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The soil microbial methylome: A tool to explore the role of epigenetic memory in driving soil abiotic legacy effects

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ABSTRACT

Epigenetics is a phenomenon whereby a stable hereditable change in gene expression can occur without changing the DNA sequence. DNA methylation (the addition of a methyl group to specific nucleotides in specific DNA motifs) is the most studied epigenetic mechanism and is widely observed in both eukaryotic and prokaryotic cells. We hypothesise that the soil methylome may play an important role in the manifestation of soil abiotic legacy effects, whereby temporary exposure of soil microbial communities to particular environmental conditions influences future soil microbial function. These abiotic legacy effects are important because they underpin the delivery of key ecosystem services in response to global environmental change. Third generation long-read sequencing technologies, such as Pacific Bioscience Single-Molecule Real-Time sequencing (SMRT-seq) and Oxford Nanopore sequencing provide an opportunity to study methylome heterogeneity in complex microbial communities. The simultaneous measurement of epigenetic, transcriptional, and microbial community composition changes may lead to the development of biomarkers of historic environmental stress and a greater understanding of the role of the soil methylome in the resilience of soil microbial communities to future environmental perturbations. It is therefore timely to add the meta-epigenetic layer to the multi-omics analysis of the soil microbiome to advance our understanding of soil abiotic legacy effects.

1. Soil abiotic legacy effects

The temporary exposure of soil to particular abiotic (non-living) conditions, such as elevated temperature or moisture, has a long-term impact on the subsequent delivery of ecosystem functions by the soil microbial community after these conditions have ceased (Adekanmbi et al., 2022; Canarini et al., 2021; Meisner et al., 2018). We refer to these phenomena as soil abiotic legacy effects. Soil abiotic legacy effects are important in the context of environmental change. Exposure of a soil to fertilizers and pesticides, extreme temperatures, or prolonged drought or flood may affect its future ability to perform functions that support key ecosystem services such as nutrient cycling (Cavagnaro, 2016), crop yields (Nguyen et al., 2018), greenhouse gas emissions (Xu et al., 2020, 2021), and resisting exotic plant invasions (Meisner et al., 2013).

2. Biological mechanisms currently used to explain legacy effects

Until now, soil legacy effects have largely been explained by shifts in soil physical or chemical properties, including nutrient or substrate availability (Pold et al., 2017), or by shifts in the composition of soil microbial communities (Cordero et al., 2023; Jurburg et al., 2017). It has been suggested that microbiome data can be used to predict future ecosystem processes (Correa-Garcia et al., 2022). However, it is already widely acknowledged that soil microbial community composition alone (the soil metagenome) cannot fully explain soil microbial functions because gene expression levels depend on environmental conditions (Jansson and Hofmockel, 2018) and multiple post-transcriptional modifications ultimately influence the delivery of functions (Nachtergaele and He, 2017). Nevertheless, soil microbial transcriptomics has been applied to quantify the delivery of microbial functions under current environmental conditions (Malik et al., 2020; Roy Chowdhury et al., 2019). But the ability to predict which genes may be expressed in the future, in response to different environmental conditions, is currently beyond our reach because some legacy effects may be stored in epigenetic memory rather than being due to the genome, the transcriptome, or post-transcriptional modifications.

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3. What is epigenetics?

"An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger et al., 2009). Differentiation of cells and tissues in multicellular eukaryotes is maintained through epigenetic mechanisms, including DNA methylation, histone modifications, changes in interphase chromatin accessibility, long non-coding RNAs, and others (Allis and Jenuwein, 2016). The role of epigenetics have been extensively studied in mammalian development (Reik et al., 2001), ageing (Pal and Tyler, 2016), and cancer (Dawson and Kouzarides, 2012). It also plays an important role in a wide range of other processes, such as phenotype determination in insects (Alhosin, 2023), adaptation to pollutants in a range of animals (Head, 2014), and stress response in plants (Akhter et al., 2021). Soils contain a rich biodiversity of both bacteria and fungi (Anthony et al., 2023) but, importantly, many of the eukaryotic epigenetic mechanisms are not applicable to prokaryotes, which don't have histones and nucleosomes. However, DNA methylation (the addition of a methyl group to specific nucleotides in specific DNA motifs) is widely present in both fungal (eukaryotic) and bacterial (prokaryotic) cells, which dominate the soil microbiome (Bahram et al., 2018). There are striking differences in methylation mechanisms and functions between eukaryotes and prokaryotes. We provide here an introduction to bacterial and fungal DNA methylation, but refer readers to Sánchez-Romero and Casadesús (2020) and (Nai et al., 2021), respectively, for a more comprehensive explanation.

