Science

RESEARCH ARTI

Cite as: M. Klapper *et al.*, *Science* 10.1126/science.adf5300 (2023).

Natural products from reconstructed bacterial genomes of the Middle and Upper Paleolithic

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Major advances over the past decade in the field of ancient DNA are providing access to past paleogenomic diversity, but the diverse functions and biosynthetic capabilities of this growing paleome remain largely elusive. Here, we investigated the dental calculus of 12 Neanderthals and 52 anatomically modern humans spanning 100 kya to the present and reconstructed 459 bacterial metagenome-assembled genomes (MAGs). We identified a biosynthetic gene cluster (BGC) shared by seven Middle and Upper Paleolithic individuals that allows for the heterologous production of a class of previously unknown metabolites we name paleofurans. This paleobiotechnological approach demonstrates that viable biosynthetic machinery can be produced from the preserved genetic material of ancient organisms, allowing access to natural products from the Pleistocene and providing a promising area for natural product exploration.

Microbial natural products play key roles in cell signaling, communication, defense, and microbial evolution, enabling the interaction between microorganisms within communities and with their abiotic environments (*1*, *2*). Because of their high structural and functional diversity, they are an ideal source of therapeutic drugs, antimicrobials, and other functional compounds (*3*, *4*). Characterizing the natural products encoded in biosynthetic gene clusters (BGCs) and synthesized by ancient microbial communities would provide both insights into past microbial lifestyles and access to previously hidden chemical, structural, and functional diversity. However, the direct detection and characterization of metabolites from ancient sources has met only limited success (*5*). Small molecules are unlikely to persist in archaeological samples over long periods due to leaching and their propensity to degrade and undergo modification through time. With the advent of paleogenomics it is now becoming feasible to access the vast structural and functional diversity of ancient low-molecular weight natural products by instead resurrecting biosynthetic pathways from ancient metagenomes.

Since the publication of the first draft genome of an ancient bacterium in 2011 (*6*), major advances in paleogenomic technologies have resulted in the characterization of more than 1,700 ancient microbial genomes, partial genomes, and metagenomes from diverse pathogens, commensals, culinary taxa, and microbiota (*7*). However, knowledge of the diverse functions and biosynthetic capabilities encoded within this growing microbial paleome lags behind. To date, most studies in the field of ancient microbial research have focused on sequence alignment to databases of reference genes or genomes, thus restricting findings to known taxa and their close relatives (*8*, *9*). Recent de novo assembly of authentic ancient DNA (aDNA) has shown that reconstruction of ancient metagenome-assembled genomes (MAGs) is possible (*10*–*13*). However, further optimization of assembly methods and exploration of deeply sequenced ancient metagenomes is needed to understand the unknown functional space and biosynthetic capabilities encoded in the microbial paleome. The development of computational tools for BGC prediction from (meta)genomes has accelerated natural product discovery by identifying gene targets for heterologous expression

(*14*–*16*). However, this requires high-quality genomic data consisting of long contiguous sequences (contigs), which are difficult to assemble from fragmentary aDNA (*17*) using currently available methods (*11*–*13*).

De novo reconstruction of ancient metagenome-assembled genomes (MAGs)

To search for ancient BGCs, we selected dental calculus metagenomic datasets previously published (*9*) from 12 Neanderthals dating to ca. 102,000–40,000 ya, 34 archaeological humans dating to ca. 30,000–150 ya, and 18 present-day humans (Fig. 1A and dataset S1a) and generated additional sequencing data for a subset of 23 individuals predating 8,000 ya to improve metagenomic sequence coverage within this set (dataset S1b). We obtained for each individual between 3.1 and 338.3 million DNA molecules (dataset S1c), with a mode fragment length of 30–48 bp for archaeological sequences (mean, 30 bp) (Fig. 1B and dataset S1d). We removed five low quality samples with fewer than 5 million sequences, and, considering assembler performance on ultrashort sequences, performed de novo assembly of the metagenomic datasets using MEGAHIT (*18*) for Neanderthals and ancient humans and metaSPAdes (*19*) for present-day humans. For MEGAHIT assemblies, we observed a persistent artifact in which damage-related miscoding lesions were overrepresented in contig sequences, and we corrected this error by replacing the damaged allele extracted from the assembly graph with the allele inferred by freeBayes (*20*) from short-read alignment to the contig sequence (fig. S1). We obtained 522,874 and 305,133 contigs greater than 1 kb in length for the present-day and ancient metagenomes, respectively, of which 21,070 and 8,165 contigs exceeded 10 kb (dataset S2a). Although on average the de novo assembly of present-day dental calculus metagenomes resulted in longer contigs (fig. S2 and dataset S2b), the assembly of a subset of deeply sequenced Pleistocene metagenomes (PES001, EMN001, and PLV001) performed similarly to present-day metagenomes (Fig. 1C), and the vast majority of contigs belonging to these individuals were inferred to be of an ancient origin using pyDamage (fig. S3 and dataset S2c) (*13*).

