**Codon bias, nucleotide selection, and genome size predict *in situ* bacterial growth and expression in rewetted soil**

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ABSTRACT

Trait-based frameworks are a staple in ecology for assessing life-strategies and community dynamics, and have been widely used in the field of microbial ecology for disentangling complex community dynamics. Despite the proliferation of microbial genomics, how growth and life-strategies are encoded in soil bacterial genomes remains unclear. In soil bacteria, traits such as codon usage bias and genome size have been linked to growth—often through indirect measurements. Here, we utilize metagenome-assembled genomes in combination with metatranscriptomics and 18O-water stable isotope probing to track genomic traits associated with the activity and growth of soil microorganisms over the course of one week following soil rewetting after a prolonged dry period. After rewetting, we found that the predominant traits of fast-growing bacterial taxa was high levels of codon bias in ribosomal protein genes and lower GC content. Similarly, we found that high levels of codon bias corresponded to stronger upregulation of ribosomal protein genes. Nucleotides requiring less energy to produce were more common at synonymous sites in fast-transcribing taxa, and were weakly correlated to faster growth. We also observed higher growth rates in bacteria with smaller genomes, indicating the important role reduced genome size may play in bacterial growth in response to sudden change in nutrient availability. Together, these results provide *in situ* evidence that genomic characteristics dictate growth rate and transcription in soil, and provide insight into the fitness advantage of these traits in soil bacteria.

INTRODUCTION

Rewetting is an ecologically important phenomenon where microbial traits associated with growth and activity may play an important role for stabilizing soil carbon (C). In ecosystems characterized by dry and wet seasons, the first rain event following the dry season results in the rapid mineralization of soil carbon (known as the Birch Effect) (1), which accounts for a large portion of the annual soil C loss from respiration (2). The rapid stimulation of microbial activity is driven by both the release of water stress as well as an influx in the bioavailability of carbon compounds sourced from osmolytes (3–5), microbial necromass (6, 7), slaking of microaggregates (8), and increased connectivity in the soil matrix (9, 10). The microbial response to rewetting therefore has important consequences for the fate of soil organic carbon. However, which traits control the growth and activity of this response remains unknown.

Trait-based frameworks have long-been used in ecology to help understand complex community structures (11, 12), and this approach has gained considerable attention in microbial ecology (13, 14) where advancements in sequencing technology have expanded the ability to probe previously unexplored microbial community structures. The advantage of studying soil bacteria using traits is two-fold. First, since traits are the product of evolutionary forces, understanding the distribution of traits answers fundamental questions concerning the evolution and selection for certain functions (15). Second, considering the complexity of microbial community datasets, traits serve as valuable metrics for assessing community dynamics across scales (16, 17) and with ecosystem function (14). Traits associated with growth are of particular importance, as growth is often central in life-strategy frameworks (14, 18, 19), functional metrics such as carbon use efficiency (20), and understanding changing community dynamics.

Trait-based analyses using genomic data can generally be separated into two broad categories: functional traits (those which describe specific function) and genomic traits (those which do not describe a specific function; e.g., GC-%, genome size, codon usage, etc.). Functional traits are often observed through functional gene composition and the expression of these genes under a set of environmental conditions. During growth, expression of specific genes varies considerably depending on function (21) and environmental constraints (22). Ribosomal genes are highly upregulated during growth, and the number of rRNA gene copies in a genome positively correlates with maximum growth rate. Although the link between rRNA levels and both growth and activity (23) are inconsistent, fast-growing bacteria tend to have a greater number of rRNA gene copies – a useful trait dimension used in microbial ecology trait-based frameworks (18, 19, 24). Although specific functional genes have been shown to be associated with growth in soil microbial communities (25), this is often under a specific set of conditions that could favor specific nutrient acquisition strategies or metabolic pathways, and therefore might not be predictive outside of that environment.

