

Supplementary Information:

Microbial aerotrophy enables continuous primary production in diverse cave ecosystems

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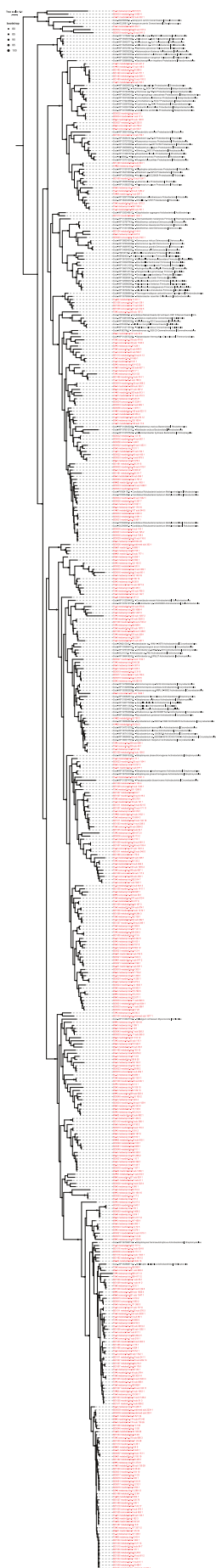
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These authors contributed equally to this work.

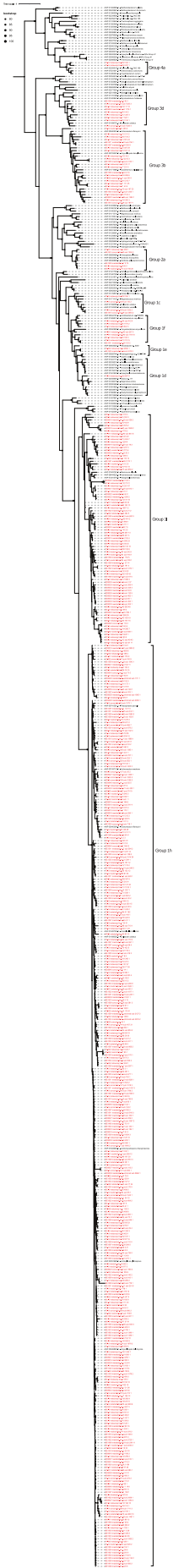
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Supplementary Figures

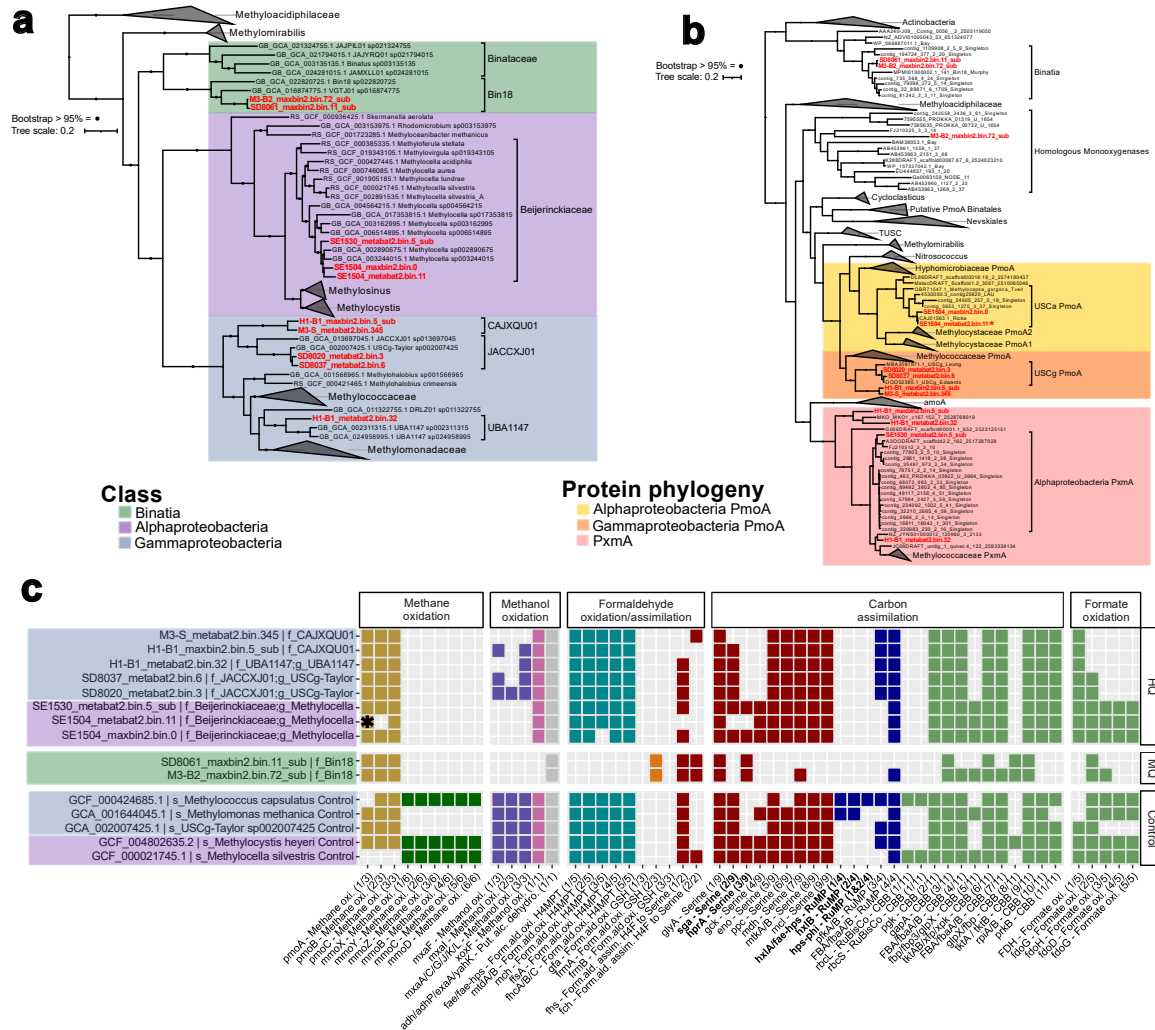
Supplementary Figure 1. Maximum-likelihood phylogenetic tree of carbon monoxide dehydrogenase large subunit (CoxL) amino acid sequences, rooted at midpoint. The tree shows sequences from this study's MAGs alongside (red) representative reference sequences (black). The tree was inferred using LG+F+I model post selection, used all sites, and was bootstrapped with 1000 replicates.