3.1. Bacterial DNA methylation

The majority of bacterial DNA methylation occurs at N6-adenine (6 mA) (Beaulaurier et al., 2019). Most prokaryotic DNA methylation is involved in defence rather than in differentiation (Gao et al., 2023; Seong et al., 2021). Like in eukaryotes, prokaryotic DNA methylation is catalysed by DNA-methyltransferases, which recognise and modify specific DNA motifs (Seong et al., 2021). However, the DNA-methyltransferases (and the correspondent methylation motifs) vary widely between bacterial species (Blow et al., 2016). Most of the bacterial DNA-methyltransferases are matched with endonucleases, which cut at the same DNA motifs, if it is un-methylated (Roberts et al., 2003). Such combination of matched DNA-methyltransferases and endonucleases are called Restriction-Modification systems. The main cellular function of Restriction-Modification systems is defence, since it allows for selective cleavage of foreign DNA (Seong et al., 2021). However, Restriction-Modification systems may also play a role in other cellular processes, such as virulence or the rate of mutagenesis (Vasu and Nagaraja, 2013). At the same time, some bacterial DNA-methyltransferases lack the matched endonucleases. They are called "orphan" or "regulatory" DNA-methyltransferases (Blow et al., 2016; Seong et al., 2021). Although these orphan/regulatory bacterial DNA-methyltransferases do not contribute to cellular defence, they can affect bacterial gene expression, potentially shaping long-lasting heritable phenotypes (Anton and Roberts, 2021).

3.2. Fungal DNA methylation

Fungi possess the ability for multicellular organisation, which requires epigenetic mechanisms to establish different cell types from the same genotype (Jeon et al., 2015; Madhani, 2021; Nagy et al., 2020). Epigenetics has been extensively studied in some fungi species (e.g. *Saccharomyces cerevisiae* or *Neurospora crassa*) which were used as reference models for eukaryotic cellular biology (Aramayo and Selker, 2013; Chou et al., 2023; O'Kane and Hyland, 2019). Most of the initial epigenetic studies in fungi were focused on histone modifications, rather than on methylation. It was even considered that *S. cerevisiae* and some other fungi with predominantly asexual reproduction had lost part of their DNA methylation machinery (Zemach et al., 2010). However, it was later shown that DNA methylation is present in *S. cerevisiae* (Pai et al., 2022). Moreover, a recent survey of multiple fungi species has shown that fungal genomes contain a diverse set of DNA methyltransferases, and the dominant DNA methyltransferases differ between major fungal clades. A major function of DNA methylation in fungi involves silencing of the transposable elements (Nai et al., 2021). In contrast to prokaryotes, in certain environmental conditions fungi can commit to meiosis, which has profound effects on the DNA methylation patterns (Barry et al., 1993; Grognet et al., 2019; Hartmann et al., 2021; Jovanska et al., 2024). Another distinct feature of fungi is the existence of different lifecycle forms, such as single cellular forms (yeast, spores) and filamentous multicellular forms (hyphae, mycelium). It had been shown that epigenetic mechanisms are involved in the switch between the yeast and hyphal forms in human fungal pathogens (Mishra et al., 2011), and the levels of methylation may differ between mycelium and spores (Nai et al., 2021). However, epigenetics of the complex filamentous mycelial forms dominant in soil have scarcely been studied. Thus, the remaining questions about soil fungi epigenetics may include the mechanisms of differentiation of the filamentous mycelial forms, epigenetics differences between different components of hyphae, and the possible epigenetic contribution to the stable mutualistic interactions between fungi and plants.

3.3. The diversity of DNA methyltransferases in fungi and bacteria

Studying soil epigenetics requires an understanding of the complexity and diversity of DNA methyltransferases present in soil microorganisms. In this regard, it is important to note that the nomenclature of bacterial DNA methyltransferases is different from the nomenclature of DNA methyltransferases in fungi. Eukaryotic DNA methyltransferases are classified into five groups: DNMT1, DNMT2, DNMT3/DRM, Masc1/RID, and DNMT5. These groups differ in their functions and domain structures. DNMT1 is involved in *maintenance methylation* (copying the methylation pattern from the hemi-methylated strand at the replication fork). Masc1/RID are the main fungal-specific DNA methyltransferases responsible for *de-novo methylation*. DNMT5 are involved in maintenance methylation and possibly in RNA-directed DNA methylation. DNMT2 is specialised in the methylation of DNA-tRNA complexs, whereas DNMT3 is absent in fungi (Nai et al., 2021). In contrast to fungi, bacterial DNA methyltransferases are classified jointly with endonucleases (in the context of the Restriction-Modification system), forming four distinct types (Roberts et al., 2003). Type 1 complexes are assembled of five subunits: one responsible for recognition, two for methylation, and two for cleavage of the target motif. Some of the Type 1 complexes may become orphan by missing the cleavage sub-units. Type 2 enzymatic systems function as pairs of separate proteins targeting the same motif: one endonuclease, and one methyltransferase. Most of the orphan DNA methyltransferases belong to the Type 2 category. Type 3 enzymes are assembled of two subunits: one for methylation and one for cleavage (coded by so called mod and *res* genes respectively). Type 4 includes endonucleases targeting modified (e.g. methylated) DNA motifs (Oliveira and Fang, 2021).

