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Soil microbiome engineering for sustainability in a changing environment

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Janet K. Jansson ✉, Ryan McClure & Robert G. Egbert

Recent advances in microbial ecology and synthetic biology have the potential to mitigate damage caused by anthropogenic activities that are deleteriously impacting Earth's soil ecosystems. Here, we discuss challenges and opportunities for harnessing natural and synthetic soil microbial communities, focusing on plant growth promotion under different scenarios. We explore current needs for microbial solutions in soil ecosystems, how these solutions are being developed and applied, and the potential for new biotechnology breakthroughs to tailor and target microbial products for specific applications. We highlight several scientific and technological advances in soil microbiome engineering, including characterization of microbes that impact soil ecosystems, directing how microbes assemble to interact in soil environments, and the developing suite of gene-engineering approaches. This Review underscores the need for an interdisciplinary approach to understand the composition, dynamics and deployment of beneficial soil microbiomes to drive efforts to mitigate or reverse environmental damage by restoring and protecting healthy soil ecosystems.

To feed Earth's growing human population that is estimated to reach 8 and 9.8 billion by 2025 and 2050, respectively[1], new solutions provided by microorganisms are required. One promising approach to sustainably improve crop production and soil health is to harness the beneficial properties of soil microorganisms (Fig. 1). Certain soil microorganisms can be harnessed to promote plant growth by acting as fertilizers or pesticides, thereby reducing reliance on synthetic chemicals. These microorganisms may also be used to improve plant productivity in marginal soils, such as saline or alkaline soils, and/or during periods of drought[2]. In addition, soil microorganisms have been used for remediation of organic and heavy metal pollutants in soil[3,4]. Because soil microorganisms are integral to carbon cycling and sequestration, they also hold potential for capturing and storing atmospheric carbon belowground[5].

Our planet is currently experiencing unprecedented anthropogenic-induced changes, such as climate change and introduction of pollutants, that are having dramatic impacts on soil ecosystems and the beneficial services that they provide. Examples of climatic changes that impact crop-production systems include rising seawater levels in coastal areas, increases in areas of saline and alkaline soils, changes in precipitation patterns that result in more intense periods of flooding and drought, and increased wildfire frequency and intensity[6]. Increased global temperatures cause permafrost thaw at the poles, resulting in changes in landscapes, together with associated shifts in aboveground vegetation and belowground microbial communities[7]. Thawing permafrost also releases additional carbon dioxide and methane to the atmosphere as microbial activity increases, leading to feedback cycling of increased warming as atmospheric carbon drives a greenhouse effect[8]. Other negative anthropogenic impacts that are compounding those directly caused by climate change include deforestation, intensive agricultural practices that result in soil erosion and loss of carbon[9,10], and exposure of soil ecosystems to pollutant chemicals[3,11]. The increased stresses placed on natural and managed soil ecosystems from anthropogenic impacts have spurred research into alternative approaches to reduce these negative environmental impacts.

Here, we will explore current needs for microbial solutions in soil ecosystems, report how these solutions are being developed and applied and elaborate on the potential for new biotechnology breakthroughs to tailor and target microbial products for specific

Earth and Biological Sciences Directorate, Pacific Northwest National Laboratory, Richland, WA, USA. ✉e-mail: janet.jansson@pnnl.gov