However, genomic traits (such as genome size, nucleotide frequency, and codon usage) may provide more generalized metrics predictive of activity and growth. For example, transcription and growth rate may be strongly determined by codon usage. A high alignment between gene codon frequencies and the anticodons of the tRNA pool increases the rate of both transcription and translation (26)—commonly referred to as codon optimization. The level of codon optimization of a transcript has important consequences for translation; affecting the rate of elongation, protein folding, initiation, and termination (27–29). Codon usage is also highly predictive of mRNA abundance, as a high abundance of optimized codons generally increases mRNA stability (30, 31) and has been independently shown to relate to higher levels of transcription (32, 33). Codon bias describes codon redundancy in the genetic code for a particular gene or genome, and generally correlates to the degree of codon optimization (34). High levels of codon bias in ribosomal proteins is associated with rapid growth in bacteria (35, 36) and has been increasingly used as a predictor of maximum growth rate (37, 38).

Outside of an intrinsic relationship between GC content and codon usage (26), there appears to be no relationship between doubling time and GC content in bacteria (39), and the link between GC content and growth in soil bacteria specifically remains unclear. However, the cost of nucleotide synthesis has been shown to influence transcription, where rapidly transcribed genes often use cheaper nucleotides at synonymous sites to reduce the cost of transcription (40). Since guanine requires more energy to produce than cytosine, and adenine more energy than uracil, genes with cytosine and thymine at synonymous sites tend to be transcribed faster than genes with guanine and adenine (40). At nonsynonymous sites, there is an inverse relationship between nucleotide cost and the cost of their encoded amino acid sites (40)—such that a higher frequency of more expensive nucleotides at synonymous sites is associated with higher levels of expression.

Genome size is also thought to influence growth; however, the relationship is less clear than codon usage or rRNA copy number. In oligotrophic marine environments, extremely small genomes are thought to arise in response to nutrient limitation to curb the cost of reproduction (termed genomic streamlining; 30), and often have slower growth rates than copiotrophs with larger genomes (18). Although streamlined genomes have been documented in soils (42), soil bacterial genomes generally tend to be large relative to other systems (43)—a potential result of the increased metabolic diversity required to utilize complex substrates (44). It’s been hypothesized that these large genomes might come at the expense of growth rate (45), as the increased energy required for reproduction in large genomes may slow growth. However, the evidence regarding the relationship between genome size and growth rate in soil bacteria is inconclusive (46, 47), necessitating further investigation.

In soils, a major hurdle in assessing how traits relate to growth rate has been our inability to effectively measure these traits *in situ*. Much of the work linking these traits to growth has been conducted using soil isolate data where the growth rate is often assessed from pure culture; though traits associated with microbial growth on media might not translate to free-living microbes in their natural environments (47). Further, the question of how these traits relate to growth during real-world phenomena remains.

Recent methodological and technological advancements have created new opportunities to track microbial growth *in situ*. In quantitative stable isotope probing (qSIP), the incorporation of added stable isotopes into DNA tracks the growth rate of microbes based on sequence (48). This approach has enabled valuable insights into metagenomic features and metagenome-assembled genomes (MAGs) associated with growth (7, 25, 49), and shows promise for linking measured microbial traits and growth *in situ*. In this study, we pair metagenomic, metatranscriptomic, and qSIP data to assess how genomic traits are associated with activity and growth during the rewetting of seasonally dry soil in a Mediterranean grassland. Specifically, we investigate how traits such as genome size, nucleotide selection, codon usage, and ribosomal protein gene frequency relate to growth and transcription.

METHODS

The field study was located at Hopland Extension and Research Center (HERC), Hopland, California, USA (39.00092, -123.07962), which resides on the ancestral home of the Shóqowa and Hopland people. The region features a Mediterranean climate of warm, dry summers and cool, wet winters. *Avena barbata* (wild oat grass) dominated the studied field site. This study consisted of 16 plots 3.24 m2, with rainout shelters constructed around each plot, either allowing full or 50% precipitation. A full description of the field experimental set-up can be found in *[Rina’s paper]*

Soils were collected in September 2018 at the end of the dry season and 25 days before the first rainfall of the wet season. At the time of collection the soil gravimetric water content was approximately 3%. Topsoil samples (0-15 cm, roughly 0.5 m3) were taken from 8 randomly selected plots (4 full and 4 reduced precipitation). Samples were transported to Lawrence Livermore National Laboratory where soil from each field plot was separately homogenized and sieved (2 mm) to remove large rocks and roots.