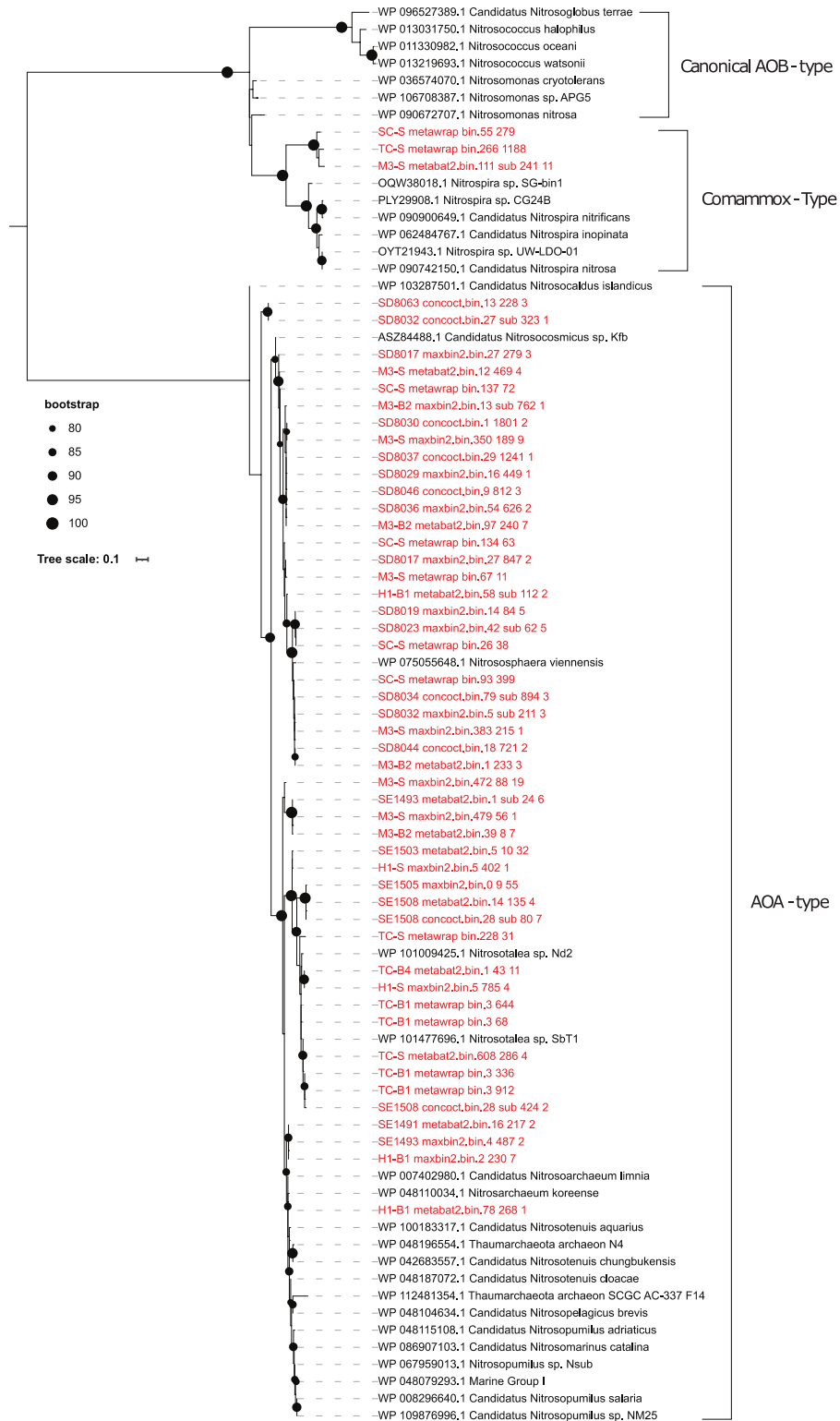
Supplementary Figure 2. Maximum-likelihood phylogenetic tree of [NiFe] hydrogenase amino acid sequences, rooted at midpoint. The tree shows sequences from this study's MAGs alongside (red) representative reference sequences (black). The tree was inferred using Q.pfam+I+ G4 model post selection, used all sites, and was bootstrapped with 1000 replicates.



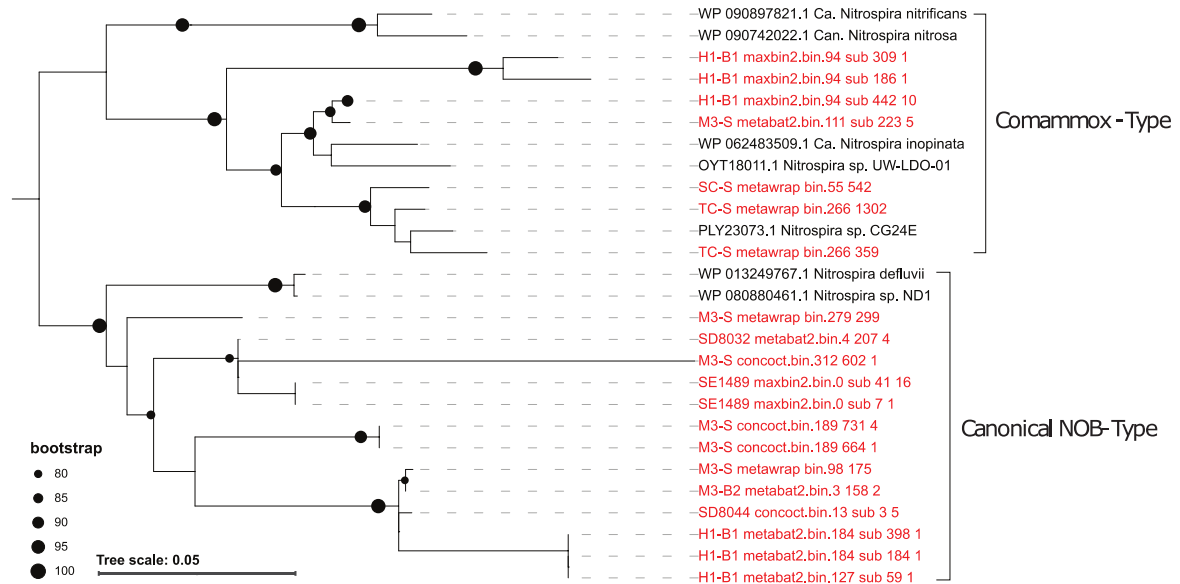
Supplementary Figure 3. Methanotroph phylogeny and metabolic potential. a, Phylogenetic tree of high-quality MAGs (completion >90% and contamination <5%) encoding metabolic potential for aerobic methane oxidation and known methanotrophs. **b,** protein phylogeny of Proteobacterial PmoA and PxA. Tree was generated with the ultrafast bootstrap approximation option using 1000 iterations and enabling the ModelFinder option (Best-fit model: LG+F+R6). **c,** Methanotroph MAG annotation to identify metabolic pathways and potential for methane carbon assimilation using DRAM and KEGG database.