Following assembly, we performed reference-free binning of all contigs longer than 1 kb (dataset S3a), followed by MAG refinement based on single-copy marker genes using metaWRAP (*21*) (Dataset S3b). We next implemented an automated pipeline to identify and remove chimeric contigs (figs. S4 and S5 and dataset S3c). Requiring >50% genomic completeness and <10% contamination estimated by checkM (*22*) , a clade separation score <0.45 by GUNC (*23*), and following MIMAG criteria (*24*), we obtained a total of 459 MAGs, of which 150 high quality (HQ) and 174 medium quality (MQ) MAGs originated from present-day metagenomes, and 25 HQ and 110 MQ MAGs originated from archaeological metagenomes (dataset S3c). However, both the modern and archaeological HQ MAGs lacked the required rRNA genes (dataset S3d), which is typical of MAGs assembled from short-read metagenomic sequencing data alone (*25*, *26*). Of the 135 archaeological MAGs, 22 HQ and 104 MQ MAGs were confirmed by PyDamage (*13*) to have characteristic age-related DNA damage (dataset S3c, q-value < 0.05), indicating that the archaeological MAGs are predominantly ancient in origin and not recent contaminants.

From the 459 MAGs, we identified a set of 226 representative MAGs (dataset S3e) by clustering at an average nucleotide identity (ANI) of 95% and performed taxonomic classification using GTDB. We then determined whether the representative MAGs were likely to have an oral origin by clustering them with all reference genomes present within the Human Oral Microbiome Database (HOMD) (fig. S6) (*27*). The majority of MAGs (75.4%) were found to be closely related to known oral taxa, although this is likely an underestimate due to the fact that several taxa not identified as oral by this method were nevertheless assigned by PhyloPhlAn3 (*28*) to taxa with oral isolation sources (dataset S3e). We observed overlap in the representative MAGs found in both present-day and archaeological dental calculus, such as the oral taxa *Arachnia propionica* (n = 16), *Flexilinea sp001717545* (n = 15), and *Lautropia mirabilis* (n = 14) (dataset S3f), but some oral MAGs were recovered only from present-day (e.g., *Capnocytophaga*) or archaeological (e.g., *Methanobrevibacter*) metagenomes. Oral MAGs were well-represented among many of the oldest Pleistocene metagenomes in our study (Fig. 1D), and at least nine MAGs of secure oral origin were reconstructed from a Neanderthal at the site of Pešturina, Serbia (*29*, *30*). These MAGs substantially exceed the age of the previously oldest reconstructed MAGs (ca. 2,000–8,000 ya) (*11*, *31*) by more than 90,000 years.

Reconstruction of authentic ancient *Chlorobium* genomes from Paleolithic dental calculus

The genus with the highest number of ancient HQ MAGs was *Chlorobium*, a genus not typical of oral microbiota or burial sediments (fig. S6), but which was detected in the dental calculus of nine individuals (dataset S4). Known members of *Chlorobium* are photolithoautotrophic, obligate anaerobic green sulfur bacteria that typically perform photosynthesis in anoxic water columns (*32*, *33*). Of the five individuals with a HQ *Chlorobium* MAG (EMN001, PES001, GOY005, PLV001, RIG001), the *Chlorobium* MAG had the highest completeness of all MAGs in the metagenome (Fig. 1D). *Chlorobium* MAG EMN001_021, originating from a ca. 19,000 ya individual at El Mirón (*34*), Spain, had a very high median contig length (N50 = 115 kb) and an exceptionally low number of contigs (n = 21) (dataset S3e). We also assembled two MQ *Chlorobium* MAGs from Upper Paleolithic humans, one from TAF017 and

another from PLV001 (dataset S3c), and we genotyped >50% of the *Chlorobium* genome at a minimum coverage of 3-fold for the Neanderthal GOY006 (dataset S4c; by mapping rather than assembly). The seven samples for which we could reconstruct at least one *Chlorobium* genome at >50% either by de novo assembly or reference-based alignment all originated from Pleistocene contexts in west Eurasia and north Africa, and were excavated from either cave sites (EMN001, PES001, GOY005, GOY006, RIG001, TAF017) or a site close to a water reservoir (PLV001) (Fig. 1 and dataset S1a). *Chlorobium* was largely absent from individuals after 10,000 ya (n = 45) other than ESA006 and ESA007 (dataset S4b) from a 19th century coastal site in Morocco (dataset S1a).

Because *Chlorobium* is not a known oral taxon, we investigated whether it could be a recent postmortem contaminant by inspecting the DNA damage patterns of the *Chlorobium* HQ MAGs in comparison to paired HQ MAGs of the securely oral taxon *Flexilinea* (NCBI taxonomy name *Anaerolinaceae* oral taxon 439) from the same individuals. The DNA sequences making up the *Chlorobium* and *Flexilinea* MAGs exhibit equivalently high frequencies of 5′ C-to-T miscoding lesions characteristic of age-related DNA damage (Fig. 1E and fig. S7) (*17*), confirming that the *Chlorobium* MAGs are of authentic ancient origin.