*Wet-up experiment*

Details of the H218O labeling can be found in Nicolas et al. 2023 and Sieradzki et al. 2022 (49, 50). In brief, soils from each of the 3 plots were separated into 12 microcosms containing 5 g of soil each, for 48 samples in total. Soils were brought to 22% gravimetric water content either with natural abundance water or with 98-at% H218O. Three replicates each of labeled and unlabeled samples from each plot were destructively harvested at 0, 3, 24, 48, 72, and 168 h post wet-up. Samples were immediately frozen in liquid-N2 and stored in a -80°C freezer.

*Metagenomic qSIP*

A full description of the DNA extraction, sequencing, assembly, binning, and qSIP calculations can be found in Sieradzki et al., 2022. DNA from 3 plots per treatment were extracted in triplicate using a phenol chloroform extraction and then pooled. Samples were then spun on an ultracentrifuge in a cesium chloride solution to create a density gradient. The contents of the ultracentrifuge tube were then separated into 36 fractions, each of which was assessed for density and DNA concentration. Fractions were combined into 5 groups along the density gradient, purified and concentrated, and then sequenced on an Illumina Novaseq platform.

Adapters were trimmed and reads were QC filtered using bbduk (51) and Sickle (52). QC filtered reads were assembled into contigs using MEGAHIT (v1.2.9; (53)), binned with MaxBin 2.0 (54) and MetaBAT2 (55), and refined with MetaWrap (56). We combined this set of MAGs with another set binned from a study at a nearby site at HERC (25) and dereplicated MAGs using dRep, which also assesses MAG completeness (57). Reads from each fraction were then mapped against the set of MAGs using BBMap (51). The mapping of reads from each fraction was used to calculate atom fraction excess (AFE) for each MAG—which can be used as a metric of growth (48). Open reading frames were identified using Prodigal (59) and annotated against the KEGG database (60) using DRAM (58).

*RNA Extraction and sequencing*

RNA was extracted from 4 samples from each precipitation treatment using a RNeasy PowerSoil Total RNA kit (Qiagen) according to manufacturer instructions. Extracted RNA was treated with RNase-free DNase (Qiagen) and stored at -80°C. RNA concentration was determined using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) and quality was assessed using a Nanodrop One Spectrophotometer (Thermo Scientific Invitrogen). Samples were sent to the Joint Genome Institute (JGI; Berkeley, California, USA), for sequencing. Paired-end 2 x 151 bp libraries were sequenced on an Illumina NovaSeq platform. Generated RNA sequences were prepared for metatranscriptomic analyses using the JGI Integrated Microbial Genomes (IMG) pipeline v.5.1.5 (61) and can be found under the GOLD project ID Gp0612223. IMG assemblies are not included in this analysis, and a more detailed description can be found in the data release (DOI: and citation).

*Metatranscriptome analysis*

Raw reads were downloaded from the JGI genome portal and were QC filtered using bbduk (51) and Sickle (52). BBmap was then used to map QC filtered reads to a concatenated reference MAG file (minid=0.95). Counts of reads mapping to DRAM-annotated genes were identified using FeatureCounts (62). Expression and normalization counts of mapped transcripts for each annotated gene (excluding rRNA genes) were generated using DESeq2 (63) using default parameters. Differential expression was calculated as the log2-fold change versus the gene expression of the control group.

*Genomic traits*

Genomic traits were calculated using custom scripts written in Python (v 3.8.2), which can be found at <will insert Github link before preprint>. To capture effective genome size across MAGs, we assumed genome size was a function of completeness—for medium to high quality MAGs we multiplied the total assembled base pairs by genome completeness. For each gene in each MAG we calculated the effective number of codons (ENC’) as described by *Novembre 2002* (64)—which uses background nucleotide frequencies to assess levels of codon bias. ENC’ values were calculated for each gene. Lower ENC’ values in a gene represent a fewer number of codons used in that gene, and therefore higher codon bias.