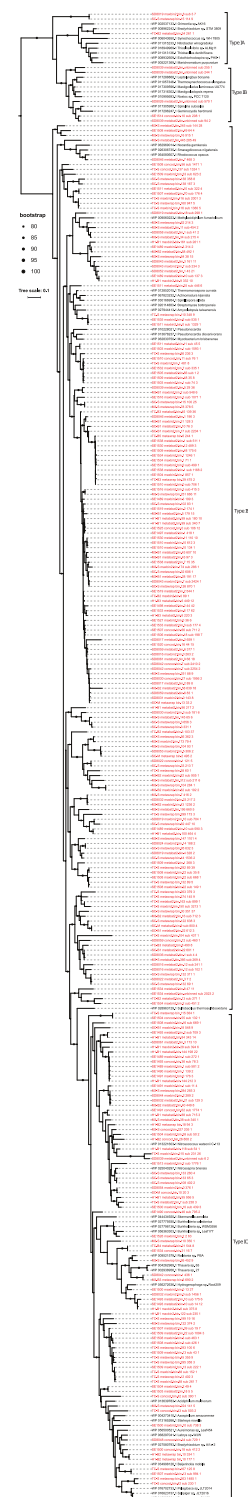
Supplementary Figure 4. Maximum-likelihood phylogenetic tree of ammonia monooxygenase subunit A (AmoA) amino acid sequences, rooted at midpoint. The tree shows sequences from this study's MAGs alongside (red) representative reference sequences (black) from both ammonia oxidising bacteria (AOB) and archaea (AOA). The tree was inferred using Q.yeast+F+G4 model post selection, used all sites, and was bootstrapped with 1000 replicates.



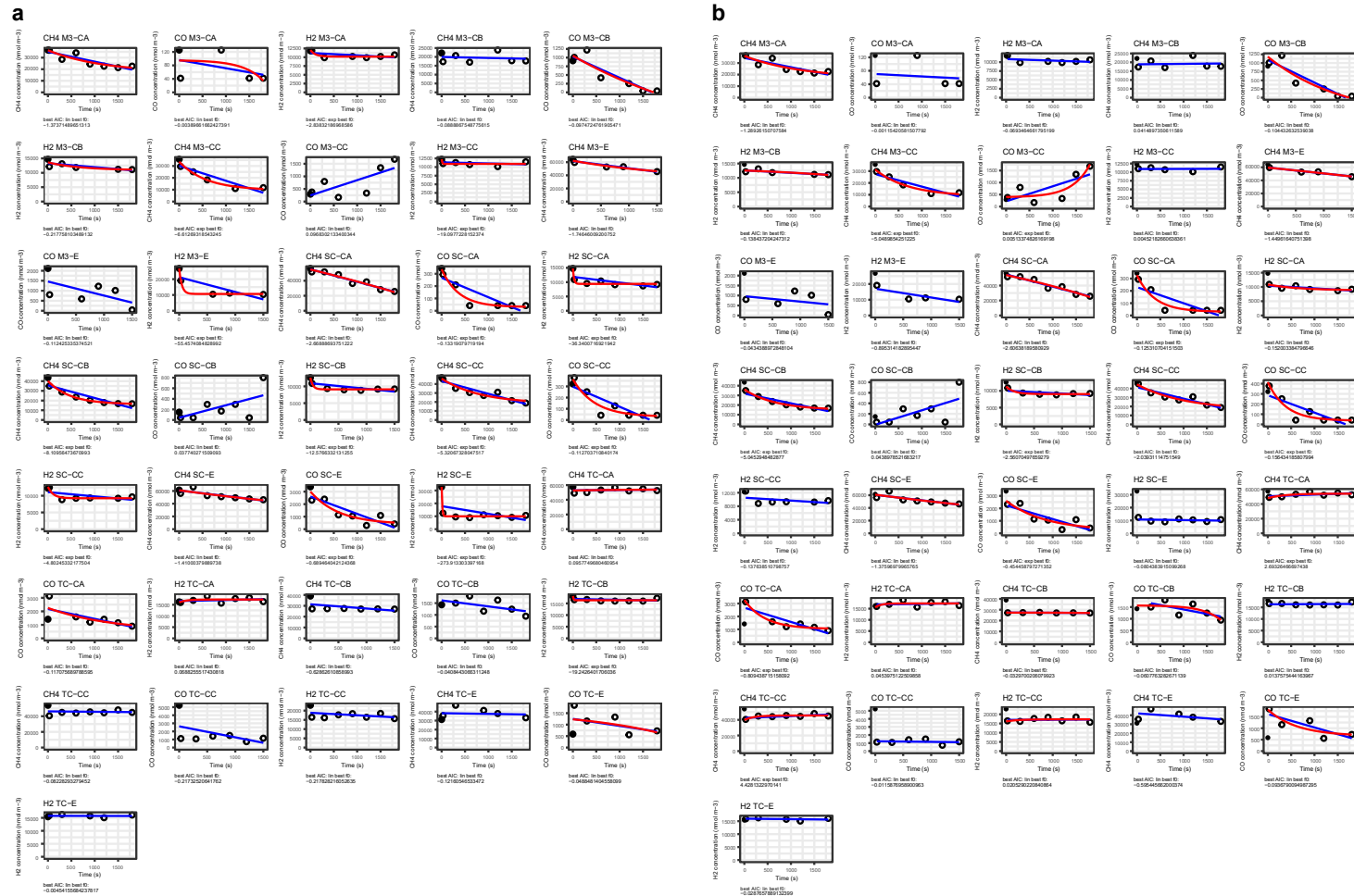
Supplementary Figure 5. Maximum-likelihood phylogenetic tree of nitrite oxidoreductase subunit A (NxrA) amino acid sequences, rooted at midpoint. The tree shows sequences from this study's MAGs alongside (red) representative reference sequences (black). The tree was inferred using the LG+I+G4 model post selection, used all sites, and was bootstrapped with 1000 replicates.