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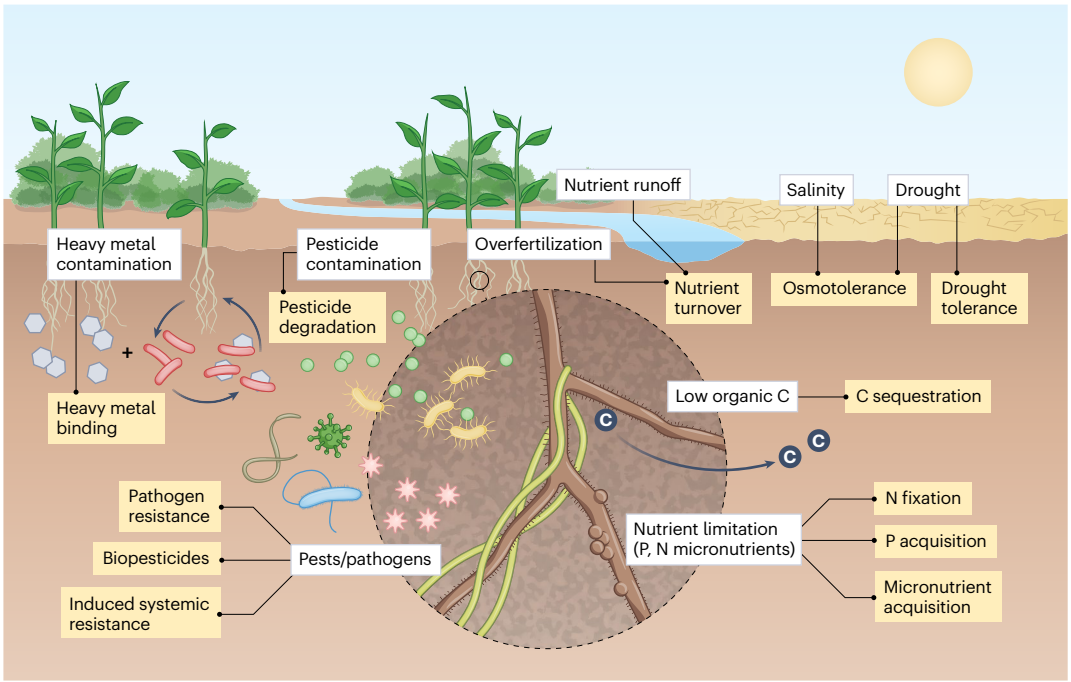

Fig. 1 | Soil microorganisms carry out key ecosystem services and have the potential to help mitigate a variety of deleterious anthropogenic impacts on soil ecosystems. White-boxed text indicates different deleterious anthropogenic impacts. Yellow-boxed text indicates different biological mitigation strategies. The rhizosphere is highlighted in the circle in the center, with associated mycorrhizal fungi (green threads), nitrogen-fixing nodule-forming bacteria (brown nodules on the root) and a variety of root-associated microorganisms that are indicated by the pink and yellow symbols. Top left, heavy metals (gray hexagons) that can be bound by heavy metal-binding bacteria (red rods) and pesticides (light green dots) that can be degraded by rhizosphere microorganisms. Bottom left, potential pests that include viruses (dark green sphere with knobs), bacteria (blue rod) and nematodes (green ribbon). C, carbon; N, nitrogen; P, phosphorus.

applications. We also highlight scientific and technological advances in soil microbiome engineering, which we classify into three pillars (Fig. 2): (1) the discovery and characterization of microbes that impact soil ecosystems, (2) understanding and directing how microbes assemble in structured communities to coexist and interact in soil environments, and (3) the developing suite of tools to genetically manipulate soil microbiomes to control composition, function and environmental persistence. An interdisciplinary approach to understand the composition, dynamics and deployment of beneficial soil microbiomes will drive efforts to mitigate or reverse severe impacts of anthropogenic activity by restoring and protecting healthy soil ecosystems.

The potential and challenges of harnessing soil microorganisms

Soil microorganisms are highly abundant and taxonomically diverse, with estimates of billions of microbial cells and thousands of species occurring in a single gram of soil[12]. However, the majority of soil microorganisms have not yet been cultivated, which makes them difficult to study. In addition, microbial abundance and community composition vary widely across different soil types and geographic regions[13]. The soil microbial community, or soil microbiome, is also influenced by environmental variables. Increases or decreases in types and functional activities of soil microorganisms can occur depending on how the environment is altered by changes in temperature, soil moisture and other variables[14].