Paleoecology and evolution of ancient *Chlorobium* species

To determine the ecological origin of the ancient *Chlorobium* species, we constructed a phylogenetic tree using all available genomes of the *Chlorobiales* order from diverse environmental sources (Fig. 2A and fig. S8). The ancient *Chlorobium* MAGs are most closely related to *Chlorobium limicola* and form a previously undescribed monophyletic clade (Fig. 2A and figs. S8 and S9). Their set of functional genes, including those required for the photosynthetic machinery, is similar to those found in modern *C. limicola* strains occurring in freshwater biofilms (figs. S10 and S14, and datasets S6 and S8). As *Chlorobium* spp. are photoautotrophs (*35*), we speculate that anaerobic colonization of the dental plaque biofilm occurred either during life or shortly after death but prior to burial through transient exposure to contaminated freshwater sources. Periodic in vivo host-mediated biofilm calcification or *Chlorobium*-mediated, light dependent dolomite formation (*36*) may have further facilitated the long-term preservation of this microbe within dental calculus. Metagenomic analyses of burial site sediments from El Mirón as well as non-human DNA obtained from a bone sample of the El Mirón individual (*37*) do not show the presence of *Chlorobium* DNA (fig. S11), supporting ante- or perimortem incorporation (dataset S7 and fig. S12).

Although related to *C. limicola*, the ancient *Chlorobium* MAGs are genomically distinct (ANI <80%) (Fig. 2B and fig. S13). Seven ancient MAGs share >95% ANI, indicating that they are members of a single species, whereas PLV001_002 had lower shared ANI values of 85–88%, suggesting that it is a different species (*38*). Thus, the ancient *Chlorobium* MAGs appear to represent two unknown, possibly extinct, *Chlorobium* species. The ancient *Chlorobium* sp. represented by the seven closely related MAGs differs from *C. limicola* and the ancient *Chlorobium* sp. represented by PLV001_002 in appearing to have lost its ability to oxidize thiosulfate, and it may have also had altered iron transport and photosynthesis capabilities (dataset S8, c and d). The additional presence of glycoside hydrolase orthologs in the ancient *Chlorobium* MAGs indicates that the ancient *Chlorobium* spp. were adapted to different growth conditions than *C. limicola*. The low diversity observed among the seven closely related ancient *Chlorobium* MAGs is not unexpected, even considering their broad geographic distribution and timespan. Wild populations of *Chlorobium* spp. are known to have extremely low sequence diversity (*39*) and to undergo genome-wide sweeps and strong purifying selection (*40*). Similarly low diversity was also observed among the HQ MAGs of the oral taxon *Flexilinea* obtained from the same individuals (fig. S15 and dataset S8e) and has been reported in well-studied ancient pathogens (*41*), such as *Yersinia pestis* (dataset S8f).

Discovery of ancient butyrolactone biosynthetic gene clusters (BGCs) with a highly similar genetic architecture

To gain insight into the biosynthetic potential of ancient microbes, we screened the ancient *Chlorobium* MAGs for BGCs using antiSMASH (*14*) and identified four different biosynthetic gene cluster (BGC) types, of which putative butyrolactone and terpene BGCs are most prevalent (fig. S16B). Butyrolactones are known signaling molecules originally discovered in *Streptomyces* species (*42*). These compounds, however, have not been previously reported in the *Chlorobiales* order. To ensure that the butyrolactone BGCs are of ancient origin, we confirmed that all respective contigs encoding the butyrolactone BGCs have characteristic age-related DNA damage (table S1) using PyDamage (*13*). An AfsA-like synthase gene, constituting the core gene of butyrolactone biosynthesis (*42*), was used as a query to understand the evolutionary origin of the *Chlorobium* butyrolactone BGC. Phylogenetic analysis of all ancient and 342 representative AfsA-like synthases from various organisms sharing a similar protein domain structure (i.e., a single HotDog domain) (*43*) showed that the AfsA-like synthases within the ancient *Chlorobium* MAGs are most closely related to those of the *Chlorobiales* sister lineage (fig. S18), suggesting vertical gene transfer.

To ascertain the fidelity of the ancient butyrolactone BGC architecture, we compared the gene synteny of the ancient

BGCs to butyrolactone BGCs across representative genomes within the *Chlorobiales* order and performed a network analysis based on pairwise sequence similarities across the entire BGC region using BiG-SCAPE (*44*). We found seven distinct gene cluster families (GCFs) that form two major gene cluster clans (GCCs) (Fig. 3A and dataset S7), of which a unique GCF consists of all ancient butyrolactone BGCs found in the highly related *Chlorobium* MAGs, as well as of one modern BGC. A comparison of the genetic architecture and sequences of the modern and ancient butyrolactone BGC regions clearly showed that all ancient and also several closely related modern butyrolactone BGCs share similar genetic features in the vicinity of the AfsA-like synthase gene (Fig. 3B; figs. S16C, S17, and S19; and table S2), suggesting correct reconstruction of the ancient BGCs.