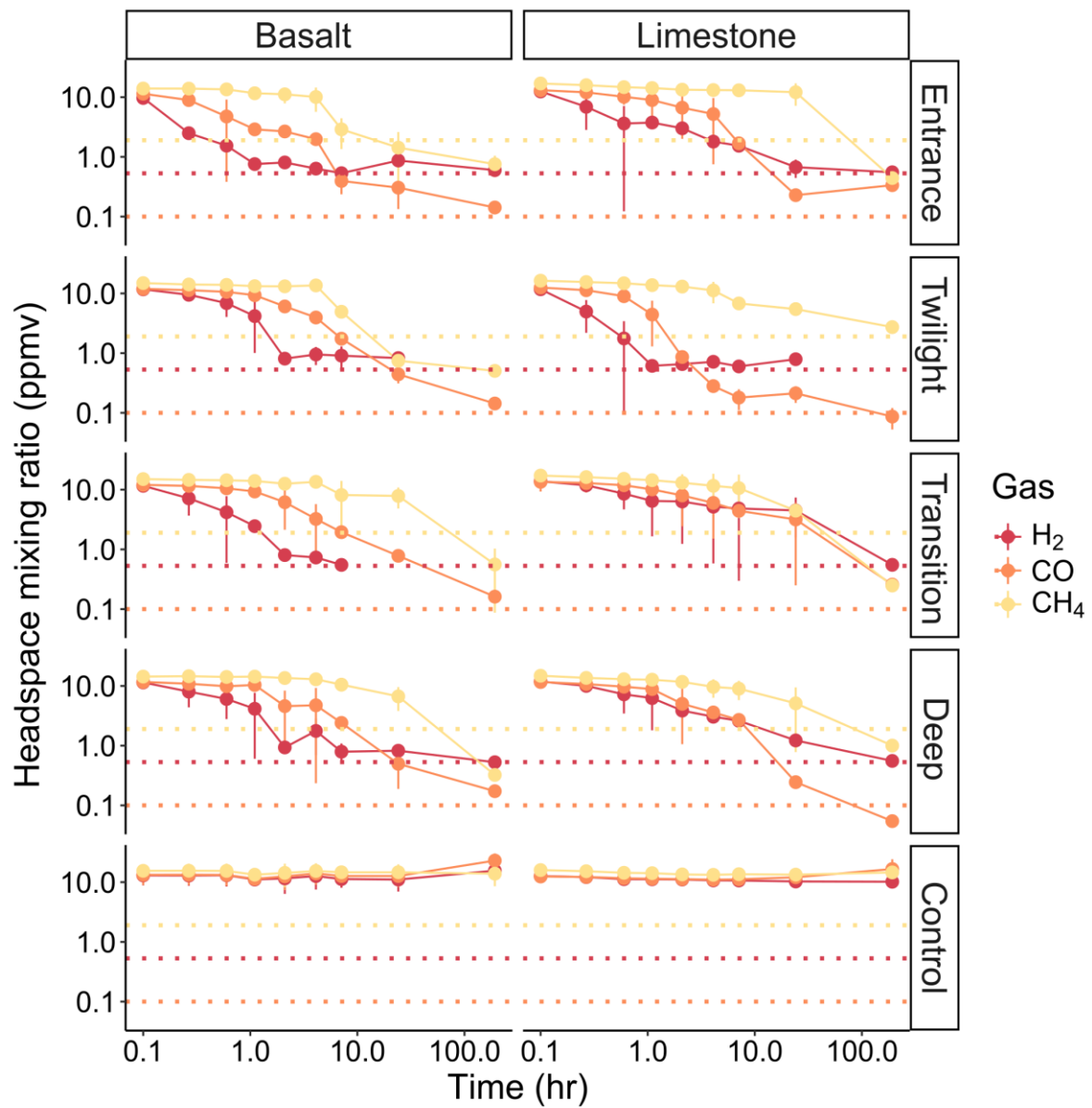
The figure displays a large, detailed phylogenetic tree of SARS-CoV-2 sequences. The tree is rooted on the left and branches out extensively. The sequences are color-coded by clade: Type A (red), Type B (green), Type E (blue), and Type G (purple). The tree is annotated with bootstrap values at various nodes, indicated by black dots of varying sizes. A legend on the left side of the tree provides a key for these bootstrap values: 80 (small dot), 85 (medium dot), 90 (large dot), 95 (very large dot), and 100 (largest dot). Below the legend, a scale bar indicates the genetic distance in substitutions per site, with a value of 0.001. The tree is divided into several major clades, labeled on the right side: Type A, Type B, Type E, and Type G. The Type A clade is the largest and most diverse, while the other clades are smaller and more distinct. The tree shows a clear pattern of divergence and convergence, with many sequences clustering closely together, indicating recent common ancestry. The overall structure of the tree suggests a rapid spread of the virus, with multiple lineages co-circulating and evolving independently.



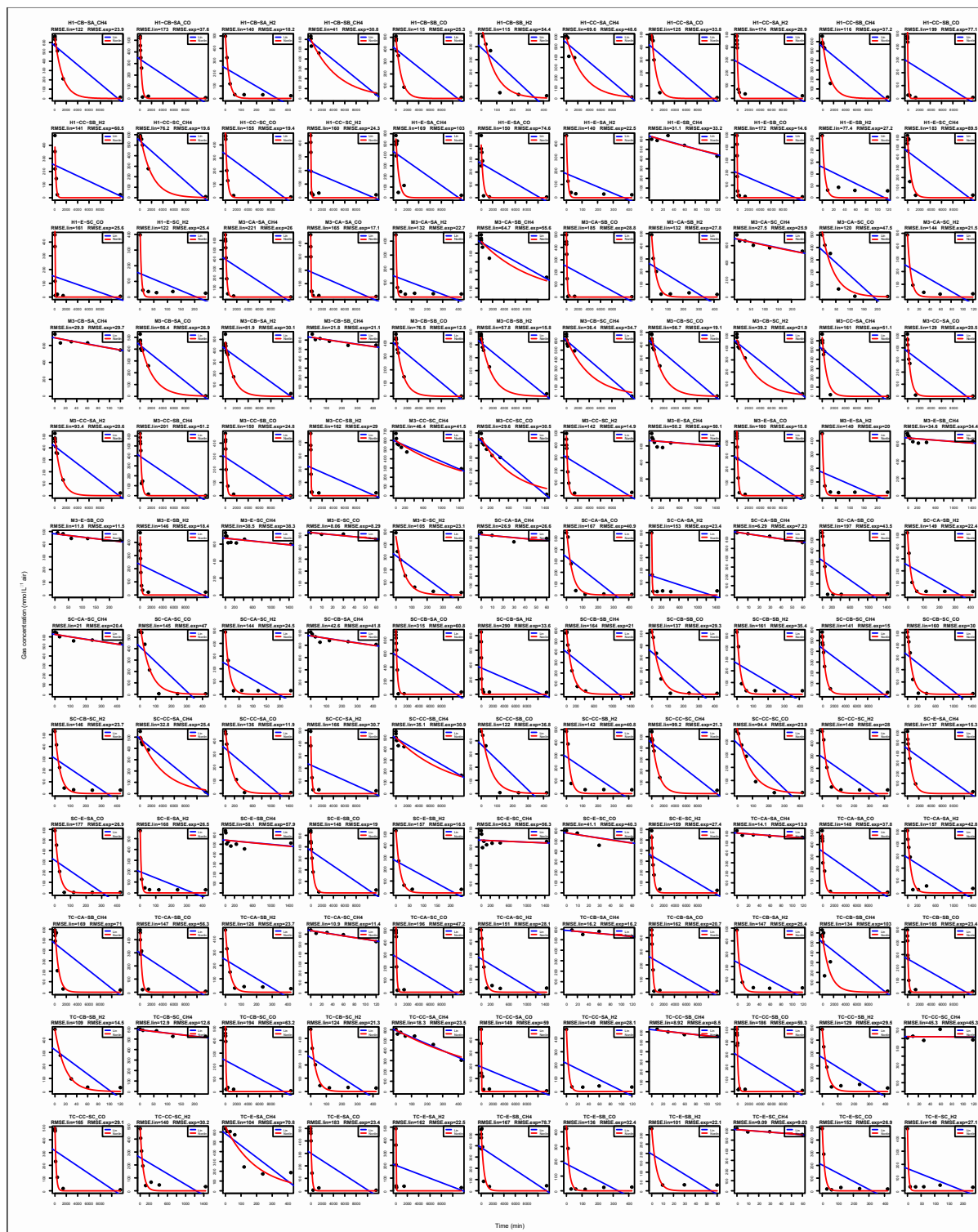
Supplementary Figure 7. Plots showing linear and non linear models of in situ flux AC measurements for H₂, CO and CH₄ across transient and equilibrium flux phases shown in panel **a** and **b** respectively. Best model fits, according to AIC for linear (lin) and non liner (exp) are given along with the flux value (nmol m² sec⁻¹).



