AMERICAN SOCIETY FOR MICROBIOLOGY | Microbiology and Molecular Biology Reviews

Applied and Industrial Microbiology | Review

A roadmap to understanding and anticipating microbial gene transfer in soil communities

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The authors declare no conflict of interest.

Published 8 April 2025

SUMMARY Engineered microbes are being programmed using synthetic DNA for applications in soil to overcome global challenges related to climate change, energy, food security, and pollution. However, we cannot yet predict gene transfer processes in soil to assess the frequency of unintentional transfer of engineered DNA to environmental microbes when applying synthetic biology technologies at scale. This challenge exists because of the complex and heterogeneous characteristics of soils, which contribute to the fitness and transport of cells and the exchange of genetic material within communities. Here, we describe knowledge gaps about gene transfer across soil microbiomes. We propose strategies to improve our understanding of gene transfer across soil communities, highlight the need to benchmark the performance of biocontainment measures *in situ*, and discuss responsibly engaging community stakeholders. We highlight opportunities to address knowledge gaps, such as creating a set of soil standards for studying gene transfer across diverse soil types and measuring gene transfer host range across microbiomes using emerging technologies. By comparing gene transfer rates, host range, and persistence of engineered microbes across different soils, we posit that community-scale, environment-specific models can be built that anticipate biotechnology risks. Such studies will enable the design of safer biotechnologies that allow us to realize the benefits of synthetic biology and mitigate risks associated with the release of such technologies.

KEYWORDS biocontainment, conjugation, gene transfer, microbiome, microorganisms, responsible innovation, soil, synthetic biology, transduction, transformation, vesiduction

INTRODUCTION

Genetically engineered microbes have the potential to help address growing global challenges, such as decreasing our dependence on chemical fertilizers in agriculture (1), mitigating soil greenhouse gas production (2, 3), accelerating pollutant degradation (4), and serving as systems for real-time sensing of threats to our health and the environment (5). However, the use of microbial technologies bears the risk of engineered DNA release into open environments and uptake by environmental microbes. Once introduced into a microbial population, genes can spread far beyond their point of origin, as exemplified by the spread of antimicrobial resistance genes (6). Gene transfer is central to both realizing the benefits of synthetic biology and mitigating the risks arising from intentional efforts to program and deploy soil microbes or following the unintentional release of engineered microbes. Currently, we do not understand gene transfer in soil sufficiently to model the frequency of this process *in situ*. There is a need for environmental microbiologists, soil ecologists, social scientists, and synthetic biologists to come together to study gene transfer in the environment and emerging genetically engineered microbial biotechnologies.

Soil characteristics influence gene transfer because soil is a heterogeneous material that exhibits considerable variability across biological, chemical, and physical parameters. A single gram of soil contains $\geq 10^4$ bacterial species and up to 10^{10} virus particles per gram (7–10), with soil biomass often being >50% fungi (11). Also, the metabolic activity of soil microbes varies in space and time, with hot spots and moments of respiration occurring that are stimulated by organic matter input (12, 13). In addition, soil physical properties that affect cell-cell interactions can vary by many orders of magnitude, such as grain size, aggregation, and permeability (14, 15). Soil chemical properties that modulate cell growth, such as hydration, nutrient content, and pH, are dynamic and can change quickly across both microscales and macroscales (16). Moreover, these properties are interdependent, and further complexity arises from biological-chemical, biological-physical, and chemical-physical interactions. Understanding how this vast soil parameter space influences gene transfer will require cross-disciplinary research.

To mitigate gene transfer by engineered microbes, biocontainment systems have been developed (17). These systems primarily prevent unconstrained persistence, proliferation, and spread of microbes (18). Biocontainment measures have become

increasingly sophisticated and now include genetically encoded technologies and physical containment measures (19). Most approaches focus on minimizing the release of viable cells, rather than considering the subsequent transfer of synthetic DNA following cell death. To date, biocontainment measures have not been benchmarked and calibrated across soil types and conditions. It remains unclear how best to implement these systems in a manner that effectively controls gene transfer across different terrestrial settings.

The purpose of this review is to discuss the parameters that underlie gene transfer in soil, our current understanding of this process *in situ*, and the benefits of developing soil standards to calibrate models that anticipate gene transfer *in situ*. The intended audiences for this review are (i) synthetic biologists who are developing technologies that could participate in gene transfer within soil, (ii) environmental scientists who would benefit from understanding emerging technologies for studying gene transfer host range, and (iii) social scientists who study responsible innovation. We first discuss how DNA can be transferred between cells, thus creating risks for altering the phenotypes of soil communities following engineered microbe introduction. We then discuss the parameters that could influence gene transfer rates and host range in soil, including the properties of the synthetic DNA used to program microbes and abiotic soil properties. We discuss potential impacts of soil on biocontainment measures and the need for models of gene transfer that predict how soil characteristics affect gene transfer host range and frequency of both native and engineered microbes. Finally, we discuss responsible research and innovation for soil biotechnologies.

MICROBIAL COMMUNITIES EXCHANGE DNA VIA GENE TRANSFER

The introduction of engineered microbes into soil microbiomes has the potential to address diverse societal challenges, such as degrading pollutants and providing fixed nitrogen for plants (20–22). Alternatively, it could create challenges by generating microbes that are antibiotic resistant or able to synthesize toxins (23, 24), and it could lead to microbial lifestyle changes, such as supporting growth in new niches (25). Central to studying the likelihood that engineered microbes will transfer their DNA to soil microbes is knowledge about gene transfer mechanisms (Fig. 1), which are reviewed in this section.

Conjugation

When a pair of bacteria come into contact, one can inject DNA into the other via conjugation. A single bacterial species can conjugate with diverse taxa (26, 27), leading to gene transfer across orders, phyla, and kingdoms (28, 29). Conjugation rates depend upon the abundances of the cells donating and receiving the DNA (30). These rates can be increased by surfaces and biofilms (31–34). Conjugation efficiency also varies with plasmid type (35), cell envelope properties that modulate cell-cell interactions (36), and defense systems that degrade foreign DNA (37). Conjugation is mediated by secretion systems (38), protein complexes that donor cells extend into recipient cells (39). The plasmids exchanged, which range from a few thousand base pairs to more than a hundred thousand base pairs (40), are classified as self-mobilizable and conjugative or non-self-mobilizable (41). Self-mobilizable plasmids can drive conjugation even when there is a fitness cost (42). In some cases, DNA exchanged via conjugation can integrate into a recipient cell's genome to secure vertical transmission (43). This type of mobile DNA is referred to as an integrative and conjugative element (ICE). In some cases, a plasmid can be conjugated to a recipient strain and then back to the original donor strain through a process called retrotransfer (44).

Transformation

Some microbes encountering extracellular DNA (eDNA) can take it up via transformation. In soil, the rate of transformation is controlled by the rate of cell death, which produces

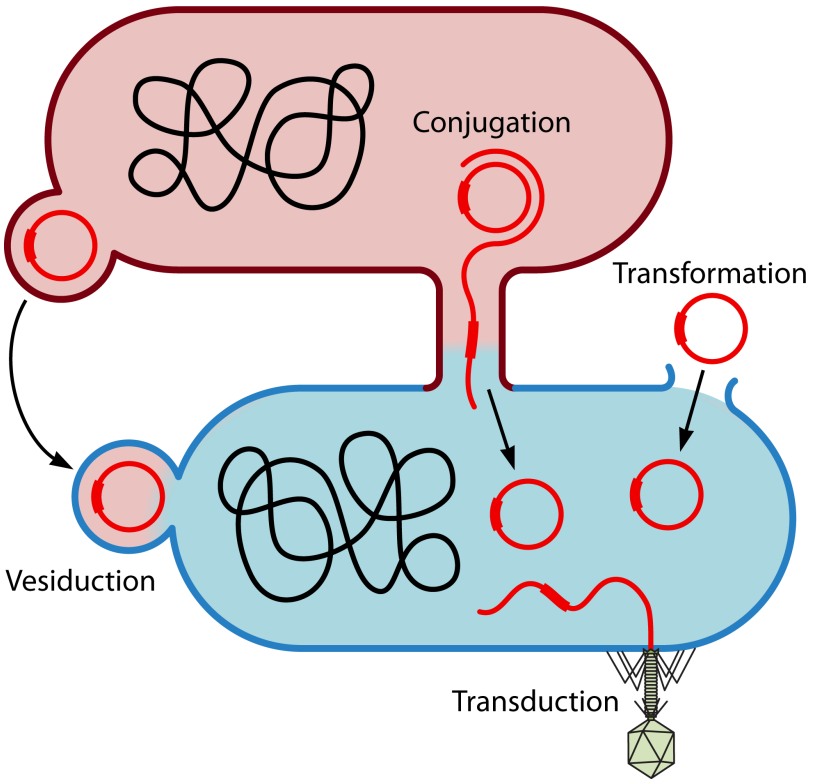

FIG 1 Gene transfer mechanisms. In nature, gene transfer can arise through four independent mechanisms, including the uptake of DNA found in the environment (transformation), infection by DNA and RNA viruses called bacteriophages (transduction), cellular exchange of DNA mediated by cell-cell interactions (conjugation), and the exchange of DNA that cells release in vesicles (vesiduction).

eDNA (45), and the rate of eDNA degradation and sorption onto soil materials, which consumes eDNA. The ability to take up eDNA, referred to as competency, varies across bacteria (46, 47). In some cases, competency involves growth arrest and complex gene expression changes (48, 49). Competent states can be induced by antibiotics, and they can confer antibiotic tolerance (50), suggesting that transformation can drive the acquisition of genes that promote survival. The efficiency of eDNA uptake varies widely, with some organisms being competent for taking up DNA having $>10^6$ base pairs (51). Competency can be induced by various physicochemical parameters, such as temperature, divalent metal ions, and extracellular signals (52, 53).