Reconstruction of ancient BGC delivers previously unobserved metabolites

In order to access the butyrolactone-type metabolite from the ancient *Chlorobium* MAGs, we reconstructed the corresponding BGC. AfsA-like synthase-based biosynthetic pathways typically involve additional modifying enzymes leading to the production of butyrolactones (*42*), butenolides (*45*), or furanoids (*46*). To validate the BGC sequence derived from metagenomic assembly of highly fragmented aDNA, we performed pairwise sequence comparisons for the three core genes (i.e., *afsA*-like synthase, oxidoreductase and *cqsS*-like sensor kinase/phosphatase gene) between all ancient *Chlorobium* MAGs. All BGC core genes are nearly or fully identical in their nucleotide and amino acid sequences (fig. S20), mirroring the high ANI shared between the respective MAGs (Fig. 2B and fig. S12).

We obtained the ancient coding sequence of the three core genes from HQ *Chlorobium* MAG EMN001_021 (which is identical to that in HQ MAG RIG001_021) (fig. S20) gene synthesis and cloned different sets of the encoded ORFs into two different expression systems under the control of strong promoters (tables S3 to S5). A derivative of the extrachromosomal broad host range expression vector pRANGER-BTB3 was used for determining the required set of biosynthetic genes in an engineered *Pseudomonas protegens* Pf-5 expression chassis. Ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS) indicated the additional presence of new compounds when expressing the key biosynthetic gene encoding an AfsA-like synthase (*plfA*) with the adjacent oxidoreductase gene (*plfB*). These products were absent when expressing each gene individually (fig. S21A). Furthermore, chassis-independent recombinase-assisted genome engineering (CRAGE) was used to stably integrate the ancient biosynthetic genes chromosomally into engineered *P. protegens* Pf-5 (table S6), and additionally into *Photorhabdus khanii* subsp. *khanii* XP03 (*72*) to verify that compound production is host-independent. Because the compounds are only produced in both mutant strains, the small molecules must originate from the heterologous expression of the ancient *plfA* and *plfB* genes (Fig. 3C and fig. S21B). From a 3 L fermentation of Pf-5 harboring both *plfA* and *plfB*, we isolated compounds with pseudo-molecular masses $[M–H]^-$ of m/z = 209.1178 and 237.1493, corresponding to the molecular formulae $C_{12}H_{18}O_3$ and $C_{14}H_{22}O_3$, respectively. Using NMR spectroscopy (figs. S26 to S32) and GC-EI-MS fragment analyses (fig. S23) we elucidated the structure of two previously unknown 5-alkylfuran-3-carboxylic acids with C7 or C9 alkyl side chains, which we named paleofuran A (**1**) and paleofuran B (**2**), respectively (Fig. 3D, fig. S22, and table S7). The chemical total synthesis of paleofuran A (**1**) in only two steps with 40% overall yield starting from 3-carboethoxyfuran (**3**) via ketone (**4**) confirmed the proposed structure (Fig. 3E, fig. S24A, and figs. S33 to S35).

As previously shown for structurally related methylenomycin furan (MMF) autoinducers (*48*), AfsA-dependent biosynthetic pathways can also lead to furanoid formation (*46*). Analogously, we found that feeding of glycerol-1,1,2,3,3-d5 led to incorporation of a single deuterium in the furan core next to the ring oxygen (figs. S24B, S25, S36, and S37). This suggests that the building blocks are also derived from a glycerol C3 unit and a C10 (for **1**) or C12 (for **2**) 3-oxo fatty acid, which would decarboxylate after condensation (Fig. 3D). Because MMFs regulate the adjacent methylenomycin BGC in *Streptomyces* species (*46*), we analyzed the vicinity of the ancient *Chlorobium* AfsA-like synthases. The adjacent genes, which are conserved in modern *Chlorobium* genomes, encode biosynthetic enzymes required production bacteriochlorophyll precursors, suggesting that the paleofurans could be involved in regulating the bacterial photosynthesis (fig. S19, table S2, and dataset S6).

Discussion

The paleobiotechnological strategy we have described here unlocks the biosynthetic potential of authentic ancient metagenomes, effectively adding a time dimension to natural product discovery. Natural product recovery not only expands our understanding of the paleome but also raises questions regarding the management, stewardship, and intellectual property of humanity's collective biological and cultural heritage (supplementary materials, section 1.1). The multiple steps implemented throughout the workflow address the known challenges of aDNA recovery from diverse sources (*17*, *49*) and highlight the benefit of deeply sequenced ancient samples for the exploration of short read-based ancient metagenomes.

The recovery of unknown, possibly extinct ancient microbial species further opens an avenue to investigate lost microbial diversity due to environmental changes and increased

selective pressures, e.g., climate change or (mis)use of antibiotics, respectively (*50*, *51*). However, given the insufficient representation of *Chlorobium* genomes in current databases, we cannot be certain whether the two ancient ecotypes are extinct or only unrecovered. *Chlorobium* members are key players in the carbon, nitrogen, and sulfur cycles (*52*) and can harvest light at very low intensities (*32*), making it likely that they were incorporated into ancient dental biofilms through transient exposure to contaminated water. Pleistocene-era cave dwelling may have increased human exposure to *Chlorobium* spp., and its high prevalence only among Paleolithic individuals suggests that the environmental microbial exposures of humans may have substantially changed over time as habitation patterns shifted in response to Holocene-era climate change.

