Corrinoids as model nutrients to probe microbial interactions in a soil ecosystem

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Earth's soils are habitats for microbial communities that drive biogeochemical cycling, plant growth, and carbon storage and persistence. The thousands of microbial species living in soil form an intricate web of interactions involving the exchange of molecules produced by different microbes. Understanding in detail how these molecular exchanges occur and how they shape microbial communities may lead to new methods to improve soil health, bioremediation efforts, and better understanding of biogeochemical processes. The overall goal of this research is to gain a deeper knowledge of the microbial interactions that drive soil community structure. However, the high functional and genomic diversity in soil microbiomes has posed a challenge for current microbiology methods to achieve this goal. **This research leverages a model group of key metabolites related to cobalamin (vitamin B_{12}), known as corrinoids, to investigate microbial interactions.**

Corrinoids are shared between different bacterial species, as they are produced by only a subset of the bacteria that use them. They are used for a variety of metabolic processes critical for bacteria in soil and other environments, such as catabolism of particular carbon sources, nucleotide metabolism, and methionine synthesis. Importantly, corrinoids are structurally diverse, and bacteria studied to date in isolation can use only some of the corrinoid forms that are available in their environment. Whether this specificity impacts bacterial growth and metabolism on a community scale had not been examined prior to this work. Based on the inherent specificity of bacteria for particular corrinoids, **the hypothesis driving this work is that corrinoids are keystone nutrients in shaping soil microbial communities**. We tested this hypothesis by examining the effects of corrinoid addition on community composition and function across multiple levels of complexity.

MAJOR RESEARCH FINDINGS

I. Corrinoid dependence is widespread in soil bacteria, but corrinoid biosynthesis is limited to a minority of the community.

We investigated whether corrinoid-producing and corrinoid-dependent microbes coexist in soil from the Northern California grassland field site at Hopland Research and Extension Center (HREC) by assessing the capacity of 503 metagenome-assembled genomes (MAGs) assembled from this site [1] to produce and use corrinoids. To our knowledge, this marks the first genome-resolved view of corrinoid use and production in a soil site.

The most prevalent corrinoid-dependent enzymes in both archaeal and bacterial MAGs are methionine synthase (MetH), the last step in methionine production, and methylmalonyl CoA mutase (MCM), which is involved in propionate and branched-chain amino acid metabolism. Nine additional corrinoid-dependent processes are also represented in the bacterial MAGs; the archaeal MAGs are dominated by MetH and MCM in addition to corrinoid-dependent methyltransferases, used in methanogenesis (Fig. 1A).