Transduction

Bacterial viruses, called bacteriophages, are abundant in soil (9, 10). They range in size from ~10^3 to >10^5 base pairs and use diverse capsids to encapsulate their genetic material (54–56). Phage-microbe interactions that underlie transduction have been established for a limited number of phages (57, 58), and anti-phage defense systems have been identified (59). Transduction is classified as generalized, specialized, or lateral. With generalized transduction, bacterial DNA is packaged into virus particles as part of virus replication (60). With specialized transduction, phage and adjacent host DNA are cut out of the bacterial genome and packaged into virus particles (61). With lateral transduction, host genomic DNA and adjacent phage DNA undergo replication together before being packaged into phage particles (62). Gene transfer rates vary across each mechanism, with lateral transduction presenting the highest rates (63).

Vesiduction

Prokaryotes can bud off portions of their membrane to create extracellular vesicles that may at times mediate DNA exchange through a process called vesiduction (64, 65). In a recent study, one plasmid was implicated in driving its own transfer by vesiduction (66). Vesicles also shuttle diverse biomolecules across the extracellular space and may at times transfer DNA incidentally. In both gram-negative and gram-positive bacteria, vesicle formation is linked to cell lysis (65), as well as processes that promote membrane curvature and budding (67–69).

CELLULAR CHARACTERISTICS AFFECT GENE TRANSFER

Gene transfer frequency and host range are expected to depend upon a wide range of biological parameters (Table 1), such as the DNA used to engineer microbes, the biomolecules encoded by that DNA, the microbes targeted for programming, and the composition of the microbiome where engineered microbes are deployed. In this section, we discuss the ways that each of these parameters might modulate gene transfer. We also discuss gaps in our knowledge about how engineered microbe and soil microbiome characteristics might influence gene transfer.

DNA considerations

With plasmid DNA (Fig. 2), gene transfer frequency is linked to DNA mobility (41). Non-self mobilizable plasmids can only be exchanged by cells whose chromosomes encode conjugation machinery, through non-specific transduction and vesiduction, or via transformation following cell death and DNA release (70). In contrast, conjugative plasmids are self-mobilizable because they encode the machinery that facilitates their own transfer (71). The integration of synthetic DNA into a genome is expected to decrease the likelihood of gene transfer compared to plasmids (72). However, gene transfer could still occur. In cases where the synthetic DNA is proximal to phage lysogens in the chromosome, synthetic DNA can be transferred via transduction (62, 63). At times, this transfer could be efficient, mobilizing chromosomal DNA at rates exceeding conjugation (63). Similarly, synthetic DNA integrated near ICEs may be mobilized

TABLE 1 Biological soil properties that could affect gene transfer risk[a]

Category	Knowledge gaps that could affect gene transfer
Species engineered	• Cell surface receptor regulation of cell-cell, cell-DNA, and cell-virus interactions. • DNA methylation and host-defense systems that affect DNA stability. • Effect of microbial relatedness on mobile DNA host range. • Propensity of an engineered microbe to colonize a soil community.
Mobility of engineered DNA	• Community gene transfer capabilities of different DNA transfer origins. • Efficiency and host range of mobile DNA replication origins. • Non-specific gene transfer of genome-integrated DNA. • Piggybacking of non-self mobilizable DNA via host conjugative machinery. • Self-mobilization of DNA through conjugation and transduction.
Fitness effects of engineered DNA	• Metabolic burden of engineered DNA across cell types and soil conditions. • Toxicity of gene circuits transferred to native community members. • Functions of engineered DNA in targeted host and community members. • Expanded metabolic niches for targeted host and community members. • Changes in the sensitivity of the microbiome to antimicrobial control agents.
Soil community interactions	• DNA super spreaders, such as phages capable of lateral transduction. • Gene transfer network connectivity across the soil microbiome. • Host defense system prevalence and diversity. • Likelihood of gene transfer between native community members.

[a]Four biological soil properties could affect gene transfer frequency and host range when an engineered microbe is introduced into a soil microbiome. For each category, the parameters benefiting from future gene transfer research are noted.

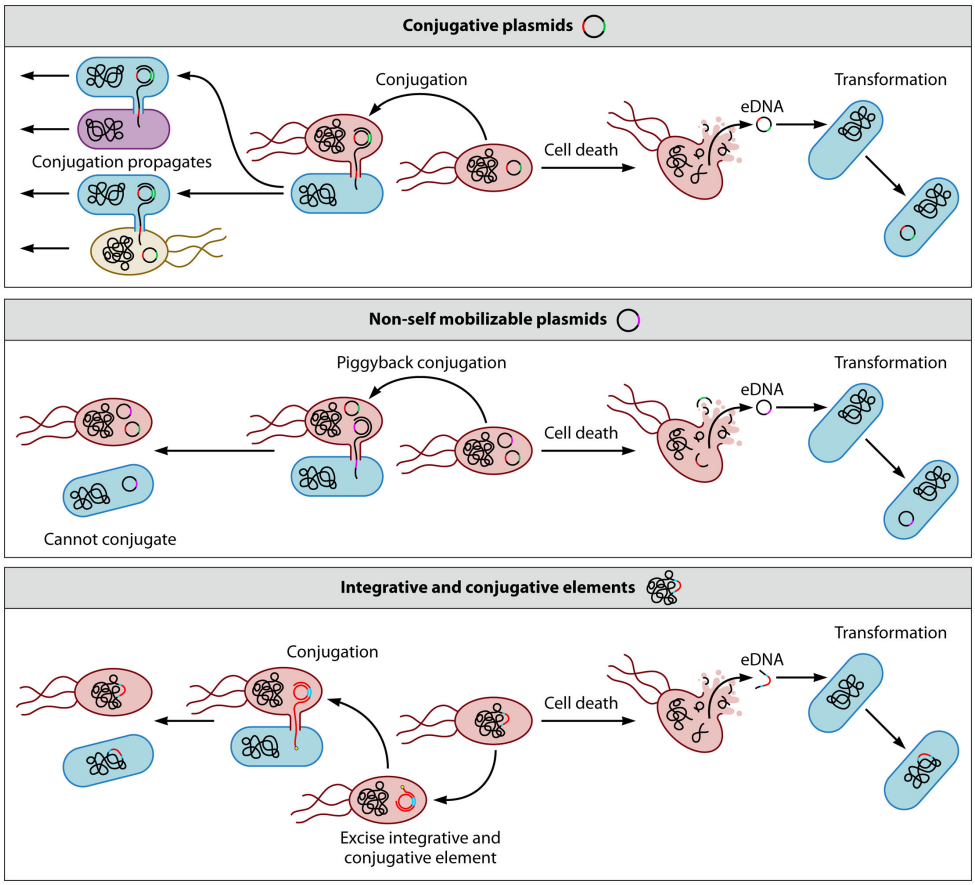

FIG 2 Biological processes that control gene transfer. Conjugation frequency is expected to vary across conjugative plasmids, mobilizable plasmids, and integrative and conjugative elements. (Top) Conjugative plasmids contain all genes required for mediating DNA exchange, so recipient cells acquiring conjugative plasmids can subsequently transfer them to another cell in a soil microbiome. (Middle) Non-self-mobilizable plasmids lack some of the genes required for self-mobilization. These plasmids can be conjugated by cells that contain the machinery required for mobilization, e.g., by cells already containing conjugative plasmids, via a process called piggyback conjugation. (Bottom) Integrative and conjugative elements must be excised from chromosomes prior to gene transfer. For each type of DNA, transformation can mediate gene transfer following cell death when engineered DNA is released into the environment.

(43). This transfer can be minimized by avoiding integration proximal to gene transfer hotspots (73).

Fitness effects

Non-native genes introduced into a microbe could affect gene transfer (74). Gene transfer frequency is expected to increase when engineered DNA expresses biomolecules that allow bacteria to increase the bioavailability of scarce nutrients, proliferate, and occupy new ecological niches. This selective advantage could occur when programming cells to degrade organic pollutants or when programming cells to alter nutrient cycling in soils (4, 75). Gene transfer frequency may be lower when engineered DNA encodes biosensors (5) since sensing technologies are designed to control information flow rather than metabolic capabilities. The DNA introduced into engineered microbes can create burdens on cellular resources and decrease growth (76, 77). This fitness burden can purge engineered microbes from a population (78) or degrade engineered DNA circuits (79). In cases where engineered DNA limits growth and persistence, DNA spread may be decreased. The extent to which this occurs in soil has not been well established. However, some risk for gene transfer remains as selfish genetic elements can

drive gene transfer despite fitness costs (37, 80). Also, mutation and recombination could decouple deleterious and beneficial genes (74).

Cell chassis engineered

The organism targeted for engineering is expected to influence gene transfer in soil (Fig. 3). Microbial fertilizers have been detected in soils years after application (81, 82). The evolutionary relationships between an engineered microbe and members of a soil microbiome could contribute to the likelihood of gene transfer. One study found that closely related microbes can transfer genes with high frequency (83). In some cases, this gene transfer is thought to arise because of high sequence similarity between those genes, which facilitates genomic integration (84–86). Another study found that taxonomic relatedness is not always a good predictor of gene transfer (87). Differences in DNA methylation systems within donor and recipient cells can also impact fitness and gene expression following gene transfer (88). Similarly, host defense systems against foreign DNA, which can be influenced by DNA methylation, may affect gene transfer. Defense systems are frequently exchanged by gene transfer and can differ between closely related organisms (89, 90). The extent to which defense systems affect gene transfer host range and frequencies across different soil community members remains poorly defined.

Molecular-scale considerations

The sequences, structures, and interactions of the cell surface biomolecules in native soil microbes and engineered microbes should be considered when trying to predict gene transfer. Conjugative pili use components of the recipient cell envelope to mediate cell-cell interactions, and these interactions contribute to gene transfer efficiency (91). Central to this interaction is molecular recognition between surface-displayed proteins encoded by plasmids in donor cells, such as TraN, and outer membrane proteins on microbial cell surfaces in communities (92). A recent study revealed how structural variation in TraN underlies mating pair stabilization and conjugation species specificity (93), illustrating how the exact sequences and structures of donor cell proteins could affect gene transfer efficiency and host range in communities. In addition, molecular recognition is critical to plasmid replication since the origin of replication must be recognized by the recipient cell replication machinery (94, 95). Similarly, bacteriophages depend upon molecular recognition for gene transfer. The attachment of phage to a recipient cell requires specific molecular interactions with surface receptors (96, 97).