Soil microorganisms include bacteria, archaea, fungi, protists and their respective viruses. Together, soil microorganisms interact to perform roles that are essential for normal ecosystem function. These roles include cycling of carbon and other nutrients, support of plant growth, degradation of pollutants and others. Some microbial populations carry out specific keystone functions for agriculture production and/or ecosystem sustainability. For example, some species of rhizobia fix atmospheric nitrogen and provide it in a form that can be used by plants and other microorganisms[1]. Other species carry out specific steps in cycling the resulting nitrogenous compounds[15] and other essential nutrients. Some soil microorganisms decompose carbon compounds that comprise soil organic matter. However, most functions carried out by specific microorganisms in soil remain largely uncharacterized. This knowledge gap is primarily due to the difficulties in studying soil microorganisms due to (1) the physical heterogeneity of the soil matrix, (2) the high taxonomic and chemical diversity, with thousands of species and metabolites and millions of potential interactions, (3) the difficulty in studying microorganisms at the microscale at which they function in situ, and (4) the fact that most soil microorganisms have not yet been cultivated in a laboratory setting, and their functions therefore remain uncharacterized. Because uncultured microorganisms may have beneficial functions that are not yet characterized, they represent a potential opportunity that could be further explored[16].

Despite these challenges, several methods have been developed and applied for the study of microorganisms in soil. Cultivation on solid or liquid medium has traditionally been used to obtain specific microbial isolates. The advantage of cultivation is that it facilitates biochemical and physiological studies of the isolated strains. Advances in culture-based approaches have helped to increase the number of known soil microbial isolates. For example, dilution-to-extinction culturing was successfully used to isolate hundreds of heterotrophic bacterial taxa from soil[17]. Microcultivation is an approach that relies on in situ systems that mimic the natural environment to favor growth of difficult-to-cultivate microorganisms. This was used to isolate new strains that degrade oil from contaminated soil[18]. In addition, a high-throughput cultivation approach, 'culturomics', was recently developed that uses machine learning and robotics to rapidly obtain isolates as colonies on microtiter plates[19]. A culturomic approach may also be used to identify soil taxa.

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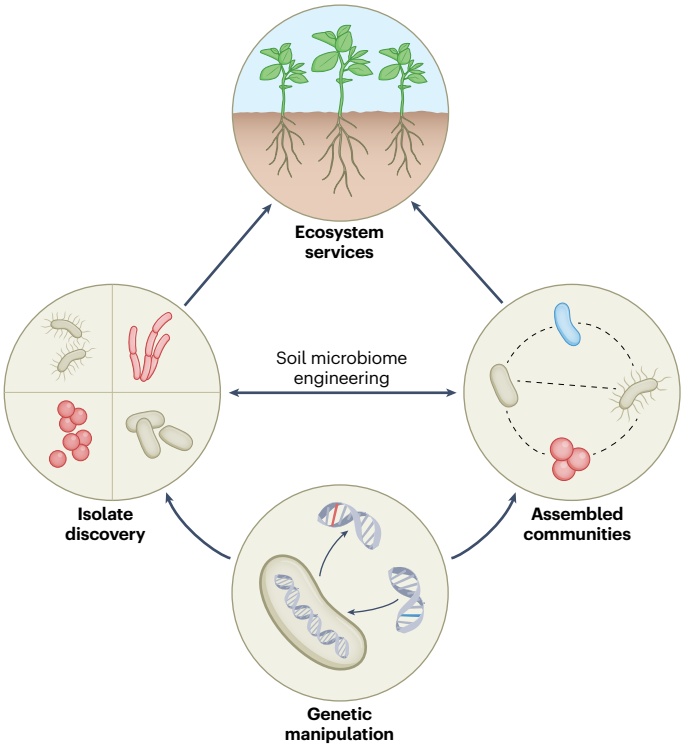

Fig. 2 | The three pillars of soil microbiome engineering are isolate discovery, assembled communities and genetic manipulation. The interplay among these pillars will lead to advances in providing soil ecosystem services, including (1) mapping microbial functions to organisms and genes, (2) improved ecosystem function through the establishment of naturally evolved and synthetic microbial communities enhanced by engineered isolates, and (3) promoting biosafety and biosecurity goals by understanding colonization and containment dynamics at the ecosystem scale.