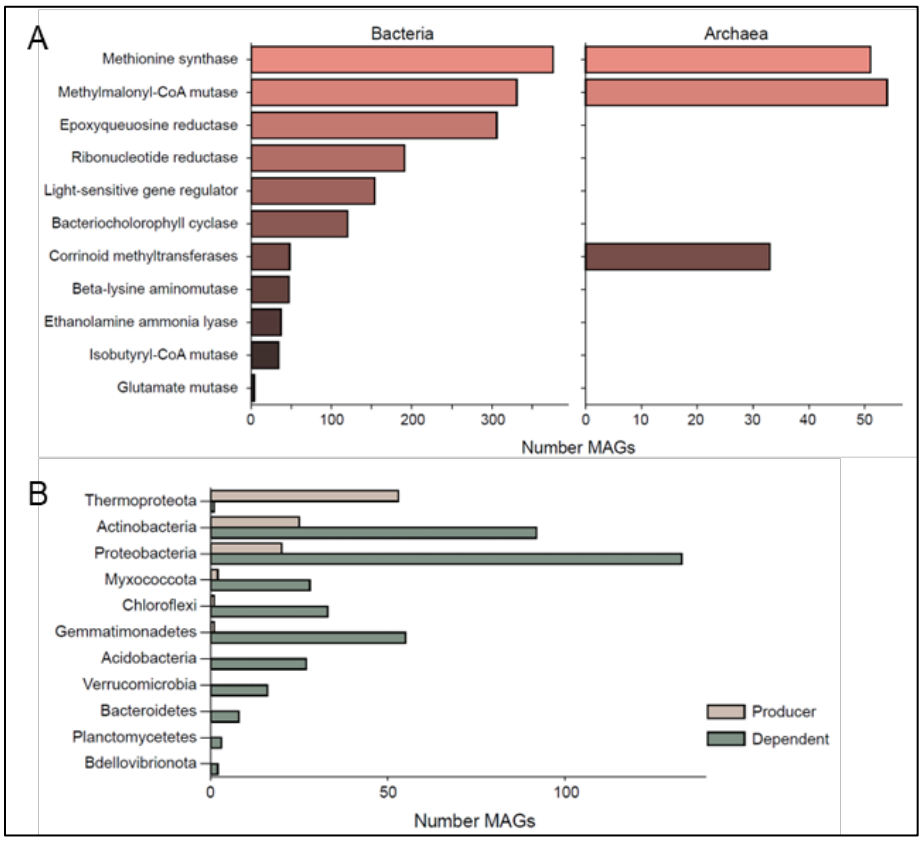

Figure 1. Analysis of corrinoid production and dependence in soil MAGs. A. Corrinoid-dependent processes encoded in soil MAGs split by bacteria (left) and archaea (right). B. The number of MAGs classified as corrinoid producer or corrinoid-dependent is shown for each phylum.

We assessed the extent to which corrinoid-based interactions may occur by predicting which MAGs represent corrinoid "producers" – capable of producing corrinoids for their metabolism – or "dependents" – reliant on external corrinoids for corrinoid-dependent processes, based on our previously established genomic pipeline. The majority of MAGs (79%) were predicted dependents, while 20% were classified as producers and less than 1% (two MAGs) as corrinoid-independent. These classifications are not evenly distributed across phyla; the archaeal lineage Thermoproteota contains the most predicted producers, followed by Actinobacteria and Proteobacteria (Fig. 1B). *This metagenomic analysis provides strong evidence that corrinoid-based metabolism and interactions are highly prevalent in this soil community.*

II. Soil contains a deep reservoir of corrinoids tightly adhered to the soil matrix.

To understand the extent to which corrinoids might be limiting nutrients for microbes in soil, we quantified the total amount of corrinoid in HREC soil. We used our *E. coli* bioassay that can accurately measure corrinoid levels even in crude extracts. Our initial attempts to extract corrinoids from soil by a recently published method [2] failed. This and ten other methods, all using organic solvents, also failed to extract measurable corrinoids. Even when 50 g of soil was spiked with up to 1.5 nM cobalamin before extraction, no corrinoid was detected in methanol-based extracts, suggesting that soil adsorbs corrinoid and the adsorbed corrinoid cannot be recovered effectively by organic extraction. We eventually found that performing multiple successive extractions with an aqueous, phosphate-buffered solution on autoclaved soil recovers the vast majority of corrinoid from soil (Fig. 2). In total, our measurements show that

this soil contains 41 pmol corrinoid per gram, of which 95% is cobalamin based on our LC-MS analysis (Hallberg et al., 2022 [3]). **Thus, we estimate that this soil contains approximately 10^4 corrinoid molecules per microbial cell, a level sufficient to support maximal growth of cultured bacteria**. However, given that cobalamin strongly adsorbs soil, the extent to which these corrinoids are accessible to the microbes that require them remains unclear.

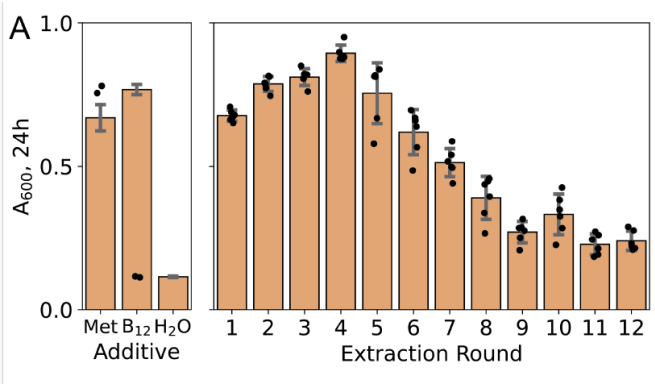

Figure 2. Extraction of corrinoids from soil. The amount of corrinoid present in 12 successive extractions of soil with an aqueous solution containing 700 mM potassium phosphate was measured by an *E. coli*-based bioassay. Controls are shown on the left.