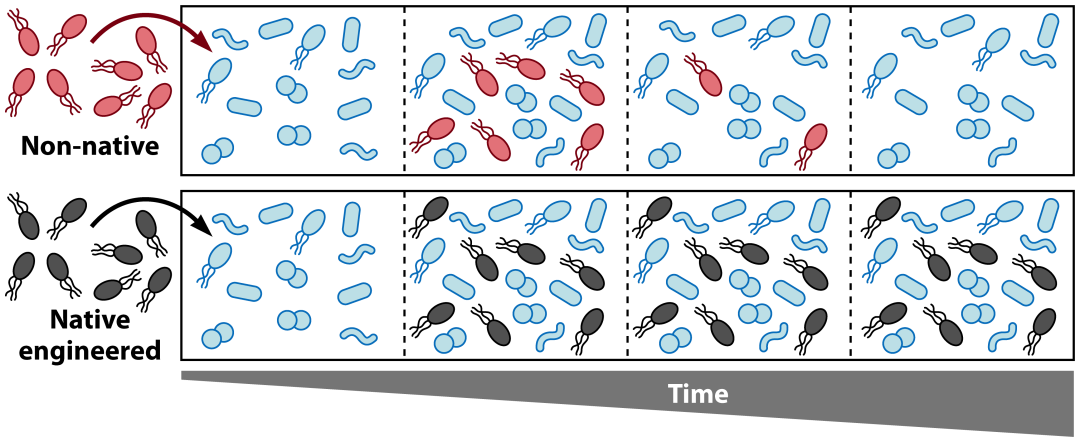

FIG 3 The persistence of engineered microbes in soil communities. (Top) When non-native microbes are engineered and introduced into a microbiome, environmental persistence can be transient if that microbe does not express genes needed to live in that niche. (Bottom) When microbes derived from the microbiome are targeted for engineering, the environmental persistence of the engineered microbe is expected to mirror that of the microbe targeted for engineering. Cellular engineering could control environmental persistence phenotypes in soil by altering cell fitness.

Thus, when engineered microbes have receptors with high similarity to those infected by phage in a soil community, the likelihood of gene transfer via transduction may be increased. For example, phage tail fibers bind to specific proteins and sugars on bacterial surfaces to facilitate injection of their genome into cells (98, 99). Phage proteins also interact with cytoplasmic proteins, like RNA polymerase, to control host processes like transcription (100). These observations suggest that it will be critical to consider the role of both intracellular and extracellular molecular interactions when building gene transfer models, as structural changes in the proteins that regulate these interactions will influence gene transfer.

Community context

Soil microbes could amplify the dissemination of DNA from engineered microbes. Natural gene transfer processes that occur within soil communities are expected to control this potentiation (101). To understand how this potentiation could increase gene transfer, there is a need to catalog the diversity of mobile genetic elements in soil communities and their host range and efficiencies as soil conditions change. Soil microbes with mobile DNA that supports gene transfer at exceptionally high rates could increase the likelihood of synthetic DNA escape from engineered microbes (51, 63). Certain microbes could act as super spreaders, increasing the dispersal of DNA used to program engineered microbes even in cases where their abundance and diversity are low in a soil community. The spread of DNA through a community is ultimately dependent upon physical contact between the donor and recipient cells as well as the network of gene transfer processes that connect the potential donors and recipients (80). Other community properties are expected to modulate gene transfer, such as the diversity of microbial immune systems (80, 102).

Biological knowledge gaps

We do not yet understand the role that different biological parameters play in controlling gene transfer among native soil microbiome members, such as those parameters listed in Table 1. Currently, there is a need to understand how the sequences of components in different mobile DNA, such as origins of replication in plasmids, affect the rates and host range in different biomes. The generation of big data on plasmid host range across diverse microbiome samples could allow for the development of improved models for gene transfer host range in complex communities and ultimately artificial intelligence that anticipates mobile DNA host range, as was transformative in establishing protein sequence-structure relationships (103). In addition, there is a need to predict how engineered microbes will persist following introduction into a community. To address this question, there is a need to understand how engineered microbes interact with other community members since cell-cell interactions underlie the propensity of an engineered microbe to colonize a soil community and participate in gene transfer. In parallel, there is a need to establish how DNA methylation and host-defense systems affect gene transfer, as they control the likelihood that DNA from an engineered microbe will persist following transfer into native soil microbes. A complicating issue that should be explored in future studies is how engineered microbe persistence varies as the composition of the active microbial community within a given geographic location changes with environmental conditions, which can occur seasonally (104, 105). Such studies should also consider potential interactions with dormant microbes.

Upon the introduction of an engineered microbe into a soil, it will be critical to understand how the synthetic DNA used for engineering affects cell fitness. In addition, it will be important to understand how the engineered DNA affects the fitness of the microbes in the soil community that have the potential to acquire that DNA through gene transfer. While the stability and fitness costs of engineered gene circuits have been studied in the laboratory (78), similar studies have not been performed in soils. Soil microbe generation times can vary dramatically *in situ*, with microbes in dryland biocrusts presenting replication times ranging from days to months following hydration

events (106). These dryland microbes exhibit behaviors that vary with growth phases following hydration, which will be important to consider when designing studies that examine the stability, fitness, and gene transfer of engineered cells. Such studies should evaluate engineered system performance in the early phases of hydration, when cells are transitioning out of their dormant phase by generating energy and repairing DNA, and then in the main hydration phase, where microbes acquire carbon and energy (106). Also, it is unclear how the chemicals synthesized by engineered pathways might affect the metabolic niches that can be occupied by engineered microbes and the soil microbes that acquire engineered DNA via gene transfer. Finally, there is a need to understand gene transfer already occurring in soil microbiomes. Members of a soil community could serve as DNA super spreaders following an initial transfer of DNA from an engineered microbe. Until recently, research tools have been limited in their ability to specifically monitor the rates and host range of gene transfer in microbiomes. As new tools emerge for measuring gene transfer host range, which are described in the section titled "Measuring and modeling gene transfer in soil," there is now an opportunity to begin deeply probing the gene transfer networks that exist in soil communities prior to the introduction of engineered microbes. The creation of high-resolution information about gene transfer host range across a wide range of soil microbiomes has the potential to provide unprecedented insight into the biological controls on gene transfer.

SOIL PROPERTIES MODULATE GENE TRANSFER

While many soil properties could affect gene transfer (Table 2), we cannot yet use these soil characteristics to anticipate gene transfer host range and efficiency *in situ*. Because the transport of all soil material, including DNA, is driven by hydrology, water properties and transport processes are expected to be important parameters in determining gene transfer frequency (Fig. 4). The key parameters determining the amount of water in soil and biofilms are temperature and precipitation, while the parameters controlling water movement are texture, structure, and porosity, which together determine water movement speed, termed hydraulic conductivity. In this section, we discuss the effects of abiotic soil properties on gene transfer in order of anticipated importance and conclude with gene transfer knowledge gaps in soil.

Soil texture and structure

After precipitation and climate, which determine whether water is present in soil, the soil matrix itself is likely the most significant control on gene transfer through its regulation of water movement and retention. The key soil physical properties that will impact water are soil texture and structure, which control porosity. These properties are expected to have a large influence on the cell-cell, cell-phage, cell-DNA, and cell-vesicle interactions that underlie gene transfer (107, 108). In addition, they are expected to affect the hydration and persistence of microbes in biofilms, which are prevalent in soil and have been implicated as hot spots of gene transfer (109, 110). To a secondary degree, nutrients (111), mineralogy (112), and temperature (113) can also influence gene transfer.

Texture can be understood as the size of soil particles, which is formally defined by the proportion of sand, silt, and clay in a soil (114). Structure, on the other hand, describes the aggregation of grains to create larger particles. In soils, these aggregates are called peds, structures that persist through wetting and drying cycles (115). The physical arrangement of soil peds determines porosity and hydraulic conductivity (116). As such, this physical arrangement is expected to act as a major control on the flow of cells and DNA within soil as well as water and nutrients that are critical to cell fitness in biofilms. Not surprisingly, this structure can partition small groups of soil microbes into spatially isolated compartments (117), which can extend the persistence of mobile DNA beyond that observed in larger well-mixed communities (118). Soil structure and texture are determined by the materials that comprise soils, with coarsely textured soils leading to high hydraulic conductivity and finely textured soils having the potential to be lower in hydraulic conductivity. While clay soils are made of particles that are approximately six

TABLE 2 Abiotic soil properties that could affect gene transfer[a]

Category	Properties that could affect gene transfer
Physical characteristics	• Hydrologic connectivity (hydration) • Structure (aggregates and peds) • Temperature (gradients) • Texture (clay, silt, and sand)
Chemical composition	• Gradients (chemical and redox) • Ion exchange capacity (cation and anion) • Organic matter (percentage and functionality) • Macronutrients and micronutrients • pH and salinity
Macroscale processes	• Fungal hyphae growth (networks of liquid) • Management (tilling vs no tilling) • Metabolic hot spots (rhizosphere) • Pore creation by fauna (worms) • Transport (erosion, dust storms, and wheels)
Amendments	• Animal manure • Chemical fertilizer (NPK containing) • Pyroligneous acids (wood acid) • Pyrolyzed organic matter (biochar) • Wastewater biosolids

[a]For different abiotic soil properties, such as physical and chemical characteristics, parameters are noted that could contribute to gene transfer frequency and host range. For each category, the soil properties that would benefit from further study are listed.

orders of magnitude smaller than sandy soils (119), not all clay soils have low porosity and low hydraulic conductivity. This occurs because clay soils can be highly structured, with ped aggregate sizes determining pore connectivity. Nevertheless, clay soils with poor structural development can lead to very low porosity and low hydraulic conductivity (120).

To date, there have been limited studies investigating the effect of soil structure and hydrologic connectivity on gene transfer host range and efficiency (117, 118, 121–123). Soil water properties have been shown to correlate with DNA movement and stability.