DNA sequencing is an approach widely used to determine the composition of soil microbiomes[20]. Specifically, amplicon sequencing of 16S rRNA genes for bacteria and archaea and 18S rRNA genes or intervening sequences for Eukarya is routinely undertaken[20]. Sequencing bypasses some of the limitations of cultivation because it captures the entire community, including cells that are inactive and/or dormant. However, a drawback is that dead cells may also be represented in the resulting sequence data. Another disadvantage of this approach is that it is only able to identify taxonomic signatures. This does not directly provide information on their functional potential. By contrast, metagenomic sequencing of DNA has the advantage of ascertaining the taxonomic and functional–genomic structure of the soil microbiome. However, metagenomic approaches are still challenging for certain soil types and for identifying low-abundance but potentially important species[21]. Higher-throughput sequencing can help with the depth-of-coverage issue, but this can lead to other problems related to high computational demands for large datasets[22]. In addition, quantitative analysis is challenging in the absence of absolute-abundance data. Most sequencing-based studies primarily rely on relative-abundance data. This can make it difficult to distinguish the actual abundance changes of a specific taxon from changes due to one species reducing in abundance, leading to greater fractional abundance for other community members[23].

Many of the same issues that hinder DNA sequencing also apply to microbiome RNA sequencing (metatranscriptomics). Additional challenges include the fact that RNA is generally more unstable than DNA, is more difficult to extract from soil and is composed primarily of ribosomal RNA, which can make sequencing more difficult for more-informative messenger RNA transcripts that indicate function (reviewed in ref. 24). Depth of coverage is especially critical for metatranscriptomics, as individual genes are expressed at levels spanning orders of magnitude, thereby making identification of expressed genes that are transcriptionally regulated and/or from low-abundance species even more challenging. A promising technique that overcomes several of these issues is to incorporate stable isotopes (for example, ^{13}C or ^{3}H) into DNA or RNA during incubation under specific conditions. Subsequent extraction and sequencing of the heavy-labeled DNA or RNA provides information on specific microbial populations that are growing or incorporating specific substrates[25].

Study of community proteins (metaproteomics) provides information about specific microbial functions in soil. For a protein to be expressed, it must have already passed regulatory bottlenecks during transcription and translation. Therefore, detection of a protein provides an additional level of confidence that a function conferred by a particular protein actually occurred in the system of study. However, coverage issues and extraction challenges can also affect recovery and analysis of proteins expressed by soil microbiome constituents used for metaproteomic analyses[26]. Another option for detection of specific proteins produced by microorganisms is to use activity-based probes[27]. These are molecules that mimic enzyme targets and bind to them, allowing for their subsequent isolation and analysis[28]. If the enzymes of interest are intracellular, the producing microorganisms can also be isolated, thereby providing information on which taxa are expressing a function of interest[27]. Even when overcoming these challenges, obtaining high-quality omics data is only the first step toward gaining an understanding of microbial communities and functions in soil. Computational annotation and bioinformatic interpretation of omics data are also substantial challenges. This is largely due to the lack of fundamental knowledge regarding soil microbial processes. Many of these processes are carried out by species that are either completely unknown or, if they have been identified, our understanding of their fundamental biology is only partial, thus leading to gaps in our interpretation of data and the role of certain species in driving observed outcomes.

Several bioinformatic tools have been developed for interrogation (DRAM)[29] and integration (MEMPIS, XCMS) of omics datasets[30,31]. The increasing availability of omics data provides an opportunity to mine the soil microbiome for specific species, genes, enzymes and/or functions that can be exploited for engineering applications. For example, metagenomes from saline soil were mined for genes that encode tolerance to salt stress[32], from which a new cellulase was cloned from a sugarcane soil metagenome[33], and a new esterase was isolated from a plant rhizosphere soil metagenome[34]. Several studies have also used amplicon sequencing to target specific microbial populations for isolation, such as previously uncultivated *Verrucomicrobia* spp. from the potato rhizosphere[35]. Ideally, microorganisms with desired functions can be used as microbial inoculants for environmental-engineering applications in soil. Here, we will discuss different microbial-inoculation strategies that have been developed, tested and, in some cases, applied.