III. Corrinoid structure significantly influences microbial community assembly.

We next examined whether corrinoids that vary in lower ligand structure – known to impact the growth of individual microbes – can elicit changes at the community level. To that end, we established soil-derived enrichment cultures containing a panel of in-house extracted and purified corrinoids (Fig. 3A). We seeded 84 enrichment cultures – six replicates of seven corrinoid conditions in two basal media – with a soil suspension and diluted the cultures 10-fold into fresh medium every 7 days. The results of 16S rRNA gene amplicon sequencing demonstrate that corrinoids impact community composition and assembly. These enrichments stochastically separated into two distinct community types, each dominated by members of different genera, as observed previously by others [4]. The enrichments dominated by

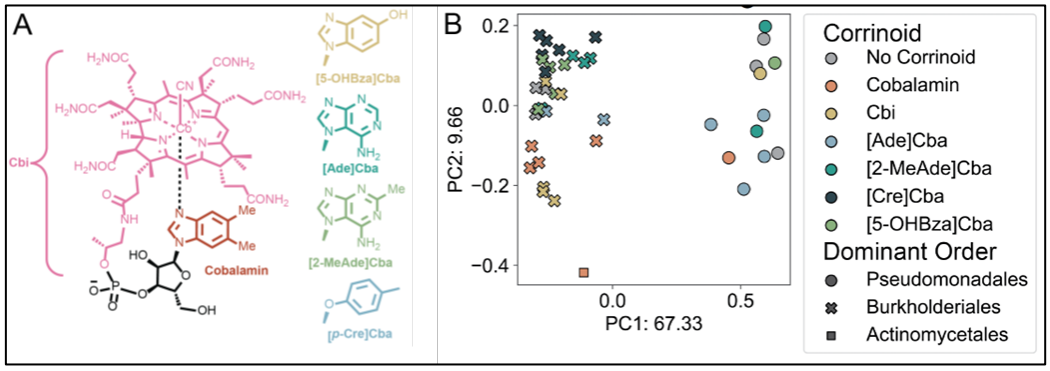

Figure 3. Analysis of soil-derived enrichment culture response to corrinoids. A. Structure of cyanocobalamin (vitamin B_{12}) and lower ligands of related corrinoids used in this study. Cobinamide (Cbi), a precursor, is shown in pink. All other corrinoids differ solely from cobalamin in the identity of the lower ligand. B. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity of enrichments at the order level grouped by corrinoid condition at 2 weeks. The full set of enrichment cultures does not cluster by corrinoid, however Burkholderia-dominated enrichments, when analyzed separately show significant clustering (ANOSIM, $R = 0.2929$, $P<0.05$; PERMANOVA, $R = 1.7148$, $p < 0.05$).

*Burkholderia we*re significantly impacted by corrinoid amendment ($p \leq 0.05$, ANOSIM or PERMANOVA) (Fig. 3B). **This result supports the major hypothesis of this research, namely that corrinoids are key mediators of microbial community assembly.**

This hypothesis was further supported by the results of a separate experiment in which we measured the effect of addition of corrinoids directly to soil. Based on 16S amplicon sequencing, we found that corrinoids significantly alter the community, and that different corrinoids elicit distinct effects on community composition. Principal coordinate analysis of Bray-Curtis dissimilarity matrices of relative abundances by order showed significant differences (ANOSIM and PERMANOVA, $p \leq 0.05$) across corrinoid conditions three days after treatment, with soil amended with cobalamin clustering most closely with unamended soil. Addition of [2-MeAde]Cba, [Cre]Cba, [Ade]Cba, and [5-OHBza]Cba, and to a lesser extent, Cbi, showed profiles distinct from unamended soil and the cobalamin treatment (Fig. 4A). This treatment effect observed at day 3 was largely lost by day 50 (Fig. 4B), suggesting that one-time corrinoid addition is a significant disturbance from which the community can rebound. **Together, these results demonstrate that addition of corrinoids other than cobalamin – the most prevalent corrinoid detected in this soil – significantly, but transiently, impacts soil community composition.** This finding could lead to new applications that develop nutritional amendments to modulate microbial community composition. We are in the final stages of preparing a manuscript describing the results from sections I-III.