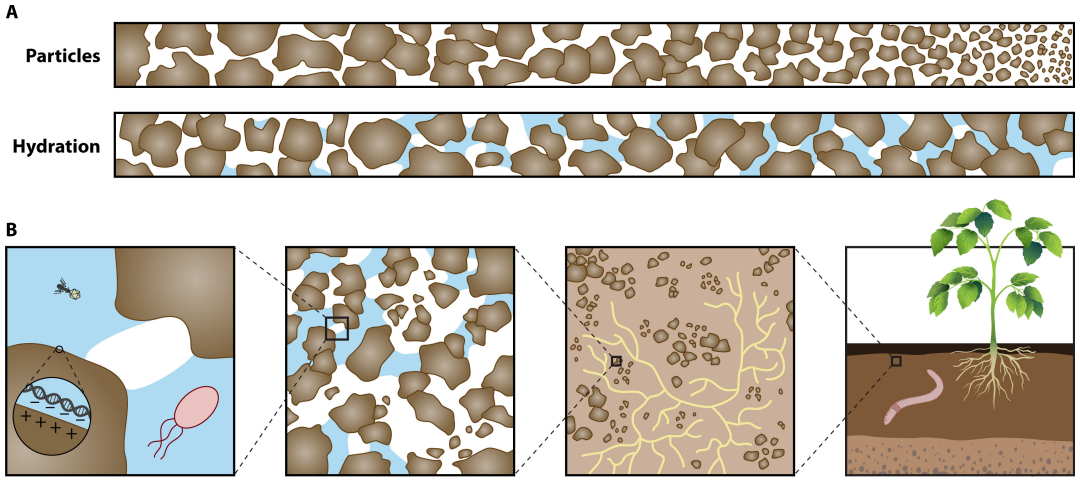

FIG 4 Mechanisms by which soil physical properties are expected to affect gene transfer. (A) Soil particle size and hydration, which dynamically vary, are expected to be major controls on gene transfer *in situ*. (B) At micron length scales (left), particle size distribution controls the connectivity of hydrated pockets containing microbes, eDNA, and phage. At longer length scales (right), fungal hyphal networks (yellow), soil fauna (pink), and plants (green) connect different microenvironments. These organisms can increase the mixing of microbes, DNA, and phage found in distal microenvironments.

High soil water content has been found to favor DNA degradation (45), while drier soils stabilize DNA (124). Similarly, soil flooding negatively influences the persistence of antibiotic resistance genes (45, 125, 126). One study revealed that hydration is inversely correlated with transformation rates (45). Another study found that conjugation in soil correlates with water holding capacity (121). These data illustrate the lack of a unifying model that describes how soil hydration and texture control gene transfer. To address this challenge, the research community will need to develop robust metadata descriptions for the soils used to perform gene transfer experiments, as such data are critical for intercomparisons of different studies. Hydrologic connectivity is also expected to control the transport of engineered microbes from the topsoil, where they are used as biological fertilizers, across the landscape to tributaries or through the vadose zone into groundwater. In addition, engineered microbes could be transported across large distances through dust movement, soil erosion, tilling, and transport via the wheels of agricultural machinery. Our understanding of gene transfer in soil remains too limited to anticipate how the longer-scale movements of engineered microbes might affect gene transfer across spatially separated microbiomes.

Soil mineralogy

The soil matrix can bind to cells, phage, and DNA, affecting their movement and interactions. Soil organic carbon is the primary source of ion exchange capacity (127), while minerals act as a secondary source. Some clay minerals (e.g., kaolinite, montmorillonite, and goethite) carry charges that interact strongly with DNA. These charges may influence the transformation rates by acting as a sink for free DNA and facilitating cellular uptake (128). For example, clay can absorb up to 200 µg of DNA per gram (129). DNA released into soil is typically degraded over a week (45, 130, 131). However, it can persist for months (132). In some cases, mobile DNA introduced into soil via manure can persist for up to half a year, depending on the sand and clay content (125). Gene transfer rates are also impacted by the clay mineral identity (133).

Soil chemical properties

Gene transfer could be influenced by soil chemical properties, such as pH, electrical conductivity, organic matter content, macronutrients (N, P, K, Ca, S, and Mg), and micronutrients (Fe, Zn, Cu, B, Mn, Mo, and Cl). One mechanism through which these properties can impact gene transfer is by modulating weak charge interactions of cells, phage, and DNA with the soil matrix and with one another, or by affecting the growth and stress of individual cells. Soil pH determines the balance of charges in a soil system and is expected to be the most significant chemical parameter controlling charge interactions. Soil organic carbon contributes the majority of cation and anion exchange capacity in soils (127), making it critical to understand potential charge interactions between the soil matrix and cells, phage, and DNA. Studies examining gene transfer via conjugation in soil have revealed that some types of inorganic matter promote conjugative transfer (134). Soil organic matter can also absorb genetic material, and therefore, high levels of soil organic carbon content may at times decrease the movement of genetic material in soils. Given the complexity and heterogeneity of soil organic carbon, it remains unclear if there are generalizable effects on gene transfer.

Soil chemicals and nutrients in the aqueous phase are expected to influence cell growth and gene transfer since growth rates affect the frequency of conjugative gene transfer. In addition, the capacity for transformation can change rapidly following adaptation to salinity changes (135), and transformation frequency can increase when cells experience starvation for a macronutrient, such as C, N, or P (136). Similarly, sodium chloride concentration and conductivity correlate with the abundance of antibiotic resistance genes and mobile genetic elements across soils (137). Also, soil nutrients represent the third largest geographical variable for predicting antibiotic resistance gene abundance in soils (138), genes that are frequently spread via gene transfer. Furthermore, manure represents a hotspot in soil for antimicrobial gene transfer due to the nutrients

and chemical antibiotic residues that may exert selective pressure on cells (139). As these observations often represent static microbiome snapshots, it is unclear whether these trends arise from horizontal or vertical transmission of DNA.

Soil gradients add a layer of complexity when considering gene transfer in soil. Chemical gradients can arise proximal to plant roots as they produce nutrients for their associated microbiomes, thereby generating biogeochemical hot spots. Also, redox gradients occur with increasing depth in soil and across soil aggregates. These gradients can present complex spatiotemporal dynamics due to soil irrigation and weather. Irrigation may lead to transient hot spots surrounding plant roots that change with plant growth stage. In soils that become saturated with water during the winter, microbial gene transfer may be insulated depending upon snowpacks. In the spring, thawing could induce hot spots of gene transfer and transport. The effect of these gradients on gene transfer remains poorly defined in soil.

Transport processes

A variety of transport processes could affect gene transfer in soil. For example, fungi forming hyphae provide networks of liquid films that support bacterial migration (140). Furthermore, the transfer of plasmids that typically occurs near the soil surface can be driven to greater depths by earthworms, which facilitate interactions between microbes that optimally persist in physically separated niches (141, 142). Soil fauna also plays a role in the creation and maintenance of soil pore structures, contributing to the movement of soil water (143). Beyond these transport processes, plants create hot spots for microbial growth by offering carbon-rich niches that increase microbial gene transfer compared with bulk soils (144–148). Furthermore, it is important to consider gene transfer in the context of the food chain. Gene transfer can occur in the guts of animals that transport and disperse microbes and their mobile DNA through the environment. Currently, our understanding of how these transport processes affect gene transfer in soil is in its infancy.

Biofilm considerations

There is a strong connection between biofilm formation and gene transfer in soil (149, 150). Within biofilm communities, which are prevalent in soils (151), gene transfer is generally thought to be higher than that with planktonic cells (34, 152). This trend is thought to arise because biofilms promote conjugative plasmid transfer compared to that observed with planktonic microbes (34, 152) and enhance transformation efficiency (153, 154). In soil, gene transfer is expected to be promoted in biofilms because of the high moisture content and cell density compared with other soil niches (155–157). At times, however, biofilms may hinder DNA dispersal beyond established biofilms (158), creating spatial constraints on gene transfer. Biofilms also control soil texture by contributing to the formation of mineral-associated organic matter (159), which affects water movement and retention in soil. These texture changes may affect gene transfer by influencing the movement of cells that detach and separate from biofilms, which can be dispersed to establish new biofilms (160, 161). Similarly, phage infection can lead to dead zones of organic matter within soil, which affects soil interactions with water, distribution of nutrients, planktonic cell movement, and microbial community structure (162). Currently, there is a need to better understand how biofilm heterogeneity influences different gene transfer processes, how gene transfer processes in biofilms affect the formation of mineral-associated organic matter, and how changes in mineral-associated organic matter feedback to affect planktonic cell movement and the establishment of new biofilms.

Soil knowledge gaps

Our understanding of abiotic controls on gene transfer in soil is still in its infancy. Table 2 lists the physical and chemical characteristics of soil that have the potential to influence

gene transfer *in situ*, as well as macroscale processes and soil amendments. Understanding the ways that these diverse soil properties affect gene transfer remains challenging because studies typically perform gene transfer measurements across a small number of soil conditions. One way to overcome this challenge in future studies is to systematically vary soil properties in gene transfer experiments by using artificial soils (163). Such soils can be tuned to mimic a wide range of soil physical properties by varying grain size and aggregation, as well as soil chemical properties by changing minerals, pH, organic carbon, and nutrient content (164–169). Currently, there is a need for the community to develop soil standards for gene transfer studies that sample a defined set of physical and chemical soil parameters, so that studies with different microbes and communities can be compared. Additionally, there is a need for soil amendment standards. Standards have been developed for some soil amendments, such as biochars (170), but these have not been leveraged for gene transfer studies. Synergistic with such standards would be the development of model soil communities for studying gene transfer (171). Communities with controllable composition represent powerful tools for studying how gene transfer risk changes with biodiversity and environmental parameters, such as temperature, pH, and micronutrients.

Beyond standards, there is a need to measure and benchmark gene transfer host range and frequency across soils from diverse geographic and ecological settings and compare these results with those from synthetic soils. Some community-level resources have been developed and can be used immediately to guide such soil gene transfer studies. Databases have been established to catalog bacteriophage and plasmid information across different soils (172–174), such as host taxonomy, genetic elements, and geographic distribution. Global surveys of mobile DNA in soil suggest that much of this mobile DNA diversity remains unexplored (175), highlighting how these atlases can be used immediately to guide studies of gene transfer using emerging tools (176). By pursuing such studies, the broader research community can generate data across a greater diversity of soils having different abiotic and biotic complexity, knowledge that will be critical for developing the next generation of gene transfer models.