Characterization of specific isolates for improved plant performance

Traditionally, microbial inoculants for soil applications have relied on single organisms with beneficial functions that are isolated in culture, grown in large volumes and applied to the field. A classic example is the use of rhizobial inoculants to provide fixed nitrogen to legume crops in place of synthetic fertilizers[1]. Arbuscular mycorrhizal inoculants have also been widely applied[36] to supplement provision of nutrients to plants, especially phosphorus in P-limited soils. Other fungal inoculants include biocontrol strains of *Trichoderma* that attack plant-pathogenic fungi[37].

Several methods have been developed for cultivation of beneficial microorganisms for environmental applications. For example, members of the barley root microbiome were isolated using a trap–bait approach[38]. This approach used clay chips to capture most of the

microorganisms present in the rhizosphere. Potentially beneficial strains can also be isolated by selective enrichment. For example, beneficial rhizosphere microorganisms were isolated following enrichment on plant root exudates[39].

Microbial inoculants for applications that benefit plants are often targeted to the root zone (rhizosphere) and are thus referred to as plant growth-promoting rhizobacteria (PGPR). Successful examples include the use of rhizobial inoculants to enhance soybean yields in Kenya[40] and Brazil[41]. Additional applications of PGPR include alleviation of abiotic stress in plants, promotion of plant growth by increasing nutrient availability and production of plant hormones and siderophores[1]. For example, auxin biosynthesized by microorganisms is thought to be involved in beneficial plant–microbe interactions either indirectly via signaling pathways or directly by changing the root architecture and growth of the host[42]. Auxin from microorganisms (indole-3-acetic acid) also has several positive effects on plant growth, including promoting formation of root hairs and lateral roots[43]. Other PGPR have been applied to mitigate plant salinity stress in a variety of studies (reviewed in ref. 44). For example, a strain of *Bacillus licheniformis* could improve the water-use efficiency of maize plants when compared to uninoculated plants[45].

Although several microbial species have been commercialized for delivery as PGPR to crop fields, their effects on plant performance are often inconsistent[1]. In some cases, the applied strains perform poorly in the field environment in which they must compete with locally adapted microorganisms[46], and therefore the desired increases in crop yields are not realized[47]. For example, tracking the abundance of inoculants added to promote growth of maize plants showed that levels of inoculants peaked at 50–100 d after inoculation but began to fall thereafter[48]. Alternatively, some single-strain inoculants have even been shown to survive for years. This ability is not well understood and depends on the environment and type of inoculant, among other factors. For example, a single fungal strain added to soil was detected up to 15 years after the initial inoculation[49]. A similar result was found with a *Bacillus* strain that was inoculated into soil and detected up to 3 years after application[50].

One of the major complications with application of PGPR is that individual formulations with a specific mode(s) of action may therefore limit their application to specific locations and/or soil and plant types[1]. Introduction of non-native inoculants can also have unintended consequences on the local soil microbiome and the ecosystem functions they carry out[51]. This was found to be the case for some single inoculants of *Rhizobium* that led to long-lasting changes in both the bacterial and fungal communities of the soil[52,53]. In addition, certain widely used fungal inoculants, such as arbuscular mycorrhizal fungi, have not been adequately assessed for their potential ecological impacts[54]. The introduced fungi may behave as invasive species if applied in high enough concentrations. Because the diversity of soil fungi is known to positively correlate with ecosystem services, an imbalance could have negative repercussions[55]. Therefore, selection of field inoculants should not only be based on their beneficial properties but also on their potentially negative impacts on the soil ecosystem.