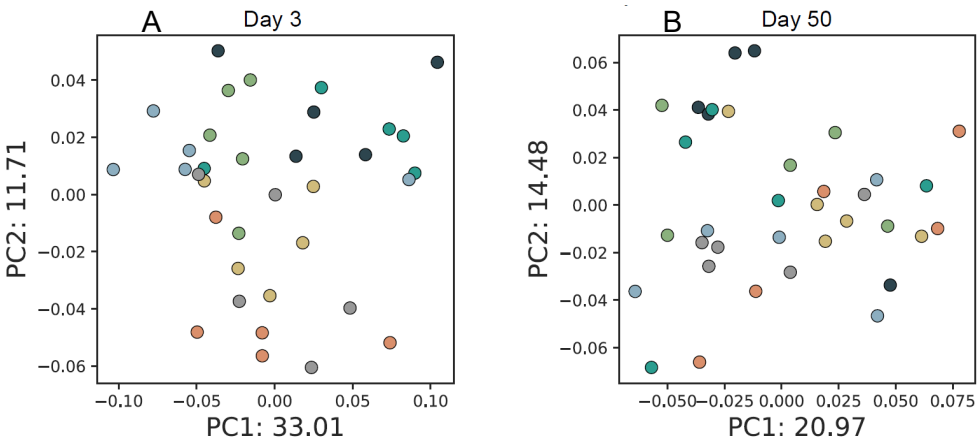

Figure 4. Impact of corrinoids on soil microcosms. Principal coordinate analysis of Bray-Curtis dissimilarity for order-level taxonomy on day 3 (left) and day 50 (right). Each PCoA shows the first two principal coordinates with the percent variance explained. The colors correspond to the corrinoid treatments shown in Figure 3.

IV. Analysis of metagenomes and metatranscriptomes from corrinoid-treated soil-derived enrichment cultures reveals specific functions influenced by corrinoids.

In addition to the role corrinoids serve as enzyme cofactors, corrinoids are also known to influence gene expression. For example, genes involved in corrinoid biosynthesis and corrinoid-independent alternative enzymes are frequently downregulated by corrinoids [5]. Thus, we hypothesized that addition of a corrinoid would influence gene expression, and that metatranscriptome analysis would reveal potential mechanisms of corrinoid-mediated changes

in microbial abundances. We assembled metagenomes and metatranscriptomes from soil-derived enrichment communities following shifts from one corrinoid condition to another. Of the total genes significantly differentially expressed in the metatranscriptome, 12% were annotated as ribosomal proteins, 10% as transporters, and 5% as transcriptional regulators. In three cases, genes for a corrinoid-independent ribonucleotide reductase (RNR) and corrinoid-dependent methionine synthase had significantly lower expression in the presence of particular corrinoids, suggesting that, like many bacteria studied in pure culture, the presence of corrinoids can repress genes for corrinoid-independent counterparts to corrinoid-dependent functions in a community context (Fig. 5).

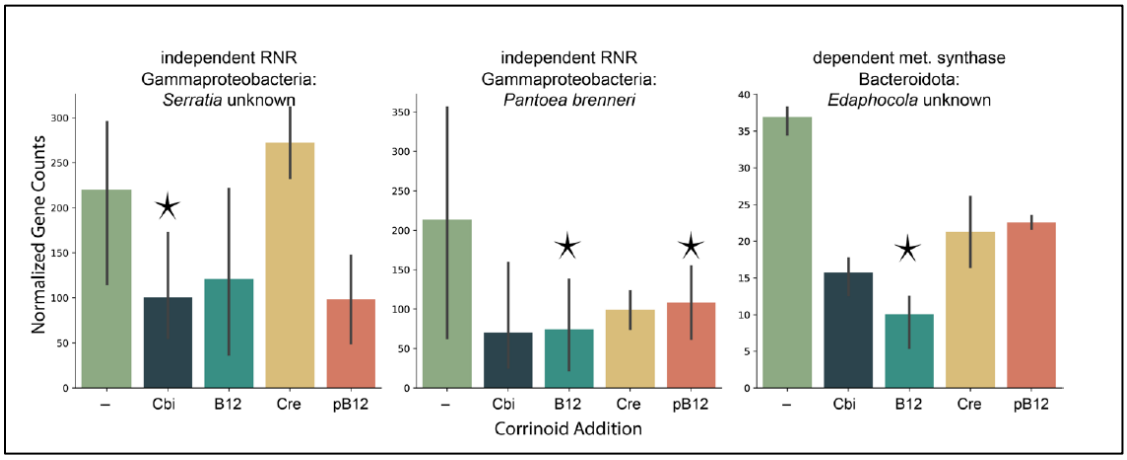

Figure 5. Differential gene expression of corrinoid regulated processes. Each graph shows a gene that showed significant differential expression in at least one corrinoid treatment condition compared to the unamended enrichment 3 hours after corrinoid addition. The genes shown are corrinoid independent ribonucleotide reductase (RNR) and a corrinoid-dependent methionine synthase. Stars indicate statistically significant differences from the unamended condition.