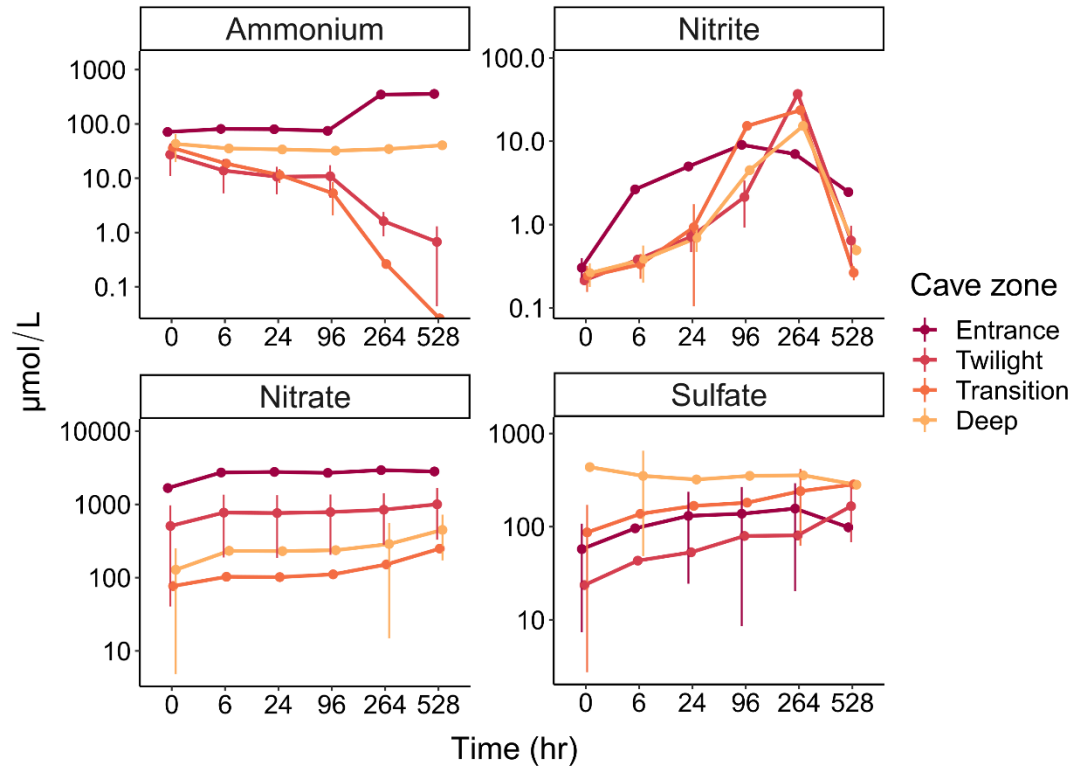
Supplementary Figure 8. The average measurements and standard deviation (n=44) of gas chromatography traces for bulk soil incubations and controls are shown, indicating uptake from ~10 ppmv to below average atmospheric levels for each gas (dashed line).



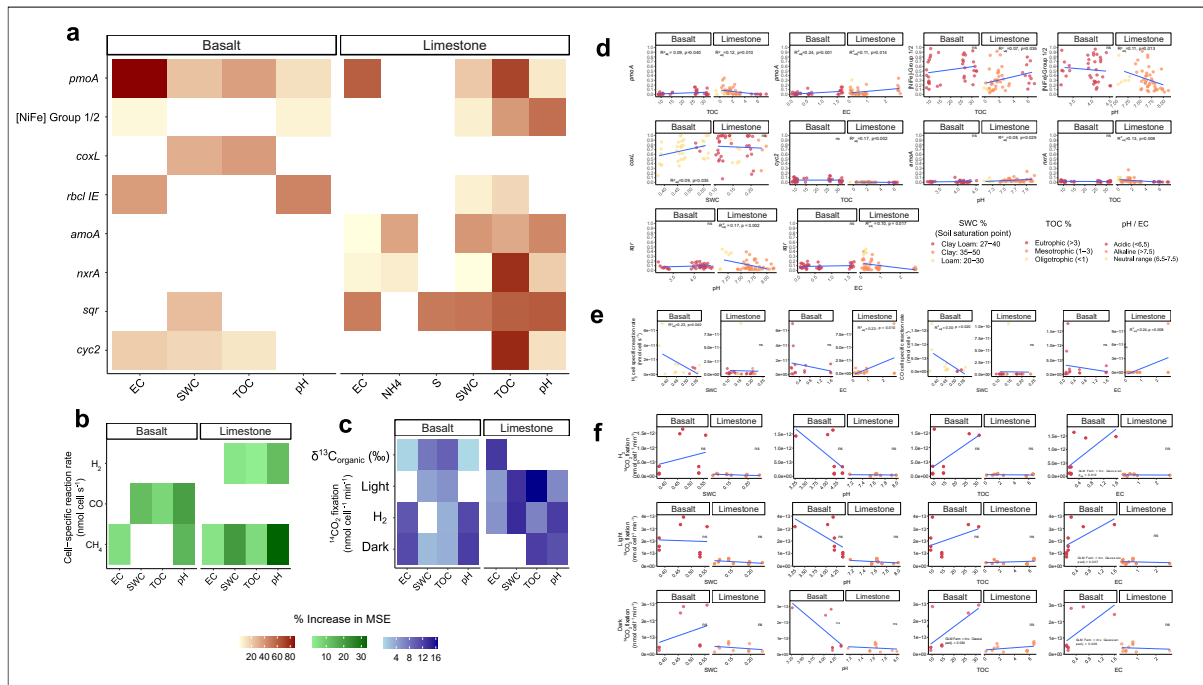
Supplementary Figure 9. Plots showing linear and nonlinear models fitted to trace gas oxidation for each sample. Root Mean Squared Error (RMSE) values are used to assess predictive accuracy. RMSE measures the average magnitude of prediction errors, with lower values indicating better fit.