Assembly of natural and artificial microbial communities

Although most soil inoculants to date comprise single isolates, there is increasing interest in application of communities of microorganisms ('consortia'), to provide greater resilience to stress and to improve their ability to survive competition from resident microorganisms to enable them to persist and perform their intended functions. Multi-strain inoculations also allow for a greater breadth of ecological functions to be added to the soil microbiome and a larger palette for genetic manipulation of functions that can be introduced to soil. Development of microbial consortia for soil applications proceeds along one of two routes: (1) combining isolates into synthetic communities ('SynComs') and (2) deriving naturally enriched natural communities with reduced complexity ('NatComs') (Fig. 3). SynComs are typically built from a relatively simple (2–5-member) pre-existing collection of isolates to facilitate their formulation and environmental application and to aid in studies of interspecific interactions[56–59]. Technological developments for building SynComs include high-throughput screening of potential combinations of species[60] and selective culturing. Once the optimal species are identified, they can be combined into specific formulations to carry out a given environmental function. SynComs built from characterized isolates offer several benefits over less-characterized systems. As they are built from individual isolates, the community composition is already known. In addition, the individual isolates can be characterized separately for their performance and genomic data. The role of each member can be defined using leave-out experiments or genetic manipulation[61]. An example of a SynCom for soil is a collection of three isolates from the soybean rhizosphere that was named the hitchhikers of the rhizosphere (THOR)[62]. The individual constituents of THOR were selected based on their potential influence in the rhizosphere and their root-distribution patterns. Only isolates that were genetically tractable were selected. THOR has since proven to be a valuable model community for understanding microbial community interactions in a genetically tractable system. Another example of a SynCom is a community of 16 species built from soil surrounding switchgrass plants[63]. While defined SynComs do have some benefits, their main drawback is that sets of isolates must first be collected, purified and studied in isolation. Only species that grow in pure culture in a laboratory setting are practical for SynComs. Furthermore, the species that are combined may not represent species that naturally interact, which may reduce their ability to persist together in the soil environment.

An alternative approach for development of mixed inoculants is to enrich microorganisms from the soil environment in which community members have naturally evolved to interact with each other (NatComs) (Fig. 3). A selective enrichment process combined with a dilution approach can be used to reduce community richness and to select for communities with a specific desired trait or combination of traits[64]. For example, Model Soil Consortium 1 (MSC-1) was enriched from soil using chitin as a substrate[65]. The resulting community contained approximately 30 members that demonstrated several positive interactions during chitin decomposition.

NatComs have some key advantages over SynComs; the major one being that, as they are allowed to develop naturally, they are more likely to represent native interactions. The user or developer has little input on which species are retained or lost in the NatCom as this is driven primarily by microbial ecology. In some cases, communities can be developed that have advantages of both NatComs and SynComs. This is the case for Model Soil Consortium 2, which is a combination of eight isolates derived from the MSC-1 NatCom[66]. As this consortium is a combination of individual isolates, it has elements of a SynCom. However, because the isolates were selected from a naturally evolved community (MSC-1), this implies that the interactions between community members are more likely to reflect those driven by microbial ecology in nature rather than an arbitrary assembly of soil isolates. Examination of Model Soil Consortium 2 has shown that only a subset of species contributes to the metabolism of abundant carbon and nitrogen sources, likely sharing these breakdown products with other members of the consortium[66]. These results suggest that the ability to metabolize a major nutrient source is not the only factor determining whether a species will be successful in a community context. Rather, this study suggested that the fundamental niche size of a species (how many carbon sources it can metabolize) is the main determinant of success, at least as measured by abundance.

One of the main advantages of reducing the soil microbiome complexity is the ability to better delineate possible interactions between species that give rise to the functions of the greater soil microbiome. Sequencing data based on the 16S rRNA gene are normally used to