V. Analysis of a newly generated soil bacterial culture collection reveals metabolic compatibility between corrinoid producers and corrinoid-dependent bacteria.

Despite numerous recent advances that use sequence-based approaches to investigate microbial community functions, experimental analysis of cultured microbes remains the definitive approach for determining function. We therefore sought to determine the ecological roles of microbes with respect to corrinoids by isolating and characterizing soil bacteria from a single study site at HREC. We performed the limiting dilution method in 384-well plates containing media with one of six different corrinoids (Fig. 3A) or no corrinoid. After extensive curation, our collection contains 161 axenic bacterial isolates comprising four phyla and 31 genera, of which **37 isolates are considered to be novel species** (<98.6% 16S sequence identity).

We applied growth-based assays and the *E. coli* corrinoid detection bioassay described above to characterize each isolate as a corrinoid producer, dependent, or independent. The majority were producers, and among these, we extracted and analyzed corrinoids from 11 isolates. All were shown to produce cobalamin, consistent with cobalamin being the dominant corrinoid in this soil ([3] section I). Further, despite the fact that the isolates were cultured on different corrinoids, all could use cobalamin and most preferred it, including the example shown in Fig. 6. (Results from sections V and VI are described in a preprint: Alvarez-Aponte et al. [6].)

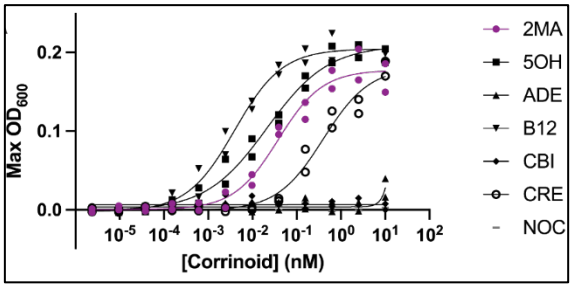

Figure 6. Corrinoid dependence in the isolate collection. A representative corrinoid dose-response curve for isolate 2MA_012 is shown. The corrinoid in which the isolate was recovered, [2-MeAde]Cba (abbreviated as 2MA), is shown in purple; cobalamin (B12) is considered the most preferred because it is the corrinoid that supports growth at the lowest concentrations.

VI. Phylogenetic analysis of corrinoid ecological roles reveals that corrinoid metabolism is conserved in a subset of genera, enabling taxonomy-based metabolic predictions.

One of our goals is to be able to predict corrinoid metabolism based on genome sequence or taxonomy to the extent possible. To that end, we analyzed our previously published corrinoid metabolism classifications for over 11,000 bacterial species and overlaid the results on a phylogenetic tree to distinguish between the competing hypotheses that 1) corrinoid production, dependence, and independence show strong phylogenetic trends, enabling predictions of corrinoid metabolism based on taxonomy, or 2) corrinoid metabolism categories are phylogenetically interspersed, making it impossible to infer corrinoid-related ecological roles based solely on taxonomy. Our results partially support both hypotheses. We found that the corrinoid producer, dependent, and independent traits are conserved in 47 out of 85 genera. Thus, for these genera it is possible to predict corrinoid metabolism with high confidence. Indeed, nearly all of our experimental results align with these phylogenetic predictions [6].

VII. Genomic and phenotypic analysis of a novel soil bacterium shows dependence on corrinoids for core functions.

We investigated more deeply the general characteristics and corrinoid-related features of one isolate, a novel species of the genus *Pedococcus*, phylum Actinobacteria, named 5OH_020. To our knowledge, this was the first reported organism isolated on the corrinoid [5-OHBza]Cba, which is commonly produced by methanogenic archaea. Phenotypic characterization showed it is corrinoid-dependent. This is consistent with our analysis of its genome sequence, which showed it lacks corrinoid biosynthesis genes and encodes two corrinoid-dependent enzymes (published in Green et al., 2022 [7]).

TRAINING OF SCIENTISTS AND DISSEMINATION OF RESEARCH RESULTS

Through the activities described above, a postdoc, two PhD students, and an undergraduate student received training in research and related activities including writing and giving presentations. All of the trainees attended conferences and most presented their work in posters and/or talks. Further, a graduate student engaged in recruitment of new scientists from underrepresented backgrounds, thus contributing to the development of a future diverse workforce.

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