To determine the relative bias of ribosomal protein genes, we calculated ΔENC’ (36):

Where ENC’all is the effective number of codons for the whole genome, and ENC’ribo is the effective number of codons of the ribosomal protein genes. The ΔENC’ values therefore represent the degree of bias in ribosomal protein genes relative to the rest of the genome, where higher ΔENC’ values represent greater relative codon bias.

Nucleotide frequencies and skews were determined at synonymous and nonsynonymous substitution sites on ribosomal protein genes according to nucleotide degeneracy detailed in Chen et al. 2016. Synonymous site nucleotide frequencies were calculated from fourfold degenerative sites, and nonsynonymous frequencies from sites where any substitution would change the encoded amino acid. GC and AT skew were calculated as:

And:

The cost of amino acid synthesis—measured as the total number of phosphate bonds used for synthesis—was derived from Akashi & Gojobori 2002 (65).

*Analysis and model selection*

All statistical analyses were conducted in R version 4.2.1 (66) and visualized with the ggplot2 package (67). MAGs were grouped by transcriptional response according to when each MAG was most transcriptionally active, as measured by the total number of upregulated genes. We divided these responses into 4 groups: early responders (most active 3 h post wet-up), middle responders (most active 24, 48, 72 h), late responders (168 h), and sensitive (down-regulated post wet-up). Differences in genomic traits between transcriptional response groups were determined using an analysis of variance (ANOVA). Tukey’s HSD was used for pairwise comparison between groups. Relationships between traits and growth rates were determined using multiple linear regression. Model comparison was conducted using Akaike information criteria (AIC)—with a threshold of -Δ 4, indicating an improvement of a model with the addition of a parameter.

RESULTS AND DISCUSSION

Determining the relationship between life-strategies and traits is a central focus in soil microbial ecology—yet it has often been a struggle measuring both the metrics of life-strategies as well as the traits themselves. Many trait-based frameworks include response-metrics such as growth rate and transcription that, until recently, were not possible to study *in situ*. Further, genomic traits were previously difficult to study directly due to sequencing and computational constraints. To help close this gap between traits and life strategies, we assessed the relationship between genomic traits of metagenome assembled genomes (MAGs) and their *in situ* growth rates as determined through quantitative stable isotope probing (qSIP), as well their transcriptional responses as determined by mapping metranscriptomes back to MAGs.

We focused on assessing the relationship between growth rate and genomic traits most often associated with growth in the literature—specifically, codon bias, genome size, and GC content. Atom fraction excess (AFE) values were used as the response variable, with higher levels of isotopic enrichment being used as index of growth. Since we had several measures of codon bias, we ran an initial set of model comparisons to assess which metric of codon bias best predicted growth rate so as to reduce the number of redundant variables. As indicated by R2 and AIC values, codon bias of the ribosomal proteins (ENC’ribo) better predicted AFE values than the codon bias of the whole genome (ENC’) or the codon bias of ribosomal proteins relative to the whole genome (ΔENC’). We therefore used ENC’ribo in model comparisons moving forward. In comparisons between traits, ENC’ribo predicted growth better than estimated genome size, GC content, or ribosomal GC content (Supplemental Table 1). This demonstrates that codon bias can effectively predict growth not only in pure cultures, as previously established, but also in complex soil microbial communities responding to a natural pulse phenomenon. Although no broad-scale analysis of codon usage bias has been conducted in soils, previous work has found that in soils with lower C, higher pH, and less rainfall, there tends to be a higher redundancy in the codon usage in the bacterial population (68). High codon bias could be a fundamental life-strategy trait for soil communities experiencing transient water and nutrient availability, enabling quicker responses to sudden pulse events. This highlights the importance of codon usage as a critical trait-dimension when considering the adaptation of soil bacteria to changing precipitation patterns in the context of climate change.