Individual fungal and bacterial species contain only a sub-set of the full repertoire of all possible DNA methyltransferases described above. The available DNA methyltransferases define the motif and functional specificity of the different taxonomic groups. Thus, some fungi (e.g. *Ascomycota*) mainly contain Dim-2 DNMT1 and RID DNMTs displaying non-CpG methylation preferences. Other fungi (e.g. *Basidiomycota*) may contain DNMT1 with RFD domain and DNMT5/RAD8 preferring canonical CpG methylation. And some fungi (e.g. *S. cerevisiae*) for a long time were considered lacking the capacity for methylation at all (Nai et al., 2021). The diversity of motifs and DNA methyltransferase types present in different bacterial groups is even more pronounced, providing the basis for the defence against foreign DNA within the Restriction-Modification system (Roberts et al., 2003) and for regulation

of many other cellular functions (Seong et al., 2021).

4. The potential role of the soil microbial methylome in adaptation to stress

Non Restriction-Modification system DNA methylation has been implicated in several key bacterial cellular processes, such as division, adaptation to stress, phase variation, dormancy and others (Anton and Roberts, 2021; Gao et al., 2023). Thus, it was shown that DNA methylation may govern progression through the bacterial cell cycle (Collier et al., 2007). Microbial phase variation (reversible variation of the protein repertoire in a part of a microbial population) is an important mechanism of microbial adaptation; most studied in the context of bacterial surface proteins and immune response evasion (Casadesús and Low, 2013). It has been shown that phase variation could be mediated through mutations in hypervariable regions in the vicinity of DNA-methyltransferases, allowing for coordinated genome-wide shifts in methylation patterns and a corresponding switch between the repertoires of expressed antigens (Beaulaurier et al., 2019). Such heterogeneity of methylomes in a bacterial population may underpin microbial community resilience because it results in sub-populations better able to tolerate future stress. Walworth et al. (2017) demonstrated epigenetic regulation of a wide range of metabolic pathways in the globally distributed marine cyanobacteria *Trichodesmium erythraeum*. A negative correlation was observed between the methylation of m5C motifs and the transcription of some core energetic, carbon, and signalling processes that is highly conserved across three spatially separated *T. erythraeum* isolates, demonstrating a clear link between DNA methylation and phenotypic plasticity. Knocking out a known methylation specificity-determining gene in *Helicobacter pylori* (a human bacterial stomach pathogen) resulted in changes in the transcriptome which also suggests that changes to the methylome may result in phenotypic changes and ultimately drive natural selection in a phenotypically diverse population (Furuta et al., 2014).

Hu et al. (2018) demonstrated the inheritance of DNA methylation patterns through generations of a model cyanobacteria strain exposed to nitrogen starvation for 72 h and then recovery through 12 generations within laboratory culture. The study reported similar methylation patterns in the 'stressed' and 'recovered' populations, distinct from the 'normal' population. However, methylation patterns were not correlated with the expression of six photosynthesis related genes, which were downregulated in response to the nitrogen starvation. Riber and Hansen (2021) argue, largely based on observations made in *Escherichia coli*, that DNA methylation status may result in gene expression associated with the ability of microorganisms to enter conditions of dormancy. Their argument is based on the observation that *E. coli* cells that last enter dormancy are observed to be the first to recover and grow, supporting the existence of a long-term 'memory'. They therefore hypothesise that the ability to enter dormancy is then selected for in a population repeatedly exposed to abiotic stress (e.g. starvation, drought, or exposure to antibiotics) as a 'dormancy-related memory effect'. Similarly, there are multiple examples of epigenetics involvement in fungi adaptation to stress (Zhang et al., 2024). For example, Liu et al. (2015) highlighted the role of a histone H3 lysine 4 (H3K4) methyltransferase gene in the stress response of the soil-borne plant pathogen *Fusarium graminearum*. Deletion of a gene (FgSet1) essential for H3K4 methylation influenced the resistance to cell wall damaging agents (congo red and calcofluor white) and thus the response of the fungi to stress. The mechanisms described in this paragraph may be partly responsible for the legacy effects observed when soil microbial communities are exposed to a temporary abiotic stress.