Supplementary Figure 10. The average measurements and standard deviation (n=17) of oxic slurry incubations are shown for four nitrogen species and sulfur, representing biological replicates from all caves except biofilm in Harman 1.



Supplementary Figure 11. Lithology level driver analysis combining Random Forest and linear models. Random forest % increase in Mean Squared Error (MSE) provides a measure of the importance of predictor variables. Blank tiles in the heatmaps indicate that no stable model could be established. **a**, Random Forest analysis of soil physicochemical drivers influencing the average gene copies per organism involved in trace gas metabolism (*pmoA*, [NiFe] group 1 / 2, *coxL*), ammonium and nitrite oxidation (*amoA*, *nrxA*), sulfide oxidation (*sqr*) and iron oxidation (*cyc2*) and soil physicochemical drivers. **b**, Random Forest analysis of soil physicochemical drivers influencing the cell specific reaction rates of H₂, CO and CH₄. **c**, Random Forest analysis of soil physicochemical drivers influencing the ¹³C signatures and ¹⁴C carbon fixation. **d**, Linear relationship between the average gene copies per organism (n=85) involved in trace gas metabolism (*pmoA*, [NiFe] group 1 / 2, *coxL*), ammonium and nitrite oxidation (*amoA*, *nrxA*), sulfide oxidation (*sqr*) and iron oxidation (*cyc2*) and soil physicochemical drivers with p values derived from two-sided tests in linear models. **e**, linear relationship between the cell specific reaction rates of H₂, CO and CH₄ (n=39) and soil physicochemical drivers with p values derived from two-sided tests in linear models. **f**, Linear relationships between ¹⁴C carbon fixation (n=21) and soil physicochemical drivers, with p values derived from two-sided tests in generalized linear models.



Supplementary notes

Supplementary Note 1. Phylogeny and physiology of cave methanotrophs.

Phylogeny of the PmoA sequences, encoded by the commonly used *pmoA* marker gene, indicated that several of the MAGs belong to the atmospheric methane oxidising groups USCα (Supplementary Figure 3b) and USCγ (Supplementary Figure 3). For the gammaproteobacterial MAGs, two MAGs, M3-S_metabat2.bin.345 and H1-B1_maxbin2.bin.5_sub, were classified as novel genera within the order CAJXQU01 (Figure S7a). Only one other genome within this order is in GTDB r214 (GCA_913058255.1, *CAJXQU01* sp913058255, recovered from a marine metagenome), and does not encode *pmoCAB*. Potentially these genomes are the first representatives of a new methanotrophic order in the Gammaproteobacteria. In both MAGs, PmoA sequences grouped with other gammaproteobacterial PmoA sequences (Supplementary Figure 3b), however not within the well-defined isolate methanotrophs of the Methylococcaceae and Methylomonadaceae families. H1-B1_maxbin2.bin.5_sub also encoded PxmAB of the *pxmABC* operon, a homologue of the *pmoCAB*. While *pxmABC* is found in many methanotrophs, the function is currently unknown^{1,2}. Both MAGs encode *pmoCAB* operon, *xoxF*-type methanol oxidation, and formaldehyde oxidation through the tetrahydromethanopterin (H₄MPT)-pathway (Supplementary Figure 3c).

MAG H1-B1_metabat2.bin.32 was classified as a novel species within the gammaproteobacterial family and genus UBA1147 (Supplementary Figure 3b). Three genomes, of which two encode *pmoCAB*, are present within this family in GTDB r214, (*UBA1147* sp002311315 recovered from a hydrothermal vent metagenome, and *UBA1147* sp024958995, recovered from a marine metagenome). H1-B1_metabat2.bin.32 encoded a PmoA sequence clustering with Methylococcaceae PmoA sequences, along with two copies of PxmA sequences (Supplementary Figure 3b). A complete *pmoCAB* operon, along with *xoxF*-type methanol oxidation, and formaldehyde oxidation through the H₄MPT-pathway was encoded (Supplementary Figure 3c). SD8037_metabat2.bin.6 and SD8020_metabat2.bin.3 were classified as novel species within the gammaproteobacterial genus USCγ-Taylor (Figure S7b), both encoding PmoA sequences clustering with other USCγ sequences (Supplementary Figure 3b). Both encode full *pmoCAB* operons along with *xoxF*-type methanol oxidation and formaldehyde oxidation through the H₄MPT-pathway (Supplementary Figure 3c). Calcium-dependent methanol oxidation was encoded by *mxoFI* in SD8020_metabat2.bin.3, and partially in SD8037_metabat2.bin.6 (*mxoF*). All gammaproteobacterial MAGs lacked complete

metabolic potential to assimilate carbon. Canonical gammaproteobacterial methanotrophs usually utilise the RuMP cycle. While all MAGs encoded *pfkA/B* and *FBA*, the key genes hexulosephosphate synthase (*hxlA/fae-hps*) and hexulosephosphate isomerase (*hxlB*) were not encoded. Reasons for absence of detection could be genome incompleteness, or the gene is divergent enough from the KO database representatives to not be correctly assigned. An incomplete serine cycle is present, missing the key gene HPR³.