Although ENC’ribo explained the most variance of any single metric, a multiple regression model incorporating ENC’ribo, genome size, and ribosomal GC content performed better than ENC’ribo alone (Supplemental Table 1, R2 = 0.49). Ribosomal GC content was negatively correlated with growth rate when codon bias was high (i.e., lower ENC`; Figure 1b). We believe this could be attributed to the higher metabolic cost of GC base pairs vs AT base pairs. We found that early growth at 24 h was most correlated with codon bias and GC content, and that the influence of genome size increased over time (Figure 1a).

Smaller genomes were associated with higher growth rates at later time points, particularly 72 and 168 h post wet-up. Smaller genomes may reduce the cost of reproduction, and thus might be key in responding to sudden pulses of nutrients. This idea compliments the hypothesis that large genomes with metabolic diversity come at the expense of growth rate (45), and poses a trade-off where smaller genomes may therefore be better adapted to pulse-driven systems where carbon is limited. This would also explain why smaller genomes are highly prevalent in arid and carbon poor soils (68–70), since the ability to quickly respond to the momentary availability of water and nutrients may be fundamental to survival and persistence.

This contrasts with bacteria in marine systems, where small streamlined genomes tend to have slower growth rates (41). This inconsistency between growth and genome size is potentially a reflection of fundamentally different life-strategies that both leverage simplified genomes. In marine environments, reduced complexity and a high surface area to volume ratio are a response to nutrient limitation.Combined, these traits reduce the total cost of replication, while increasing the likelihood of capturing dissolved nutrients—with less of an emphasis on rapid growth. In contrast, in this soil system, we suggest that the lower cost of replication associated with smaller genomes might allow for faster growth in response to pulses of nutrients entering the environment. In this way, the reduced cost of smaller genomes might serve both strategies well, and the relationship between genome size and growth rate might rely on life-strategy. Assessing the impact of genome size on growth therefore likely requires putting genome size in the context of other traits associated with growth, such as number of rRNA gene copies or codon bias.

High codon bias, in addition to being a predictor of growth, has also been shown to increase gene expression. Yet, it is unclear the extent to which the relationship between codon bias and growth can be attributed to faster rates of transcription. Although it is not possible to directly measure the contribution of higher transcription vs more efficient translation to growth using these data, we can assess the influence of codon bias on gene transcription and the relationship between transcription and growth. We separated transcriptional responses into 4 temporal categories according to their peak transcriptional responses—which were based on the proportion of expressed genes that were significantly upregulated at each timepoint (see methods). Early responding taxa had a higher level of codon bias (Fig 2a), and a greater upregulation of ribosomal protein genes early on (Fig 2b). The transcriptional responses followed a logical pattern with growth rate, where early transcribing taxa were better represented during early growth and late transcribing taxa were more often growing later (Supplemental Figure Xa). However, we did not find a strong relationship between the expression of ribosomal protein genes and growth rate (Supplemental Fig Xb). These results demonstrate that although the transcription of ribosomal proteins may be necessary for growth, it cannot be reliably used as a quantitative proxy for growth rate. This corroborates prior research indicating rRNA serves as a poor metric for activity (23) and highlights important limitations in the use of metatranscriptomics for quantitatively assessing certain metrics between organisms in microbial communities.

*Nucleotide selection*

Nucleotide cost was linked to transcriptional response, with early responding organisms displaying a higher AT (A-T/A+T) and GC skew (G-C/G+C) at synonymous sites for ribosomal protein genes (Fig 3 A&B). Our findings align with results from Chen et al. (2016) (40), which demonstrated that the lower cost U and C at nonsynonymous sites were associated with increased gene transcription. However, this relationship did not directly correlate with growth rate. GC skew of synonymous substitutions on ribosomal protein genes was weakly negatively correlated to growth (R2 = 0.12, p < 0.01; Fig 3C) and AT skew was not significantly negatively correlated with growth (R2 = 0.01, p = 0.06; Fig 3D). These results underscore the disparity between traits affecting transcription and those having a more pronounced influence on growth.

