This metagenomic data was sequenced on the Illumina HiSeq platform (500 cycles). Sequencing was done at NICHD, University of California, Irvine. The raw reads were deposited in the NCBI Sequence Read Archive (study PRJNA157749).

**QC Processing and Analysis.** The V3–V4 reads were processed using the QIME package (version 1.9) (69). In this study, we discarded any reads that mapped to a contaminant control was processed and did not show a band in the amplicon region. The amplicons were pooled and sequenced on the Illumina MiSeq platform using paired end 301-bp reads using the Miseq reagent kit v3 (600 cycles) (Illumina).

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onto the fragments to generate Illumina libraries, which were eluted in DNA elution buffer (Zymo). Libraries were quantified using the KAPA Illumina/UniQPrep quantitation kit, normalized on qPCR, and sequenced on the Illumina NextSeq platform generating paired end 151-bp reads using the NextSeq 500/550 high output kit v2.5 (300 cycles) (Illumina).

16S Data Processing. 16S amplicon sequence reads from 61 varnish samples and 19 soil samples were processed using QIIME2 (69) to generate feature tables containing the frequencies of each unique sequence variant per sample. Quality filtering, denoising, merging of paired end reads, and chimera removal were done using DADA2 (70). The QIIME2 q2-feature-classifier plugin was used to align the sequences against the Greengenes 13.8 database (71) and assign taxonomy. One sample that returned <2,000 total reads was omitted from downstream analyses. NMDS and ANOSIM analyses were done by calculating a Bray dissimilarity matrix using the vegan ecology package in R (72). LEfSe analysis was done using the Microbiome Analyst tool (73) with default settings. For the phylogenetic tree, this paper, sequences were aligned with the SINA aligner (74) and converted from fasta format to phylip format with SEAVIEW (75). The phylogenetic tree was constructed using PhyML (76) implemented on the website www.atgc-montpellier.fr/phyml/ with default settings, and the resulting tree was visualized using FigTree.

Metagenomic Data Processing. Taxonomic assignments of raw metagenomic reads were done using the MG-RAST analysis platform (77). Metagenome sequence read quality control, de novo assembly, and binning of MAGs was performed largely on the KBase platform (78). Combinations of read preprocessing, assembly, and binning methods were tested and evaluated based on the quality and quantity of final MAGs. Read preprocessing included no processing (raw reads), Bloom Filter Read Error Correction (79) v. 1.811 (drop_unique_kmer_reads = 1, kmer_size = 33), Trimomatic (80) v. 0.36, LEADING_3 TRABLES SLEDGINGWINDOW A 15 MINLEN 125, and BBMap (81) v 37.93 (bbduk.sh -Xmx1g, k = 15, mink = 10, ktrim = r, tbo). Processed reads were assembled with MEGAHIT (82) v2.4.2 (metalarge), MetaSPAdes (83) v1.2.4 (K-mer sizes 33, 55, 77, 99, and 127), or IDBA-UD (84) v1.0.4. Assemblies were performed with minimum contig size of 2,000 bp. All MAGs from combinations of the above setting were evaluated for quality using the CheckM software v1.0.18 on KBase with default settings. Sufficiently high-quality bins were defined using the Parks et al. (87) cutoff of completeness > 50 and contamination < 50. Bin taxonomy was assigned using GDBTk v3.0.2 using gdbtk release 49 (88). The number of bins passing this cutoff and their phylogenetic affiliations were used to compare the different parameters described above. The greatest number of quality bins was achieved with Trimomatic, MetaSPAdes, and MetaBAT2 for sample 74, and Trimomatic + BBmap mink, MetaSPAdes, and MetaBAT2 for sample 24. Other combinations of parameters produced a subset of less complete versions of the final bins, not completely different MAGs. Genome annotation was conducted using the RASTtk algorithm (89) v.0.1.1 in KBase, as well as a local implementation of the KofamScan (90) software for the KEGG database. The categorical metabolic pathways encoded by these genomes were parsed using KEGG-decoder (91), and additional analyses of genes of interest, including implementation of the KOfamScan (90) software for the KEGG database. The completeness was achieved with Trimmomatic, MetaSPAdes, and MetaBAT2 for sample 41, MetaSPAdes for sample 2, and MetaSPAdes for sample 5. Other combinations of parameters produced a subset of less complete versions of the above setting were evaluated for quality using the CheckM software v1.0.18 on KBase with default settings. Sufficiently high-quality bins were defined using the Parks et al. (87) cutoff of completeness > 50 and contamination < 50. Bin taxonomy was assigned using GDBTk v3.0.2 using gdbtk release 49 (88). The number of bins passing this cutoff and their phylogenetic affiliations were used to compare the different parameters described above. The greatest number of quality bins was achieved with Trimomatic, MetaSPAdes, and MetaBAT2 for sample 74, and Trimomatic + BBmap mink, MetaSPAdes, and MetaBAT2 for sample 24. Other combinations of parameters produced a subset of less complete versions of the final bins, not completely different MAGs. Genome annotation was conducted using the RASTtk algorithm (89) v.0.1.1 in KBase, as well as a local implementation of the KofamScan (90) software for the KEGG database. The categorical metabolic pathways encoded by these genomes were parsed using KEGG-decoder (91), and additional analyses of genes of interest, including MCOs and pili, were done using local BLAST searches for known systems and refocused echoes (96) (MW frequency, 34.8 GHz; time, 0.1 μs; repetition time, 10 ms).

Pulsed ENDOR/ESEEM spectra were recorded using a laboratory-built 35-GHz pulsed EPR spectrometer (95). All spectra were recorded at 2 K using an immersion helium cryostat. 37P, 1H Davies ENDOR spectra were recorded using the pulse sequence: τ = τ1−π − τ2−τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ − τ -
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