From the reconstructed ancient *Chlorobium* MAGs, we accessed previously unreported paleofurans. That the paleofuran BGC and its genetic vicinity remained largely unchanged over at least ~100,000 years may suggest that paleofurans play an important role in the ecology of *Chlorobium* species. This ability to reconstruct microbial genomes from well-dated archaeological contexts has important implications for refining bacterial evolution models and overcoming time resolution challenges. Our approach can, in principle, be applied to any ancient metagenomes, thus providing a roadmap for future BGC exploration and metabolite discovery. The analysis and comparison of the product spectrum of ancient and modern paleofuran BGC homologs provide key opportunities to explore BGC evolution.

In conclusion, by merging metagenomics, genome mining, gene synthesis, and metabolic analyses with the field of aDNA research, we chart a path for the discovery of ancient natural products to gain evolutionary insights on their formation and origin, as well as to inform their potential future applications.

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ACKNOWLEDGMENTS

We thank Mateja Hajdinjak for the access to the shotgun sequencing data of the toe bone of EMN001 from El Mirón; Sandra Sázelová, Jiří Svoboda, Isabelle Crevecoeur, Hélène Rougier, Patrick Semal, Dušan Mihailović, Mirjana Roksandic, Catherine Bauer, Chistoph Cupillard, Marta Díaz-Zorita Bonilla Dorothée Drucker, and Louise Humphrey for providing Pleistocene-era dental calculus for analysis; Franziska Aron for paleogenomic data generation; Zhiying Zhao and Yasuo Yoshikuni for sharing the CRAGE system with us; Andrea Perner, Nico Ueberschaar, and Heike Heinecke for MS and NMR measurements; Michael Cyrulies, Matthias Steinacker, and Uwe Knüpfer for large-scale fermentations; and Floyd Dewhirst for discussions about the ecology and metabolism of *Chlorobium*. **Funding:** This research was supported by the Werner Siemens Foundation, the Max Planck Society, the Leibniz Association, the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy EXC 2051 (Project-ID 390713860, "Balance of the Microverse"), CRC1127 ChemBioSys, B09, project number 239748522, and Leibniz Award, the DAAD graduate fellowship program supported WKA, and the Joachim Herz Foundation supported MK. **Author contributions:** CW and PS conceived and designed the project. CW and PS acquired funding and supervised the research. MK and AH administered and coordinated the research. MK established cloning and heterologous expression protocols. AH performed assembly, authentication, binning, MAG classification, genome reconstruction, pangenome and phylogenetic analyses. AI performed phylogenetic analyses, BGC bioinformatics analyses, and gDNA and plasmid sequencing analyses. MK, SC, JF, JAFY, IMV, MB contributed to bioinformatics analyses. MRGM and LGS directed the excavations at El Miron Cave and provided archaeological sediment samples. MK, IW, HS, EVB, TH, and PS performed cloning, heterologous expression, and prepared host chassis strains. MK and SZ performed isolation, purification, and structure elucidation. MK, IW, and VH performed biosynthetic studies. VH performed GCMS analyses. WKA performed syntheses. MK, AH, AI, VH, WKA, MB, SZ, IW, HS, JF, IMV, JAFY, RH, HMD, IV, CH, CW, and PS analyzed data. MK, AH, AI, CW, and PS wrote the manuscript, with the input of all coauthors. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** The accession number for all newly reported sequencing data are available from the European Nucleotide Archive: PRJEB55583. The sequences and annotations for the metagenome-assembled genomes are available on the Edmond Max Planck Data Repository (*53*). The code and files used to reproduce the analyses is available in the GitHub repository https://github.com/paleobiotechnology/EMN001_Paleofuran, archived in Zenodo (*54*), which also includes the raw NMR data, GCMS data, HPLC chromatogram data, and a material transfer agreement for CRAGE components granted on 20th of October 2020. Datasets S1 to S9 can be found under 06-figures_tables in the code repository.

SUPPLEMENTARY MATERIALS

science.org/doi/10.1126/science.adf5300
Materials and Methods
Supplementary Text
Figs. S1 to S37
Tables S1 to S7
References (*55–164*)
MDAR Reproducibility Checklist

Submitted 28 October 2022; accepted 13 April 2023
Published online 4 May 2023
10.1126/science.adf5300