BIOCONTAINMENT ATTENUATES GENE TRANSFER

The persistence of engineered microbes can be controlled in communities using a range of biocontainment approaches. To date, most approaches have been tested in relatively simple settings that lack the biological, chemical, and physical complexities of soil. To be effective in soil, there is a need to understand the performance of these systems across the vast soil parameter space and refine the best containment systems so that they present robust performance across dynamically changing soil conditions. In this section, we review current biocontainment technologies (Fig. 5) in the context of soil applications and outline knowledge gaps that would benefit from future study.

Metabolic dependencies

To control cell viability, metabolism can be addicted to a continual supply of a specific chemical (17) by knocking out one or more genes that synthesize essential metabolites (177). Such metabolic dependencies have also been created by programming cell viability to depend upon a chemical that is not readily available in the soil environment, such as phosphite (178). The performance of these biocontainment measures is expected to be sensitive to the types of microbes present in soil, as some microbes may produce the essential metabolite, and soil amendments may introduce those chemicals. Much work is needed to understand how mutations in engineered microbes and undiscovered metabolic pathways within soil microbiomes could influence cell viability in the absence of chemical supplementation.

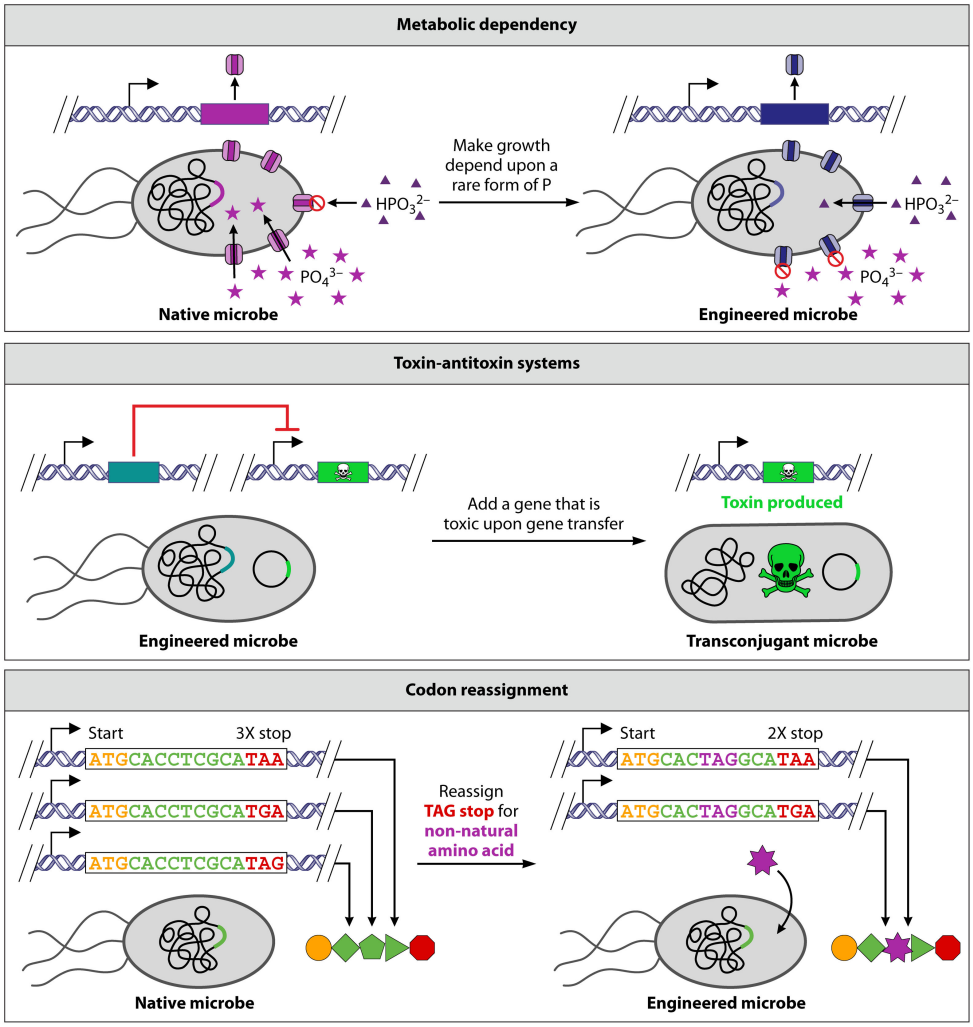

FIG 5 Biocontainment approaches for engineered microbes. (Top) Metabolic defects can be engineered so that microbes only grow when a specific metabolite is provided, such as phosphite (purple triangle). (Middle) Toxin-antitoxin systems can be used to prevent community members from acquiring DNA from engineered microbes via gene transfer. With this approach, the DNA encoding the toxin (green) is tolerated in the engineered cell due to the presence of an antitoxin. When the DNA from the engineered microbe is taken up by environmental microbes, the toxin is produced, thereby killing those microbes. (Bottom) Codon reassignment can be used to engineer cells to require a non-natural amino acid for viability. In the engineered cells with this genetic recoding, essential genes are translated into full-length proteins by incorporating non-natural amino acids via read-through of reassigned stop codons. Following gene transfer to environmental microbes, these genes produce truncated proteins.

Kill switches

Genetic circuits have been created that link specific environmental conditions to the production of toxic gene products (17, 179–183). These circuits function as kill switches, using the chemical and physical information from the environment to induce killing, such as pH (184), temperature (185, 186), and metal ions (187). These biocontainment measures can leverage Boolean logic to achieve multiple layers of control over cell persistence. For example, AND gate logic has been achieved using passcode kill switches, which only allow engineered microbes to survive when a combination of two sugars is present (188), and OR gate logic has been achieved using a Deadman switch, which kills cells when a survival signal is removed or when a kill signal is present (188).

Kill switch stability can be a challenge. The toxins produced by kill switches can be compromised by mutations that abolish function despite improvements that have been achieved by multiplexing toxins and increasing genome stability (180, 183, 188). This mutational susceptibility is thought to arise from leaky expression of the toxins when they are designed to be off, which leads to a fitness cost and selective pressure to escape that burden. To buffer against toxicity, antitoxin molecules have been expressed at low levels in engineered microbes (184, 185), and they have been combined with other biocontainment approaches (189, 190). To create kill switches with increased fidelity within soil, there is a need to identify gene circuit designs that minimize fitness burdens when the switch is meant to be off across diverse soil types and conditions. Also, there is a need for improved buffering against inactivating mutations.

Next-generation biocontainment

Genetically recoded organisms overcome some limitations of metabolic dependencies and kill switch systems by engineering microbes using genetic material that is challenging for other microbes to use. To achieve this, genomes are recoded so that growth depends on non-biological chemicals (19, 191–197). This coding can be achieved by systematically replacing a particular stop codon in the engineered microbe with a synonymous stop codon, e.g., substituting UAA for every UAG codon in the genome, and then reassigning the UAG codon to a non-standard amino acid and using those new UAG codons within coding sequences (191). When DNA from these organisms participates in gene transfer, any environmental microbe acquiring the DNA is expected to prematurely terminate translation of the recoded mRNA (191). Similar xenobiological approaches have been applied at the DNA and RNA levels, and microbes have been engineered to replace native nucleic acids with synthetic and non-standard analogs (198–200). Another strategy to decrease DNA susceptibility to gene transfer is sequence entanglement, where the open reading frame of an engineered gene is overlapped and entangled with a second gene (201). With this approach, gene transfer frequency between bacteria can be suppressed by entangling a gene used for programming a microbe with a gene encoding a toxin. A complementary strategy to next-generation approaches is physical biocontainment. Bacterial encapsulation in hydrogel beads can be used to prevent the escape of engineered cells while allowing the exchange of nutrients and small molecules with the external environment (202).

Preventing environmental DNA formation

Many biocontainment systems are designed to kill the host cell, while the genetic material is left intact and could be released following cell lysis. As discussed in the section titled "Soil properties modulate gene transfer," eDNA can be strongly adsorbed by soils (203), and it can persist in soil for months (45, 70, 130), potentially allowing gene transfer to soil microbes via transformation. Furthermore, DNA-containing vesicles produced during cell lysis may lead to gene transfer. To decrease the risk of gene transfer after engineered cells perform their designed function, genetic circuits can be programmed to degrade genetic material using non-specific nucleases (187). In addition, microbes can be engineered to induce double-stranded breaks using sequence-specific nucleases (204), which can be lethal to cells. Genetic circuits producing these enzymes can be created that are selectively activated in recipient cells following gene transfer. This phenotype can be achieved by inducing the expression of a nuclease when the synthetic DNA is transferred to a new microbe lacking the requisite methyltransferase or through the production of a toxin that is inhibited by an antitoxin in the donor cell that is absent in the recipient cell (189, 190). The extent to which DNA degradation is required to maximize biocontainment performance in soil will require further investigation across different soil types.

Dormancy challenges

Cell dormancy may at times impede biocontainment. Many soil microbes are in dormant states (205), and this dormancy is associated with a decreased susceptibility to antibiotics and stresses (206–209). With engineered microbes, dormancy has been implicated in supporting persistence in dried soil for over a year (210). Engineered microbes could become dormant within soils, allowing them to survive biocontainment and DNA degradation, thereby prolonging their persistence in soil while providing an opportunity for their engineered DNA to be transferred to native soil microbes. Some engineering strategies consider cell dormancy (211), while others do not, as survival ratios are typically measured using microbes grown to the exponential phase (19, 184, 189). *In vitro* models for dormancy have been established in mycobacteria and could be adopted to evaluate the efficacy of biocontainment systems for dormant microbes (206, 212, 213). Currently, there is a need to better understand how the dormancy of both engineered microbes and soil microbes affects gene transfer in soil as well as the performance of biocontainment measures.