There was no relationship between nonsynonymous nucleotide usage and transcription (Fig 3 A&B). We found a slight positive relationship between GC skew and growth (Fig. 3E), and a surprisingly positive relationship between AT skew and growth (Fig. 3F, R2 = 0.22, p < 0.01). This could potentially reflect the inverse relationship between nucleotide and amino acid cost. More expensive nucleotides tend to encode less energetically expensive amino acids (40), and this relationship could reflect this cost conservation. However, amino acid cost was not associated with growth (Supplemental Fig X). When we examined the amino acid composition as related to nonsynonymous substitutions, we found that AT skew most closely correlated with the abundance of lysine (encoded for by AAG and AAA)—yet it is unclear what specifically about lysine would be causing this relationship. Lysine is not notably cheaper to synthesize and has elemental ratios similar to other amino acids. This relationship may be due to features of the ribosomal protein structure, the analysis of which is beyond the scope of this study. Alternatively, the relationship could also be caused by correlations between AT skew and other genomic features, especially GC content (linear regression R2= 0.46). Further, the addition of nonsynonymous AT skew to the model outlined in Supplemental Table 1 did not explain much more variation compared to the base model.

*Limitations and future directions*

Codon bias, GC content, and genome size may be particularly relevant in pulse-driven systems, but it is still unknown how they may relate to growth in soils where water and nutrient supply are more abundant and consistent. For example, the frequency of cycles of drying and rewetting are known to influence microbial community responses to rewetting (71). Although this may be tied to shifts in community composition, it does not preclude plasticity in the physiological response of individual taxa. Frequency of rain events could potentially influence the response of these traits to rewetting events.

How genomic traits relate to growth in environments where change is gradual is also not well understood. A complete picture of the relationship between genomic traits and activity of soil bacteria requires assessing these relationships across environments and stimuli. Although the growth rates of soil bacteria under stable conditions are often difficult to measure—recent advancements in isotopic vapor labeling allow for the study of bacterial growth rates without the addition of water or nutrients (72, 73) and provides a promising avenue to study growth in environments without perturbation.

It is essential to underscore the potential of these traits for predicting growth and transcription. Even though the most predictive traits could account for approximately 50% of the variance in growth, it's important to acknowledge the limitations in terms of sample size and the potential for bias resulting from metagenomic binning. Consequently, the extent of predictive power these traits possess for forecasting growth in response to real-world phenomena remains uncertain; additional studies and data will offer more clarity on these relationships. These considerations should guide us when making conclusions about the growth potential of microbial communities.

*Conclusion*

These results highlight trait dimensions that correspond with growth rate and bacteria in soil microbial communities. Specifically, we found that faster growth was related to high codon usage bias, lower GC content, and smaller genome sizes. Transcription was associated with nucleotide synthesis cost as well as codon usage bias. These relationships offer important perspective for examining the broad scale ecological distribution of these traits, as the importance of short-term responses may play a vital role in systems characterized by pulses of nutrient availability.

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Figure 1. *Atom fraction excess (AFE) values as they related to effective number of codons in ribosomal protein genes (ENC`) and estimated genome size (Mbp) at each time point* ***(A)*** *as well as the GC content of ribosomal protein genes at 72 h post wet-up, with color indicating ENC values* ***(B).*** *Lower ENC values indicate a higher level of codon bias.*A diagram of ribosome protein

Description automatically generatedA graph of a graph showing a number of red dots

Description automatically generated with medium confidence

*Figure 2:* ***(A)*** *Codon usage bias of ribosomal protein genes colored by transcriptional response type.* ***(B)*** *Ribosomal protein gene expression over time, averaged by response type* 

A collage of diagrams

Description automatically generated

*Figure 3: Nucleotide skew as it relates to transcriptional responses and growth rate. The synonymous and non-synonymous GC (****A****) and AT* ***(B)*** *skew by transcriptional response, with color indicating site-type. Growth rate as a function of synonymous nucleotide skew for GC-skew* ***(C)*** *and AT skew* ***o)****, and nonsynonymous GC* ***(E)*** *and AT skew* ***(F)****.*