Cytochrome *c* oxidase was encoded in all gammaproteobacterial MAGs, and the cytochrome *bd* oxidase was also encoded in USCy-Taylor and CAJXQU01 MAGs, both cytochromes are characterised by high oxygen affinity⁴. Dissimilatory nitrate reduction (*narGHJI*) was fully encoded in USCy-Taylor and UBA1147 MAGs, while partially encoded in CAJXQU01 (*narGH* and *narGHJ*). Assimilatory sulphate reduction (*cysDCHJI*, lacking *cysN*) and partial dissimilatory sulphate reduction (*dsrCEF*), though lacking the key genes *dsrAB*, was encoded in UBA1147. Three MAGs, SE1530_metabat2.bin.5_sub, SE1504_maxbin2.bin.0, and SE1504_metabat2.bin.11 were classified into three different species (<95% ANI) within the alphaproteobacterial *Methylocella* genus (Supplementary Figure 3b). This genus contains the well-studied isolate *Methylocella silvestris* and the recent upland soil cluster alpha atmospheric methane oxidising isolate *M. gorgona*. SE1504_maxbin2.bin.0 encoded a PmoA sequence clustering with USCα PmoA, while SE1504_metabat2.bin.11 encoded a partial USCα-clustering sequence (67 aa, denoted by * in Supplementary Figure 3b & c) located at the edge of a short contig (2890 bp). SE1530_metabat2.bin.5_sub encoded no USCα-PmoA sequence but contained a sequence clustering with alphaproteobacterial PxmA sequences (Supplementary Figure 3b). All MAGs encoded XoxF-type methanol oxidation, and formaldehyde oxidation through the H₄MPT-pathway. While SE1530_metabat2.bin.5_sub, SE1504_maxbin2.bin.0 had the genomic potential to assimilate formaldehyde through H₄F and serine cycle, bin.11 is missing two key serine cycle genes, *sga* and *hprA* (Supplementary Figure 3), and does not encode the H₄F pathway, perhaps due to genome incompleteness. Oxidation of formate to carbon dioxide was also encoded in all MAGs. Similar to the USCα isolate *M. gorgona* the CBB cycle was incomplete and missing the RuBisCo genes in all three MAGs in contrast to the USCα-encoding MAGs of ⁵ and other members of the *Methylocella* genus⁶. Control genome *M. silvestris* encodes the high oxygen affinity *cbb₃*-type and *bd*-type cytochromes, while the *Methylocella* MAGs obtained here encode low oxygen affinity type-*o* cytochromes⁴.

CAJXQU01 MAGs were amongst the three most dominant predicted methanotrophic taxa in the cave system, along with JACCXJ01 and *Methylocella* (Supplementary Figure 3a). Carbon and energy acquisition pathways from each MAG in these taxonomic groups were

compiled, revealing broadly similar methylotrophic lifestyles (Supplementary Figure 3c). However, in contrast to CAJXQU01 and JACCXJ01, *Methylocella* MAGs from the caves encoded the complete tetrahydrofolate pathway and serine cycle to assimilate carbon from formate (Supplementary Figure 3c). Additionally, all three taxonomic groups encoded a complete TCA cycle and glyoxylate shunt, the core 3C glycolysis steps of the Embden-Meyerhof-Parnas pathway, and the pentose phosphate pathway, whilst only *Methylocella* MAGs additionally encoded the Entner-Doudoroff pathway.

In addition to the alpha and gammaproteobacterial likely new methanotrophs, copper membrane bound monooxygenases (CuMMOs), representing methane monooxygenase homologs, were identified in the MAGs SD8061_maxbin2.bin.11_sub and M3-B2_maxbin2.bin.72_sub and were classified as novel genera within the Bin18 family (Supplementary Figure 3c). Both MAGs contained PmoA-like sequences grouping with other PmoA-like sequences from the Binatia class (Figure S7b). M3-B2_maxbin2.bin.72_sub also contained an additional MMO-like sequence that grouped with other hydrocarbon monooxygenases. None of the members within Binatia have been experimentally confirmed to be able to carry out methanotrophy. Furthermore, genes for methanol and formaldehyde oxidation could not be identified, indicating that the Bin18 MAGs are most likely not capable of methanotrophy and may be oxidising other hydrocarbons.

Supplementary Note 2

Etymological Information

We propose renaming the Pseudonocardiaceae genus GCA-003244245 as '*Candidatus* Hydrogenomurus', based on the high-quality annotated genome SE1519_metabat2.bin.2 (completeness/contamination: 100%/2.79%) from Tunnel basalt caves (herein '*Candidatus* Hydrogenomurus basanatii')

Species: '*Candidatus* Hydrogenomurus basanatii' (ba.sa.na'tii. N.L. gen. n. *basanatii*, of the basalt cave (Tunnel Cave), where the bacterium was identified)

Genus: '*Candidatus* Hydrogenomurus' (Hy.dro.ge.no.mu.rus. N.L. neut. n. *hydrogenum*, hydrogen (that which produces water, so called because it forms water when exposed to oxygen); from Gr. neut. n. *hydôr*, water; from Gr. ind. v. *gennaô*, to produce; L. masc. n. *murus*, wall; N.L. masc. n. *Hydrogenomurus*, a hydrogen-using bacterium from cave wall)

Full Classification:

- Domain: Bacteria
- Phylum: Actinomycetota

- Class: Actinomycetia
- Order: Mycobacteriales
- Family: Pseudonocardiaceae
- Genus: GCA-003244245 (proposed as '*Candidatus* Hydrogenomurus')
- Species: '*Candidatus* Hydrogenomurus basanatii'

We propose renaming the Egibacteraceae genus JACCXR01 as '*Candidatus* Hydrogenocavus', based on the high-quality annotated genome M3-B2_metabat2.bin.58 (completeness/contamination: 96.56%/3.3%) from Shades of death limestone caves (herein '*Candidatus* Hydrogenocavus calcicola')

Species: '*Candidatus* Hydrogenocavus calcicola' (cal.ci'co.la. L. masc./fem. n. *calx* (gen. calcis), chalk; L. masc./fem. n. suff. *-cola*, an inhabitant; from L. masc./fem. n. *incola*, dweller; N.L. masc./fem. n. *calcicola*, of the limestone cave (Shades of death), where the bacterium was identified)

Genus: '*Candidatus* Hydrogenocavus' (Hy.dro.ge.no.ca.vus. N.L. neut. n. *hydrogenum*, hydrogen (that which produces water, so called because it forms water when exposed to oxygen); from Gr. neut. n. *hydôr*, water; from Gr. ind. v. *gennaô*, to produce; L. n. cava, cave; N.L. masc. n. *Hydrogenocavus*, a hydrogen-using bacterium from caves)

Full Classification:

- Domain: Bacteria
- Phylum: Actinomycetota
- Class: Actinomycetia
- Order: Euzebyales
- Family: Egibacteraceae
- Genus: JACCXR01 (proposed as '*Candidatus* Hydrogenocavus')
- Species: '*Candidatus* Hydrogenocavus calcicola'

We propose renaming the Actinomycetota order JACCUZ01 as '*Candidatus* Hydrogenolapis', based on the high-quality annotated genome SD8021_metabat2.bin.4

(completeness/contamination: 84.11%/1.1%) from the limestone cave Scrubby Creek (herein '*Candidatus* Hydrogenolapis speleophilus').