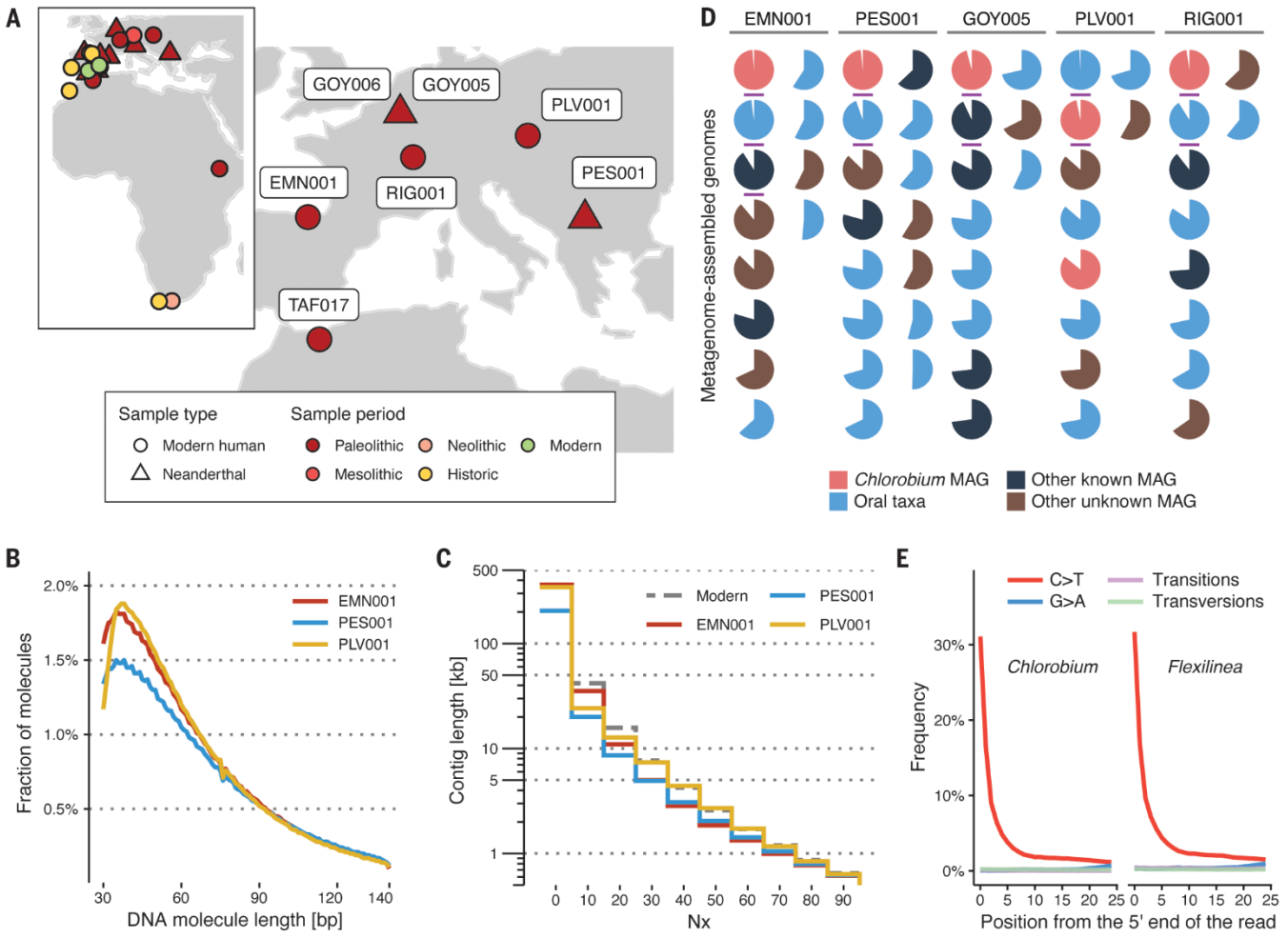

Fig. 1. De novo assembly and reference-free binning of ancient metagenome-assembled genomes (MAGs) from dental calculus samples. (**A**) Sample locations and temporal overview. Inset shows all analyzed samples, and the larger map displays seven samples for which we were able to reconstruct >50% of the *Chlorobium* genome. (**B**) DNA length distribution of EMN001, PES001, and PLV001 metagenomes estimated from PE75 Illumina-sequenced read pairs. The mode fragment length was <40 bp, and only 12.4%, 21.4%, and 12.6% of read pairs were longer than the maximum inferrable length of 140 bp (dataset S1b). (**C**) Contig length distribution of the longest contig (N0) and at the nine deciles (N10 to N90) for EMN001, PES001, and PLV001 compared to the average contig length of present-day dental calculus samples. (**D**) Summary of all MAGs recovered from dental calculus samples containing a HQ *Chlorobium* MAG. Pies indicate the completeness estimated by checkM, horizontal bars the HQ MAGs, and colors the inferred MAG source. (**E**) 5′ substitution frequency of EMN001 short-read data aligned to *Chlorobium* and *Flexilinea* contig consensus sequences. High frequencies of C-to-T transitions relative to other possible substitutions indicate age-related damage.