Biocontainment knowledge gaps

To date, microbial biocontainment systems created for bioremediation represent the best-characterized systems in soil settings (214–216). With the simplest systems, a killing function is repressed using the chemical targeted for bioremediation, such that engineered microbe death occurs when that chemical is depleted. To enhance performance, systems that combine kill switches and metabolic dependencies have been developed (217). To date, system performance has been evaluated across a small number of soil conditions, so it is not clear how their functions will vary across soil types and conditions. The exogenous molecules used for regulation may adsorb to soil matrices rich in clays, resulting in sequestration away from the target microbe, or they may adsorb to soil amendments like biochar (218). Soil microbes themselves are frequently compartmentalized in aggregates (219, 220), and engineered cells may at times become inaccessible to the chemicals used to regulate these containment measures. Soil aggregate structure controls water flow and can, therefore, limit the movement of solutes through the soil matrix (221).

Currently, there is a need to benchmark the different biocontainment strategies that have been developed across a spectrum of soil types and conditions to understand which present the necessary fidelity for field applications (222). In future studies, biocontainment should be benchmarked in soil microbiomes that sample a range of biological, chemical, and physical properties as outlined in prior sections. With kill switches, there is a need to identify gene circuit designs that minimize fitness burdens when the switch is meant to be off. Such designs are critical for avoiding inactivating mutations and system failure. Emerging strategies for biocontainment are appealing for soil applications because they have the potential to link microbial fitness to molecules thought to be absent from soil. However, these approaches cannot yet be easily applied to non-model soil microbes in a predictable and cost-effective manner. Furthermore, it is unclear how different soil types and conditions will affect the bioavailability of non-natural chemicals used to complement engineered microbe growth and how to strategically introduce these chemicals into a soil community to support the growth of the engineered microbe across the targeted environmental conditions. Also, the extent to which soil chemicals could substitute for non-natural chemicals in engineered microbes will need to be rigorously evaluated across diverse soil types and conditions. For example, in the future, it will be critical to establish if some soil microbes have the metabolic capabilities to synthesize passcode chemicals via uncharacterized metabolic pathways. Similar studies are needed with microbes having metabolic dependencies.

MEASURING AND MODELING GENE TRANSFER IN SOIL

Tools for measuring gene transfer across microbiomes are emerging that are poised to provide insight into mobile DNA host range and transfer efficiency across diverse soil types and conditions. This section describes these tools as well as models for anticipating gene transfer. These models have largely been parameterized using measurements in homogeneous growth medium that do not capture the biological, chemical, and physical complexity of soils. By performing community-scale measurements of gene transfer rates and host range across soils and microbiomes of known composition, we hypothesize that models can be generated that better anticipate the frequencies of gene transfer in soil and the attenuation of those processes in engineered microbes using biocontainment.

Community models

Quantitative models exist for predicting gene transfer in the laboratory (223). The classical model for conjugation is Levin's law of mass action (30), which predicts that the transconjugant (*t*) cell number is proportional to the number of bacteria that can receive the gene (recipient cells, *r*), the number of bacteria that can transfer the DNA (donor cells, *d*), and the intrinsic rate (γ) at which transfer occurs (Fig. 6A).

$$[t] = [d] \cdot [r] \cdot \gamma$$

Similar models exist for transformation and transduction (224, 225), and these modeling approaches have been extended to consider gene transfer across communities (226, 227). Currently, there are several challenges with using these models to estimate gene transfer in soils. They are largely parameterized using gene transfer studies that involve pairs of model bacteria *in vitro* (152), a condition that does not reflect real-world soil complexities. Also, these models cannot be applied to unculturable microbes. Multi-species models have been created by assigning unique rates of transfer terms to each species-species interaction (101). Also, models have been developed for predicting how gene transfer and ecological interactions jointly contribute to microbiome stability (228). However, these models are not grounded in biome-scale gene transfer measurements, as methods to monitor gene transfer in complex communities have only recently emerged (26, 27, 229). A grand challenge for soil microbiology is to calibrate these models using community-scale measurements of gene transfer *in situ* and refine these models to predict gene transfer risk across diverse soil types and conditions.

Abiotic parameters

The chemical and physical properties of a soil affect microbial fitness and nutrient bioavailability (13). In addition, soil properties are thought to control gene transfer by controlling cell-cell interactions (230) and the bioavailability of eDNA (130, 231), phage (232), and chemicals that stimulate gene transfer (233, 234). The extent to which these abiotic-biotic interactions contribute to gene transfer rates and host range in soil is not known. Currently, there is a need for measurements that establish which of these abiotic processes are most important to consider when improving community-scale models of gene transfer. Once such information is obtained, the abiotic parameters that impact gene transfer to the greatest extent can be used to refine and extend quantitative models (Fig. 6B through D). Synthetic soils are expected to represent a simple way to manipulate individual matrix parameters (235), while simplified model microbial communities can be used to test biotic responses and quantify gene transfer rates under a range of environmental conditions (171).

Predicting biocontainment

To benchmark the performance of biocontainment in soil, several parameters need to be measured, including: (i) the persistence of the engineered microbes and their eDNA,

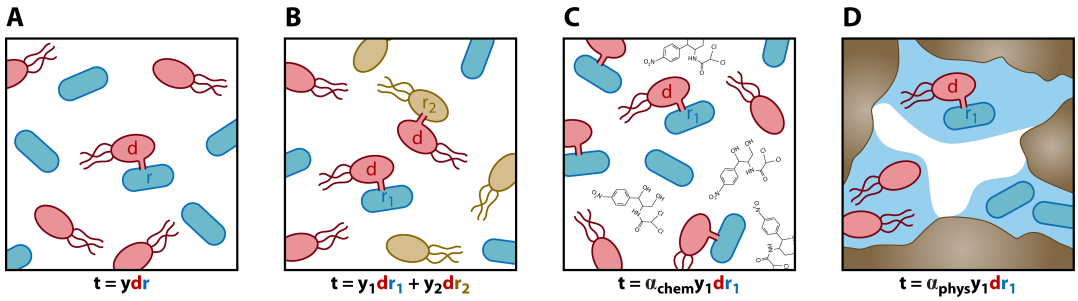

FIG 6 Models for gene transfer rates and host range in soil. (A) The Levin model for conjugation uses a rate equation to describe the concentration of transconjugants (*t*) using the intrinsic conjugation rate (*y*), the donor cell (*d*) concentration, and the recipient cell (*r*) concentration. (B) This model can be modified to anticipate how a single donor strain varies in its conjugation rate with different members of a community. (C) Chemicals that stimulate conjugation *in vitro*, such as low levels of antibiotics, are expected to affect the rate of conjugation across soil microbiomes when they are bioavailable. (D) The physical properties of soil, such as hydration, have been shown to affect conjugation rates. When modeling conjugation in soil, additional terms capturing these chemicals (a_{chem}) and physical (a_{phys}) parameters are needed.

(ii) the genetic stability of biocontainment, (iii) the transfer of DNA from engineered microbes to soil microbes, (iv) the persistence of the engineered DNA following transfer to soil community members, and (v) the subsequent transfer of this engineered DNA among soil community members. Existing measures of biocontainment largely monitor the persistence of engineered cells and stability of these systems (182, 188, 189). Such measures do not assess if DNA has been transferred to native soil microbes via eDNA transformation following engineered microbe death. A small fraction of eDNA is predicted to persist in soil (130), which may lead to gene transfer long after engineered microbes die. In such cases, biocontainment that degrades DNA before cell death will be critical. More broadly, many biocontainment systems remain poorly characterized across soil parameter space (236–238). Currently, there is a need to standardize how biocontainment systems are evaluated (239). As discussed in the section titled "Soil properties modulate gene transfer," the research community would benefit from developing a set of soil standards to enable comparisons about how different biocontainment designs attenuate gene transfer across different soil conditions.

Measuring host range

Currently, there is a need to understand how engineered cells transfer their DNA to different microbes in soil microbiomes, how well biocontainment measures function in soil, and how soil communities might potentiate gene transfer. To address each of these issues, there is a need for accurate and simple measures of engineered DNA host range in soil microbiomes. One of the simplest strategies for studying gene transfer is to code a selectable marker within engineered DNA and to enrich for community members that take up DNA using antibiotics (240). By sequencing the enriched microbes, data can be generated about gene transfer host range. However, this approach is not compatible with a large fraction of soil microbes since many cannot be cultured (241). Also, some soil microbes have intrinsic antibiotic resistance, which can lead to false positives. Visual reporters can also be incorporated in mobile DNA and enriched using cell sorting prior to sequencing (242). While this approach does not require culturing of cells following gene transfer, reporter expression can be low in some microbes because the transcription and translation initiation sequences used to control reporter expression behave differently across different soil community members.

Methods are emerging for monitoring gene transfer host range across microbiomes (Fig. 7). First, high-throughput chromosome conformation capture (Hi-C) can map mobile genetic elements to their microbial hosts by chemically crosslinking mobile and genomic DNA and then using sequencing to identify the chromosomes linked to mobile DNA (229). Hi-C has been used to monitor soil-phage host interactions, revealing an increase in lysogenic infections following soil drying (176). Second, environmental transformation

sequencing, ET-seq, records gene transfer host range by coding a transposon and transposase within mobile DNA (26). When this mobile DNA enters an environmental microbe, it randomly inserts a transposon into the chromosome, such that it barcodes microbes that participate in gene transfer. With this approach, the host range of mobile DNA is established using metagenomic sequencing. Third, RNA-addressable modification (RAM) records gene transfer using a ribozyme that adds barcodes onto 16S rRNA (27). When mobile DNA encoding the ribozyme enters an environmental microbe, the catalytic RNA is transcribed and barcodes rRNA. To identify microbes that participate in gene transfer, barcoded rRNAs are amplified to include species-specific variable regions and then sequenced to identify which microbes participated in gene transfer. A recent study showed that RAM can be multiplexed to compare the host range of multiple conjugative plasmids in parallel (27). While RAM has only been used to monitor conjugation in wastewater, it is expected to be useful for gene transfer studies in soil.