5. Methods to study the role of microbial DNA methylation in soil legacy effects

The molecular mechanisms of microbial epigenetics have been primarily studied in laboratory cultures (Sánchez-Romero and Casadesús, 2020), while studies of microbial epigenetics in environmental samples are scarce. As a result, we are not aware of examples of differential DNA methylation in response to stress imposed on natural soil ecosystems reported in the scientific literature. However, Hiraoka et al. (2019) constructed the genome of 19 bacterial and archaeal taxa collected from Lake Biwa, Japan and identified 22 methylated motifs, nine of which were not previously found in the REBASE repository, which implies a remarkable unexplored diversity of DNA methylation in prokaryote communities. Soils are the most biodiverse habitat and are likely home to 59% of the species on Earth (Anthony et al., 2023). The effect of environmental stress on soil epigenetics has not yet been studied, despite a few authors highlighting the importance of the soil epigenome and its potential to advance our understanding of gene expression in soils (Manter et al., 2017; White et al., 2017).

Rambo et al. (2019) collected sediment cores from the top 30 cm of an estuary, extracted DNA, and used a methylation-sensitive restriction endonuclease, *Hpa*II, which cleaves at unmodified CpG regions to create a methylation-dependent fragment distribution prior to Illumina sequencing. The results demonstrated differential 5 mC methylation with sediment depth at 1173 CpG sites out of 6254 CpG sites identified, including sites in chitinase genes. 9% of CpGs exhibited methylation shifts of >50% with sediment depth, consistent with a binary epigenetic on/off switch for specific genes. Rambo et al. (2019) therefore provide evidence to support the role of DNA methylation in the regulation of energetically costly biochemical processes such as chitinase activity. However, this approach has not been used in soils and would only be appropriate for known methylation motifs that match the restriction enzyme used.

Because of the nature of epigenetics, at the interface between genotype and phenotype, methylation patterns are difficult to interpret in isolation. Thus, multi-omics studies have been essential to establish bacterial methylation roles beyond foreign DNA defence. For instance, combining methylation profiling with RNA-seq revealed the impact of methylation on the transcriptional regulation in *E.coli* and *Helicobacter pylori* (Estibariz et al., 2019; Fang et al., 2012). Oliveira et al. (2020) combined DNA methyltransferase knock-outs, DNA-seq (SMRT sequencing with detection of 6 mA methylation) and RNA-seq to establish the role of methylation in sporulation and biofilm formation in *Clostridioides difficile*. Of course, the multi-omics approach has also been applied to fungi. For instance, Bonner et al. (2021) combined DNA methyltransferase-targeted mutagenesis, methylation profiling, and RNA-seq to study the effect of methylation on mycotoxin production and environmental adaptation in *Fusarium graminearum*. It should be noted that the examples introduced here were single-species human pathogens. However, we refer to these studies to illustrate that a multi-omics approach will also be essential for understanding the methylation profiles in a more complex soil microbial community.

Until recently, the primary method of studying DNA methylation involved treating DNA sequences with bisulfite to convert unmethylated cytosine to uracil prior to sequencing, but this approach is capable of detecting only methyl-cytosines, and is therefore not suitable to study the 6 mA methylome, the most abundant bacterial DNA methylation (Beaulaurier et al., 2019). The third-generation sequencing methods, such as Single-Molecule Real-Time sequencing (SMRT-seq), developed by Pacific Biosciences, and Nanopore sequencing, developed by Oxford Nanopore Technologies, have no such limitations. They can directly detect methylated bases, estimate methylome heterogeneity computationally from bulk sequencing data without prior chemical treatment, and are suitable for the detection of 6 mA (Oliveira, 2021).

PacBio employed a creative set of technology, chemistry, and physics to film the synthesis of individual DNA molecules base-by-base in real time (hence "SMRT" name: Single Molecule Real Time sequencing). PacBio SMRT sequencing can capture the methylation state from the kinetic parameters because DNA polymerase takes a longer time to incorporate base at a methylated site, which is recorded in the PacBio

movie (Flusberg et al., 2010). The Oxford Nanopore method is based on measuring the ionic current through a pore while a DNA or RNA strand passes through it. The shape and charge of individual nucleotides change the pore conductivity, creating unique patterns (called "squiggle") that can be deciphered to provide information about the sequence. Nanopore sequencing can detect methylation because the methylated bases disrupt ionic current through the pore in a specific manner (Rand et al., 2017), which can be detected by Nanopore base-callers (Bonet et al., 2022). PacBio sequencing can provide high accuracy (HiFi) reads with average base quality $Q > 20$, and up to about 25 kB length (Hon et al., 2020). Nanopore is less accurate (especially at the homopolymer runs), but it may provide longer reads (Wang et al., 2021) and detect a wider repertoire of DNA modifications, including 6 mA, 5 mC, 4 mC, hydroxy-methylation, and others, (Gouil and Keniry, 2019; Rand et al., 2017). Therefore, the choice of technology may depend on the specific study objectives.