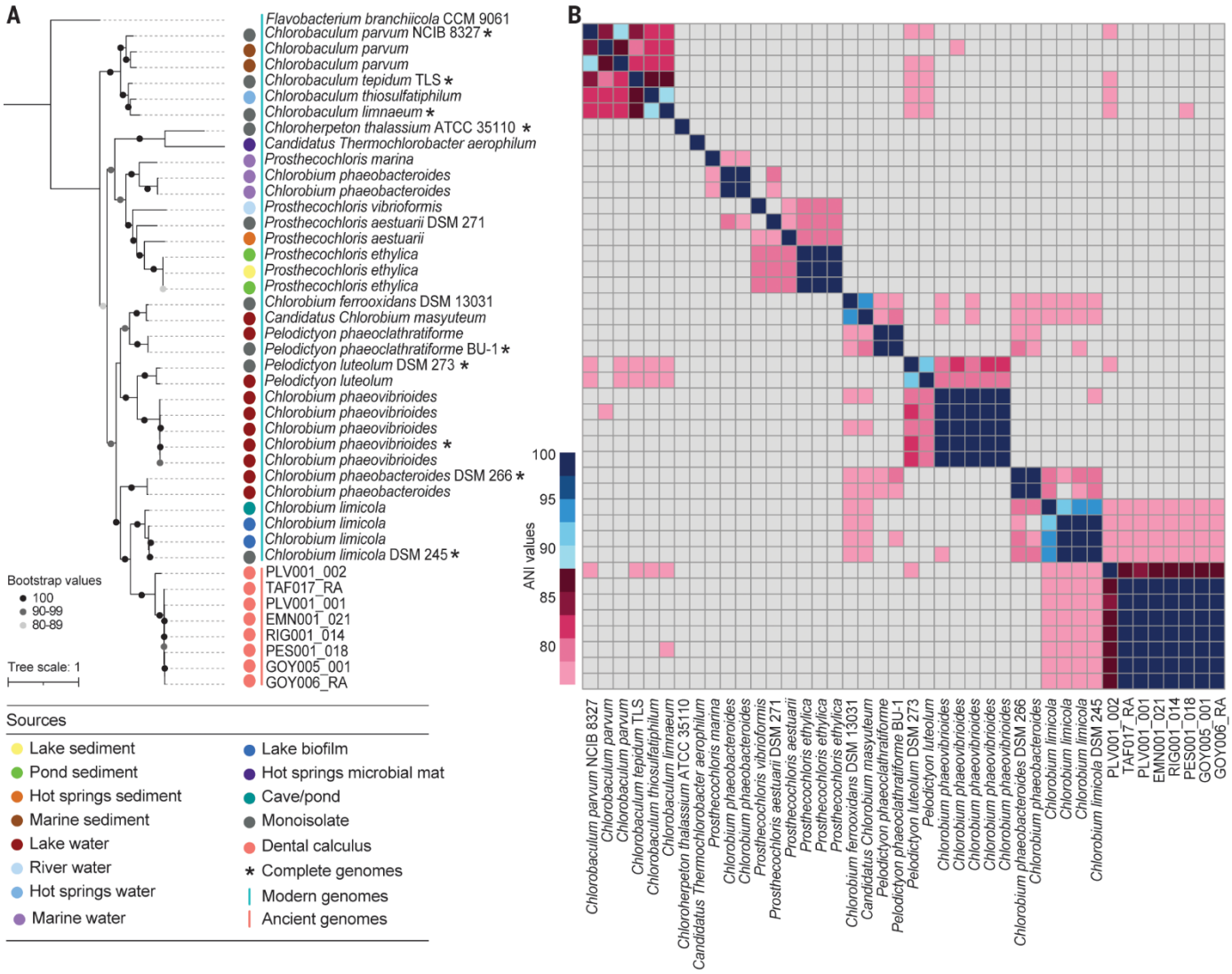

Fig. 2. Phylogeny and shared average nucleotide identities (ANI) of ancient *Chlorobium* MAGs within a subset of modern *Chlorobiales* genomes and MAGs. (A) A pruned phylogenetic tree based on the amino acid alignment of all core genes of *Chlorobium limicola* illustrating the phylogeny of the ancient *Chlorobium* MAGs compared to 34 modern genomes of *Chlorobiales* species. The color coding indicates the sources from which the organisms and modern and ancient MAGs were obtained. Clades supported by at least 80% of the bootstrap replicates are indicated by dots on the respective branch. *Flavobacterium branchiicola* was used as an outgroup to root the tree. (B) Corresponding heatmap illustrating the ANIs ≥ 74% (colored) and <74% (gray).