Monitoring dynamics

Selectable and visual reporters represent the most commonly used approaches for measuring gene transfer rates (243). These approaches have provided insight into the efficiency of plasmid transfer across soil communities (244). With most reporters, the microbes studied must be extracted prior to the analysis of the reporter signal. To allow for non-disruptive measurements of gene transfer in soil, indicator gas reporters have been developed (245). By coding these reporters into conjugative vectors, the effects of soil hydration on gene transfer can be measured (126). More recently, these tools have been improved to decrease background signals in the absence of conjugation (246). Currently, these indicator gas reporters are limited to studies of conjugation in soils that involve defined pairs of microbes, although they are expected to be useful for studying transduction and transformation dynamics. Currently, there is a need for simple strategies that enable gene transfer rate measurements across soil microbiomes *in situ*.

A roadmap for improving models

Until recently, models of gene transfer have been limited by the amount of data that can be quickly and cheaply generated to train those models. Also, data have largely been acquired using measurements performed under ideal conditions that are not representative of the soil environment. With the recent development of methods for measuring gene transfer host range in communities, there is now an opportunity to generate big gene transfer data to train models (247). Autonomous barcoding technologies, like ET-seq (26), can be incorporated into any mobile DNA of interest and used to measure gene transfer host range under ideal conditions via filter mating assays with microbiomes (248). By pairing data arising from such assays with omics information about the microbiome used for gene transfer, models can be trained to predict gene transfer host range using biological information alone in the absence of soil. To translate these models into soil, similar experimental measurements will need to be performed in soil microbiomes (249). The results of these measurements can then be paired with chemical and physical information about those soils to evaluate how transitioning from the petri dish to a soil habitat affects the host range. Currently, the major limitation with such measurements is obtaining a large enough signal from barcoding methods to track these reactions *in situ*. By performing measurements across a range of microbiomes and soils, models can be created with more predictive power. Such models will be critical to understanding gene transfer in native communities, establishing biocontainment needs for different engineered microbes, and guiding the design of biocontainment systems with the necessary fidelity.

RESPONSIBLE BIOTECHNOLOGY INNOVATION

When developing engineered microbes, it is important to consider who will ultimately be affected by those genetically engineered technologies. In this section, we discuss the

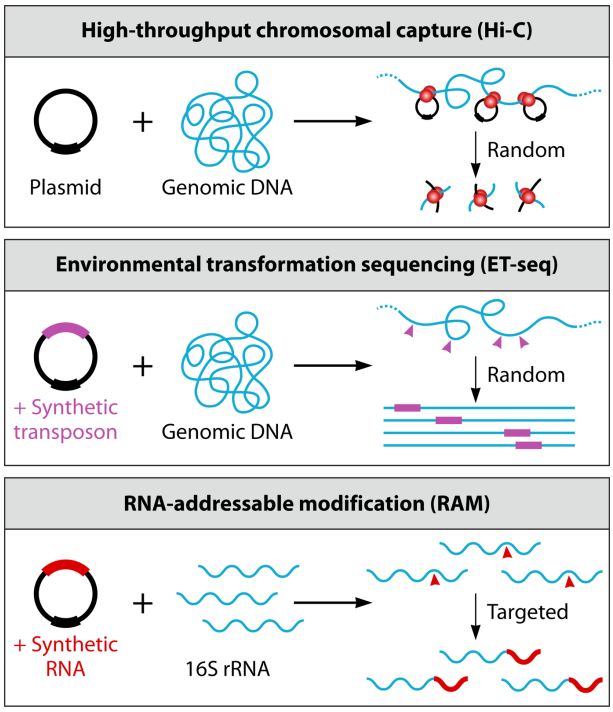

FIG 7 Strategies to monitor gene transfer host range in microbiomes. (Top) Hi-C uses chemical crosslinking to covalently attach plasmid and genomic DNA. Proteins (red) are used to promote interactions between mobile and genomic DNA, and this linked DNA is sequenced to identify which microbes participated in gene transfer. (Middle) ET-seq uses a transposase and synthetic transposon (purple) coded in the mobile DNA to modify the genomic DNA of the cells that take up the mobile DNA. Modified chromosomal DNA is sequenced to identify which microbes participated in gene transfer. (Bottom) With RAM, a catalytic RNA (red) is coded in the mobile DNA. This catalytic RNA binds and modifies a conserved 16S rRNA sequence in the microbes that take up the mobile DNA. Targeted rRNA sequencing is used to identify the organisms in the community and determine which ones participated in gene transfer.

current regulation of engineered soil microbes, how gene transfer research can play a role in risk assessment, and how this soil microbiome research will be important for responsible governance frameworks. Also, we discuss how responsible innovation raises questions about societal impact that seeks to ensure that equitable and beneficial outcomes are achieved with implementation (250–252), and how it benefits from the integration of stakeholder perspectives into the development and governance of new technologies.

Current regulation

Engineered plants have been a major driver of genetically modified organism (GMO) regulation across the globe, setting the basis for engineered microbe regulation. The European Union (EU) and the United States (US) have established two contrasting approaches (253). The EU follows a process-based approach (254), which considers the methods used to develop the product and assumes that genetic engineering carries more risk than conventional breeding (255). The EU has set up regulations for assessing the environmental release of GMOs via the Deliberate Release Directive (254), which establishes a methodology for assessing risks to the environment that may arise from GMO release in addition to post-market monitoring requirements. In contrast, the US follows a more product-based approach, which focuses on the characteristics and use of the end products. With this latter approach, GMO assessment is based on the relatedness to their natural counterparts.

On a global scale, the type and restrictiveness of regulations are highly fragmented due to countries fully or partially following these two approaches (253, 256). The rise of precise genome-editing technologies such as CRISPR allowed crops with new traits to be engineered with minimal changes to endogenous genes and without the introduction of foreign DNA (257). This advancement has led to changes in global regulation. Many countries no longer subject such genome-edited products to the same regulations as GMOs but instead consider them equivalent to conventionally bred crops (258). In the US, a renewed interest in environmental applications of GMOs has stimulated discussions within the scientific and policy community on whether regulatory frameworks and processes need to evolve so that they are more effective for governing emerging biotechnologies (259).

Recently, engineered microbes have been approved for use in agriculture. To decrease the need for energy-intensive fertilizer production, which can increase greenhouse gas production and eutrophication (260–262), companies have engineered nitrogen-fixing microbes that decrease the amount of fertilizer required in agriculture. These products have been tested with various crops (263, 264), with some products being approved in the US at the state level. These engineered microbes have been developed using intrageneric DNA manipulations, where genomes are remodeled to endow cells with new functions. With this engineering approach, DNA within a chromosome is moved around the same chromosome to program new behaviors, or DNA from another organism within the same genus is used for genetic parts. As such, the engineered cells avoid introducing foreign DNA sequences into cells. Thus, engineered microbes that are approved for use have been designed to minimize concerns about the transfer of foreign DNA to native soil microbes. One potential challenge with the use of intrageneric-focused regulations is that classifications of taxonomic relationships dynamically change with time as the research community acquires more genomic data (265). These dynamic changes could affect which microbes are subject to regulation.

Gene transfer research has the potential to shape regulations of future engineered soil microbe products, such as those developed using intergeneric DNA manipulations, where genomes are engineered using non-native DNA parts. As outlined below, using the US as an example, these regulations can be complex and involve numerous governmental agencies. The US Coordinated Framework of Biotechnology outlines regulatory policies for ensuring the safety of genetically engineered products (266–268), which distributes authority to regulate biotechnological products across the Food and Drug Administration, the US Department of Agriculture (USDA), and the Environmental Protection Agency (EPA). Each agency has its own risk assessment procedures for their respective product categories (269). The roles, responsibilities, and procedures of these agencies are evolving as new genetic engineering techniques and biological applications emerge (270–272). To assess the risks of microbes engineered using intergeneric design, where microbes are engineered using synthetic DNA that is not observed within the genus being targeted for modification, field testing is currently needed to provide insight into gene transfer frequencies and host range across different soil types and their potential ecological impacts beyond those that are intended. Some data are available for commercially relevant species (273), but much more is needed to support evidence-based regulations and decisions.

Engineered soil microbes that require federal oversight are expected to fall under the USDA and EPA. With the USDA, the Animal and Plant Health Inspection Service (APHIS) facilitates regulatory review of biotechnology products under the authority of the Plant Protection and National Environmental Policy Acts. The APHIS conducts reviews that focus on engineered microbes posing a plant pest risk. With the EPA, these biotechnologies are usually regulated by the Toxic Substance Control Act (TSCA), the Federal Food, Drug, and Cosmetic Act, and the Federal Fungicide, Insecticide, and Rodenticide Act (FIFRA). These regulatory pathways oversee different applications, such as engineered microbes developed for pesticides, biofertilizers, and bioremediation. Risk categories

that encapsulate potential hazards related to toxicity and pathogenicity are the focus of the TSCA and FIFRA risk assessment procedures (274).

Engaging stakeholders

Proactive work that is anticipatory, reflexive, and inclusive can cultivate diverse perspectives to help inform future research priorities (275, 276). Engagement provides an opportunity to align research with community priorities for soil biotechnologies and think through questions related to the relative potential benefits and risks. This includes considering how gene transfer risks relate to the potential benefits of engineered microbes (277, 278), such as enabling new strategies for recycling of waste and bioremediation (278), increasing soil health (279), enhancing ecosystem resilience in a changing climate (280), and decreasing greenhouse gas production (3). Public perception research on soil bioremediation suggests that communities may view biotechnologies more positively than other approaches for remediation (281–283). Given current uncertainties with emerging technologies that could transfer genes to soil microbes, it is important to engage with communities early in a transparent fashion and clearly frame the reward-to-risk ratio. Transparent engagement aimed at understanding community concerns and discussing uncertainties proactively can cultivate trust between technology developers and local communities to bolster beneficial societal outcomes (284). Alongside research on gene transfer in soil and biocontainment efficacy, stakeholder and public engagement research can contribute positively to achieving desired societal benefits.