Although a number of recent studies have demonstrated the utility of third generation sequencing and a multi-omics approach to study microbial epigenetics (Flusberg et al., 2010; McIntyre et al., 2019), the technology is yet to be applied to epigenetics in soils. In fact, we are not aware of any previous studies which investigate microbial epigenetics in complex ecosystems like soil. A key challenge that has prevented the development of a soil microbial methylome discipline is the need to link methylation in specific taxa with the gene expression in the same taxa. Amplicon sequencing makes possible the extraction and analysis of variable regions of DNA within a phylogenetic marker gene (e.g. 16S rRNA), or in a functional gene (Alteio et al., 2021). However, linking the function to the taxa in a complex community requires longer DNA regions than provided by short-read sequencing. As well as characterising the methylome, Oxford Nanopore and PacBio provide long-read data, which may cover entire genes or transcripts in one read, paving the way to taxa-specific detection of genes or transcripts. However, new bioinformatics tools and resources still need to be built to enable such taxa-specific microbial epigenetics analysis. Because of the complexity of the soil microbiome, the interpretation of epigenetics data may therefore require simultaneous measurement of epigenetic, transcriptional, and microbial community composition changes. In contrast to short-read sequencing, which did not allow reliable distinction of reads from homologous genes in different species, the length of Nanopore or PacBio reads may facilitate analysis of both DNA and RNA data at a species-specific level, directly relating methylation to expression. The analysis should also account for the fundamental differences in methylation mechanisms and functions between bacteria and fungi. While such multi-omics approaches are challenging, and still require new bioinformatics tools, they may be necessary to understand soil epigenetics. Thus, the opportunities to study soil microbial epigenetics are now emerging because of the fast development of new sequencing technologies and the corresponding bioinformatics tools and resources.

6. Unlocking epigenetics to better understand soil legacy effects

It has previously been shown that complex microbial communities display a high level of functional redundancy (or functional similarity) since many taxa are able to perform the same function under the same environmental conditions (Eisenhauer et al., 2023; Louca et al., 2018). A large number of studies have focused on investigating the relationships between microbial biodiversity and the delivery of ecosystem function to better understand how this functional redundancy arises (de Graaff et al., 2019; Delgado-Baquerizo et al., 2016; Trivedi et al., 2019; Wagg et al., 2014). However, most of these studies didn't account for the possible contribution of epigenetics to the apparent functional redundancy of soil microbial communities. Epigenetic heterogeneity allows genetically identical microorganisms to perform different functions when exposed to the same environmental conditions. Therefore, the heritable diversity of soil microorganisms is greater than previously assumed by their genetic or community diversity only. Epigenetics may represent a mechanism whereby taxa are able to conserve an energetically costly function within the heritable memory of a taxa and express it only under environmental conditions which offer the species a competitive advantage. So far, microbial epigenetic diversity has been largely 'invisible' when investigating the relationship between soil microbial functions and the soil microbial metagenome (or *meta*-transcriptome).

Microbial communities in the real soil environment are simultaneously exposed to multiple stresses associated with environmental change (e.g. elevated temperature, salinity, exposure to xenobiotics, drought) which interact and strongly impact soil microbial functions (Rillig et al., 2019). We propose the deployment of long-read sequencing technologies in a multi-omics fashion to elucidate the impact of environmental stresses or perturbations on genetic, transcriptomic and epigenetic diversity in soil microorganisms. This understanding may lead to the development of biomarkers of historic environmental stress (Rey et al., 2020) so that researches can identify, based on the soil methylome, the nature and extent to which soils have been exposed to deleterious environmental conditions. We hypothesise that soil microbial communities with a more heterogenous methylome, all else being equal, will be more resilient to future abiotic perturbations. We therefore consider it timely to add the meta-epigenetic layer to the multi-omics analysis of soils, to advance our understanding of soil abiotic legacy effects.

CRediT authorship contribution statement

Tom Sizmur: Writing – original draft, Conceptualization. **Alexey Larionov:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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