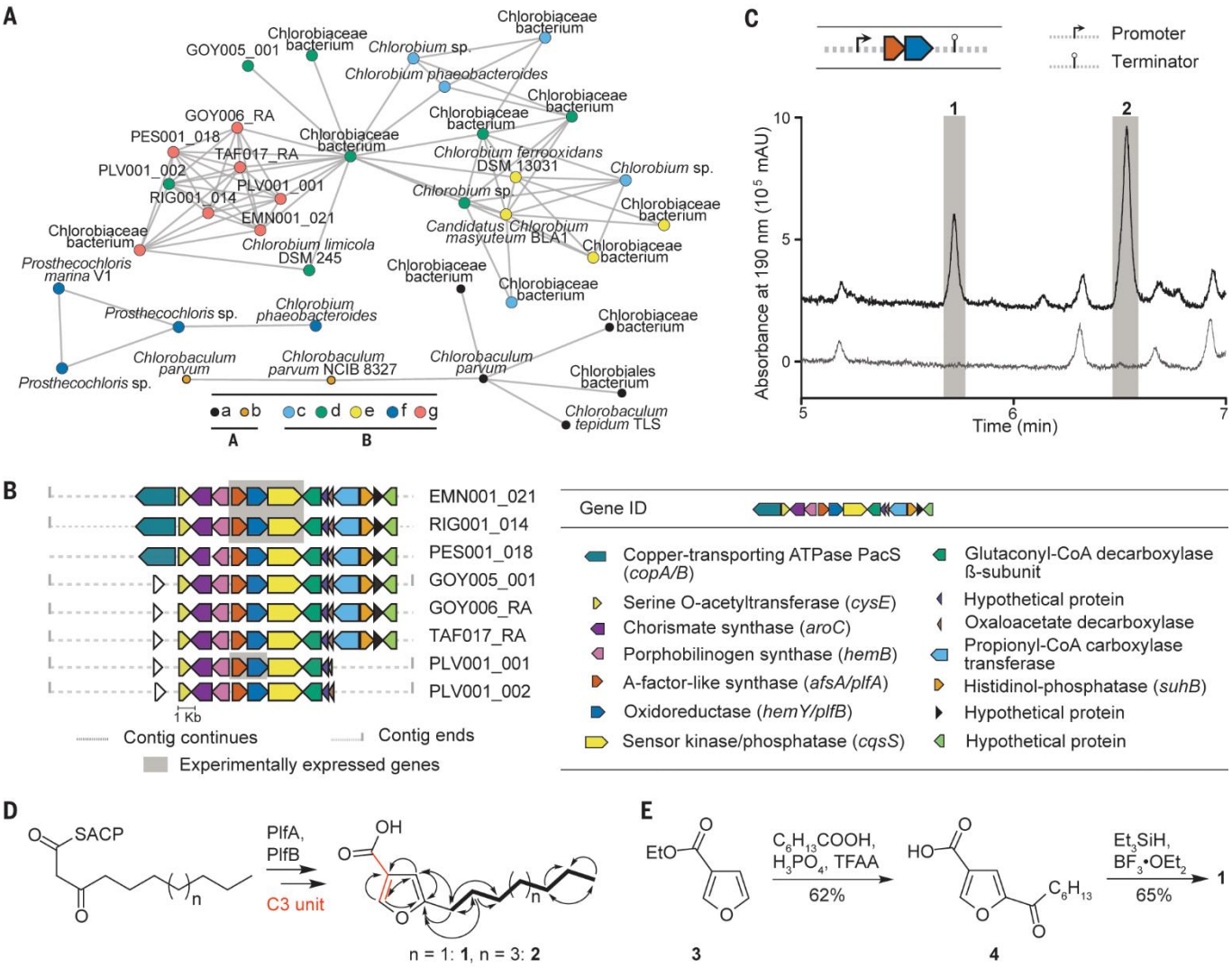

Fig. 3. Reconstruction of ancient butyrolactone BGCs to access metabolites. (A) A network analysis of ancient and modern butyrolactone BGCs using BiG-SCAPE identified 2 gene cluster clans (GCCs, A–B) including 7 gene cluster families (GCF, a–g) in the order of *Chlorobiales*. The butyrolactone BGCs from ancient MAGs fall in one GCF (g). The majority of the modern BGCs are found in different, but related GCFs. For illustration purposes, the raw pairwise distances were filtered to include only those estimated as ≤ 0.3 (supplementary materials, section 7). GOY005_001 does not appear in GCF (g) due to initial assembly truncation (supplementary materials). (B) All ancient butyrolactone BGCs share a similar genetic architecture with high sequence similarities. Heterologously expressed genes are highlighted with a gray box. Gene annotations are given with orthologs in parentheses, if available. (C) HPLC profiles at 190 nm of culture extracts of Pf-5 harboring the *afsA*-like synthase *plfA* with the adjacent oxidoreductase *plfB* (black line) and of Pf-5 host control (gray line) show that paleofuran A (1) and B (2) are only produced upon heterologous expression of the ancient biosynthetic genes. (D) Chemical structures of paleofuran A (1, $n = 1$) and B (2, $n = 3$) with COSY (bold lines) and HMBC correlations (arrows). Paleofuran biosynthesis requires both the AfsA-like synthase PlfA and the oxidoreductase PlfB, which most likely condense a glycerol-derived C3 unit (red) with an acyl carrier protein (ACP)-bound 3-oxo fatty acid, followed by a decarboxylation. (E) Scheme for the chemical synthesis of paleofuran A (1), TFAA = trifluoroacetic anhydride.

Natural products from reconstructed bacterial genomes of the Middle and Upper Paleolithic

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Science,**Ahead of Print**
DOI: 10.1126/science.adf5300