Species: '*Candidatus* Hydrogenolapis speleophilus' (spe.le.o'phi.lus. N.L. masc. adj. speleophilus, cave-loving, referring to the cave environment where the MAG was identified)

Genus: '*Candidatus* Hydrogenolapis' (Hy.dro.ge.no.la'pis. N.L. neut. n. hydrogenum, hydrogen; L. n. lapis, stone; N.L. neut. n. Hydrogenolapis, a hydrogen-oxidizing bacterium)

Family: '*Candidatus* Hydrogenolapaceae' (Hy.dro.ge.no.la.pa'ce'ae. N.L. neut. n. Hydrogenolapis, a (*Candidatus*) bacterial genus; suff. -aceae ending to denote a family; N.L. fem. pl. n. Hydrogenolapaceae, family of the genus Hydrogenolapis)

Order: '*Candidatus* Hydrogenolapales' (Hy.dro.ge.no.la'pa.les. N.L. neut. n. Hydrogenolapis, a (*Candidatus*) bacterial genus; suff. -ales ending to denote an order; N.L. fem. pl. n. Hydrogenolapales, order of the family Hydrogenolapaceae)

Full Classification:

Domain: Bacteria

Phylum: Actinomycetota

Class: Actinomycetia

Order: JACCUZ01 (proposed as '*Candidatus* Hydrogenolapales')

Family: JACCUZ01 (proposed as '*Candidatus* Hydrogenolapaceae')

Genus: JACCUZ01 (proposed as '*Candidatus* Hydrogenolapis')

Species: '*Candidatus* Hydrogenolapis speleophilus

We propose renaming the Gammaproteobacterial order JACCXJ01 as '*Candidatus* Methylooligotrophales', based on the high-quality annotated genome SD8037_metabat2.bin.6 (completeness/contamination: 96.44%/0.56%) from Shades of death limestone caves (herein '*Candidatus* Methylooligotropha calcicola')

Species: '*Candidatus* Methylooligotropha calcicola' (cal.ci'co.la. L. masc./fem. n. *calx* (gen. calcis), chalk; L. masc./fem. n. suff. *-cola*, an inhabitant; from L. masc./fem. n. *incola*, dweller; N.L. masc./fem. n. *calcicola*, of the limestone cave (Shades of death), where the bacterium was identified)

Genus: '*Candidatus* Methylooligotropha' (Me.thy.lo.li.go.tro'pha. N.L. neut. n. *methylum*, from French *méthyle*, back-formation from *méthylène*, coined from Gr. n. *methu*, wine, and Gr. n. *hulê*, wood, referring to the methyl group; N.L. pref. methylo-, pertaining to the methyl radical; Gr. masc. adj. *oligos*, little, few; Gr. masc./fem. adj. *trophos*, feeder, rearer, that which nourishes; N.L. fem. n. *Methylooligotropha*, an oligotrophic methyl-using bacterium)

Family: '*Candidatus* Methylooligotrophaceae' (Me.thy.lo.li.go.tro'pha.ce'ae. N.L. neut. n. *Methylooligotropha* a (Candidatus) bacterial genus; suff. *-aceae* ending to denote a family; N.L. fem. pl. n. *Methylooligotrophaceae*, family of the genus *Methylooligotropha*)

Order: '*Candidatus* Methylooligotrophales' (Me.thy.lo.li.go.tro'pha'les. N.L. neut. n. *Methylooligotropha* a (Candidatus) bacterial genus; suff. *-ales* ending to denote an order; N.L. fem. pl. n. *Methylooligotrophales*, order of the family *Methylooligotrophaceae*)

Full Classification:

- Domain: Bacteria
- Phylum: Proteobacteria
- Class: Gammaproteobacteria
- Order: JACCXJ01 (proposed as '*Candidatus* Methylooligotrophales')
- Family: JACCXJ01 (proposed as '*Candidatus* Methylooligotrophaceae')
- Genus: USCg-Taylor (proposed as '*Candidatus* Methylooligotropha')
- Species: '*Candidatus* Methylooligotropha calcicola'

We propose renaming the Gammaproteobacterial order CAJXQU01 as '*Candidatus* Methylocavales', based on the high-quality annotated genome H1-B1_maxbin2.bin.5_sub

(completeness/contamination: 97.84%/2.43%) from Harman basalt caves (herein '*Candidatus* Methylocavus basanatii')

Species: '*Candidatus* Methylocavus basanatii' (ba.sa.na'tii. N.L. gen. n. *basanatii*, of the basalt cave (Harman 1), where the bacterium was identified)

Genus: '*Candidatus* Methylocavus' (Me.thy.lo.ca.vus. N.L. neut. n. *methylum*, from French *méthyle*, back-formation from *méthylène*, coined from Gr. n. *methu*, wine, and Gr. n. *hulê*, wood, referring to the methyl group; N.L. pref. methylo-, pertaining to the methyl radical; L. n. cava, cave; N.L. neut. n. *Methylocavus*, a methyl-using bacterium from caves)

Family: '*Candidatus* Methylocavaceae' (Me.thy.lo.ca.va.ce'ae. N.L. neut. n. *Methylocavus* a (Candidatus) bacterial genus; suff. -aceae ending to denote a family; N.L. fem. pl. n. *Methylocavaceae*, family of the genus *Methylocavus*)

Order: '*Candidatus* Methylocavales' (Me.thy.lo.ca.va'les. N.L. neut. n. *Methylocavus* a (Candidatus) bacterial genus; suff. -ales ending to denote an order; N.L. fem. pl. n. *Methylocavales*, order of the family Methylocavaceae)

Full Classification:

- Domain: Bacteria
- Phylum: Proteobacteria
- Class: Gammaproteobacteria
- Order: CAJXQU01 (proposed as '*Candidatus* Methylocavales')
- Family: CAJXQU01 (proposed as '*Candidatus* Methylocavaceae')
- Genus: CAJXQU01 (proposed as '*Candidatus* Methylocavus')
- Species: '*Candidatus* Methylocavus basanatii'

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