Looking forward

As outlined here, gene transfer in soil is influenced by the biological, chemical, and physical characteristics of soil, which vary across geographic locations. For this reason, there is a need to establish how each of these soil properties affects gene transfer host range and efficiency involving both native soil microbes and engineered microbes being developed for soil applications. With robust measurements that calibrate microbiome-scale models of gene transfer, soil properties could be used to estimate gene transfer risk for emerging soil biotechnologies. Such models will be critical for estimating gene transfer frequencies in soils where engineered microbes are deployed and in other locations where these technologies could be unintentionally transported via erosion, dust storms, vehicle wheels, or the soles of shoes. Furthermore, there is a need for community-level discussions about the risks of emerging microbiome engineering technologies, which build upon conversations about the risks of early recombinant DNA technologies (285, 286). Rigorous gene transfer studies guided by key knowledge gaps identified through such discussions are currently needed to support effective ecological risk assessment by decreasing key uncertainties and complementing other metrics that inform risk assessment for engineered soil microbes. By overcoming these knowledge gaps, insight could be generated that helps regulators modernize their regulatory frameworks by defining field test conditions for engineered microbes that effectively quantify gene transfer risks in soil. Such a modernized framework is expected to support a more effective process to test and responsibly deploy emerging soil biotechnologies.

ACKNOWLEDGMENTS

This research was supported by the U.S. Department of Agriculture (USDA) Biotechnology Risk Assessment Grants (BRAG) program under award 2021-33522-35356, USDA Agricultural Microbiomes in Plant Systems and Natural Resources Grant FLAW-2022-09648 (accession 1030620) from the National Institute of Food and Agriculture, the Kleberg Foundation, the Rita Allen Foundation, a McSherry-Poe Award, the National Science Foundation (grants 2124307, 2227526, and 2223678), a Rice Academy Post-doctoral Fellowship, and three Science Focus Area programs funded by the U.S. Department of Energy (DOE), Office of Science, Biological and Environmental Research: Secure Ecosystem & Engineering Design (Oak Ridge National Laboratory,

ORNL), Persistence Control of Engineered Functions in Complex Soil Microbiomes (Pacific Northwest National Laboratory, PNNL), and Phenotypic Response of the Soil Microbiome to Environmental Perturbations (PNNL). These include Research Grants FWP ERKPA17 and FWP 07880. PNNL is operated by Battelle for the DOE under contract DE-AC05-76RL01830. ORNL is operated by Battelle under contract DE-AC05-379-00OR22725. This research was sponsored by the Army Research Office and was accomplished under Cooperative Agreement Numbers W911NF-24-2-0073 and W911NF-22-1-0239.

The views and conclusions contained in this document are those of the authors and should not be interpreted as representing the official policies, either expressed or implied, of the Army Research Office or the U.S. Government.

Finally, thanks to Dr. Lingchong You at Duke University for critical feedback.

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David L. Gillett completed his Ph.D. in Microbiology in 2023 under the supervision of Chris Greening at Monash University, Australia, where he studied how mycobacteria adapt to starvation by entering dormancy. During his thesis studies, he cultivated a fascination with how both environmental and pathogenic microbes thrive and survive in challenging environments. He is now driven to tackle the global challenges that these microbes are central to such as land degradation and antimicrobial resistance. To do his, he has been gaining experience in synthetic biology following his Ph.D. by working with Joff Silberg at Rice University as a Rice Academy Postdoctoral Fellow, where he is currently developing molecular tools to manipulate microbial communities.

Malyn Selinidis is currently a graduate candidate co-advised by James Chappell and Joff Silberg at Rice University in the Biochemistry and Cell Biology doctoral program. Malyn received B.S. degrees in Biochemistry and Computational Chemistry from the University of Texas at Austin as a Dean's Scholar. Since then, Malyn has been studying how soil properties affect horizontal gene transfer in soil by developing and applying synthetic biology tools in soil microbiomes. With a primary interest in environmental applications of synthetic biology, Malyn plans to continue exploring this subfield beyond graduate education.

Travis Seamons began studying synthetic biology under the advising of Tara Deans in 2018 while earning bachelor's and master's degrees in biomedical engineering at the University of Utah. His thesis work there characterized a fungal transcription factor's behavior in bacterial genetic circuits. He then joined Rice University's Systems, Synthetic, and Physical Biology doctoral program in 2023 where he began studying soil synthetic biology in Joff Silberg's group. He tracks horizontal gene transfer in soil communities and is developing new methods to program fungi in the soil. He is excited to investigate and reprogram the connections fungi make with other organisms. He finds inspiration in natural ecosystems and is happiest when immersed in greenery.

Dalton George is a Postdoctoral Research Scholar at Arizona State University, jointly appointed in the School for the Future of Innovation in Society (SFIS) and the School of Biological and Health Systems Engineering (SBHSE). He is also currently a visiting Postdoctoral Scholar at the Baker Institute for Public Policy (Rice University). Dalton completed his Ph.D. in Forestry and Environmental Resources with a specialization in Genetic Engineering and Society at North Carolina State University where his research focused on developing capacity for responsible research and innovation with emerging agricultural and environmental biotechnology. Currently, his research has expanded to study the social, ethical, and policy implications for deliberate release of genetically engineered microorganisms.

Alexandria N. Igwe earned her B.S. in biology from Howard University in 2013, M.S. in soil science from Texas A&M in 2015, and Ph.D. in microbiology from the University of California Davis in 2020. She has since held positions as an NSF Postdoctoral Research Fellow in Biology and U.S. Department of Agriculture Postdoctoral Fellow at the University of Miami and Rice University. Currently, Allie is an Assistant Professor in Biological Sciences at Virginia Tech who studies how microbes mediate abiotic plant stress and phenotypic plasticity. For the past decade, this research topic has allowed Igwe to explore interesting natural extreme environments while learning information that can support plant growth on marginal lands.

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Robert G. Egbert is a senior staff scientist in the Biological Sciences Division at the Pacific Northwest National Laboratory (PNNL). He earned a B.S. in Electrical Engineering from Brigham Young University and a Ph.D. in Electrical Engineering at the University of Washington. His research in the fields of synthetic biology and biosecurity focuses on genetic circuit design, genome editing, and functional genomics applied to understand and control the environmental persistence of engineered microbial systems.

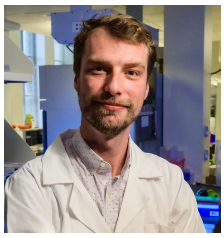
Kirsten S. Hofmockel is a Chief Scientist in the Biological Sciences Division of the Earth and Biological Sciences Directorate at the Pacific Northwest National Laboratory. She holds a joint appointment in the Department of Agronomy at Iowa State University. She earned her B.S. at Penn State in Environmental Resource Management. She went on to work as a postbac at the Smithsonian Environmental Research Center, before earning her Ph.D. in Ecology at Duke University. She has served as President of the Soil Ecology Society, and as a liaison to the U.S. National Committee for Soil Sciences. She is a Department of Energy Early Career Award recipient for research focused on how plants, microbes, and soils interact to influence carbon storage. She investigates the basic biology underpinning how soil microbial community members interact to generate beneficial ecosystem functions. She was named an AAAS Fellow AAM Fellow for her achievements in soil microbiome science.

Alicia L. Johnson is currently a Civic Science Postdoctoral Fellow at the Baker Institute for Public Policy at Rice University. She completed her B.S. in chemistry at Northwest Missouri State University and her Ph.D. in Biochemistry at the University of Nebraska-Lincoln, where she used mass spectrometry techniques and nematodes to study metabolism in drug-resistant epilepsy. During her studies, she became increasingly interested in the policy and ethical issues surrounding biotechnology, its environmental applications, and its implications for health disparities. This led her to the Baker Institute's Science and Technology Program where she now studies at the intersection of regulatory policy, environmental synthetic biology, and community engagement.

Kirstin R. W. Matthews is a fellow in science and technology policy at Rice University's Baker Institute for Public Policy and a lecturer in the Department of BioSciences at Rice University. Matthews has a B.A. in biochemistry from The University of Texas at Austin and a Ph.D. in Molecular Biology from the University of Texas Health Science Center at Houston. She is the director of the Baker Institute Science and Technology Policy Program and the Center for Health and Biosciences' Biomedical Research Program. Her research focuses on ethical and policy issues at the intersection between traditional biomedical research and public policy and emerging biotechnologies. Matthews also collaborates with Kenneth Evans and Neal Lane to understand how scientific advice is used in and provided for the federal government, including the White House Office of Science and Technology Policy and the President's Council of Advisors on Science and Technology.

Caroline A. Masiello is the W. Maurice Ewing Chair of Earth Systems Science in the Earth, Environment and Planetary Sciences department. She also served as director of the Sustainability Institute and holds joint appointments in the BioSciences and Chemistry. She received a B.A. in Physics and a B.A. in Mathematics from Earlham College, an M.A. in Environmental Science from the University of North Carolina, Chapel Hill, and a Ph.D. in Earth System Science from the University of California, Irvine. She is a biogeochemist who develops and applies new tools to understand the processes that control carbon, nitrogen, and water fluxes through the Earth system. She also works in the area of carbon market development.

Lauren B. Stadler is an Associate Professor of Civil and Environmental Engineering at Rice University. She earned a B.S. in Engineering from Swarthmore College, and an M.S.E. and Ph.D. in Environmental Engineering from the University of Michigan, Ann Arbor. Stadler is an environmental engineer whose research focuses on wastewater epidemiology, environmental antibiotic resistance, wastewater and resource recovery, and environmental synthetic biology. Her group studies horizontal gene transfer in microbial communities to better understand the spread of antimicrobial resistance and for microbiome engineering applications. Through this work, she became interested in developing technologies for tracking gene transfer in microbial communities and the responsible application of synthetic biology in the environment.



James Chappell is an Associate Professor of BioSciences at Rice University, where he holds a joint appointment in Bioengineering. He earned his B.S. in Biochemistry and a Ph.D. in Molecular Biosciences at Imperial College. He then trained as a post-doctoral scholar with Julius Lucks in Chemical and Biomolecular Engineering, initially at Cornell University and then at Northwestern University. Chappell is an RNA synthetic biologist who creates synthetic RNA regulators of gene expression, studies the portability of RNA regulators across the domain of life, generates synthetic genetic circuits capable of performing signal processing, and applies RNA-based tools for functional genomics.

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