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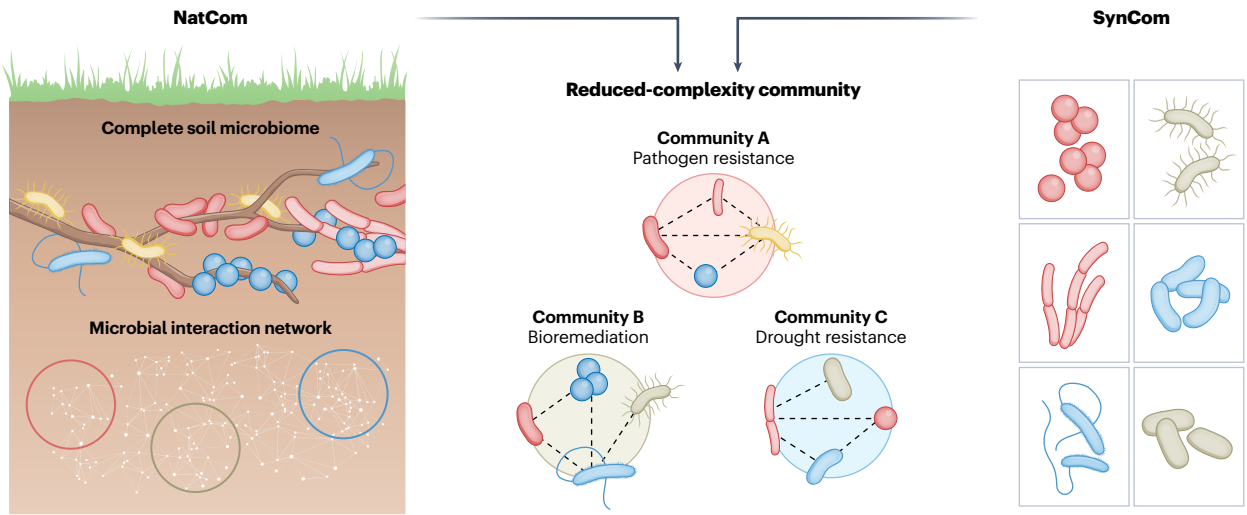

Fig. 3 | Design of tractable communities of soil microbes. The design of tractable communities usually proceeds along one of two routes. In the naturally evolved route (NatCom), naturally interacting members of the soil microbiome are enriched from soil. In a SynCom, individual isolates are combined to form a community. A SynCom leads to much greater control of the community's constituents as well as more inherent knowledge of the potential processes, but the individual SynCom members may not naturally interact and may be less stable in the environment than a NatCom.

infer a network in which taxa are linked based on their co-abundances across a range of samples and/or experimental conditions. Networks can be built using Pearson or Spearman correlations, but other network-inference tools have also been used (reviewed in refs. 67,68). Once inferred, networks have been used to predict species interactions (that is, those that are strongly correlated in abundance), the nature of the interaction (a positive correlation may indicate cooperation, and a negative correlation may indicate competition), and keystone members in a community (by identifying central nodes in the network) (Fig. 4). For example, network analysis was used to identify potential keystone species in MSC-1, such as members of the *Streptomyces* and *Rhodococcus* genera[65].

Network inference can be a powerful tool to view potential interactions between species, but care must be taken when interpreting coexpression networks. Species co-abundance networks are normally derived from macroscale amounts of soil (0.1–1 g) and thus include species that may not normally co-occur on a microbiological scale. In addition, mechanical processing of soil samples (sieving, homogenizing, bead beating, etc.) destroys the native structure of the soil. Therefore, co-occurrence networks are not in themselves definitive proof of species interactions. Indeed, new studies have suggested that interaction networks, in some cases, may be driven more by the richness of the samples rather than by individual interactions[69]. The main advantage with network analyses is that they provide an integrated and high-level view of the system that can point researchers in the right direction to pursue more targeted experiments to test specific hypotheses.

Environmental applications of soil inoculants

Soil inoculants have been developed for a variety of applications, including bioremediation of pollutants, improvement of soil fertility and support of plant growth (Fig. 1). For example, the toxic pollutant 2,4-dinitrotoluene was degraded in contaminated soil by an engineered strain of *Pseudomonas putida* in association with plants[70]. However, the majority of recent studies have instead focused on promotion of plant growth. We therefore mainly highlight that application in this Review.

There is increasing interest in designing SynComs and NatComs (Fig. 3) to promote plant growth and resilience in the face of climate change and other stress conditions. Design of SynComs for soil applications can, however, be impeded by lack of understanding of the complexity of natural interactions that can occur between community members in the environment. One approach that can circumvent this is to design SynComs based on knowledge of the core microbiome, which consists of a specific combination of microorganisms that are recruited by a given plant species (reviewed in ref. 63). For example, the core microbiome associated with 12 cultivated varieties belonging to the *Citrus* genus has been defined as *Pseudomonas*, *Agrobacterium*, *Cupriavidus*, *Bradyrhizobium*, *Rhizobium*, *Meorhizobium*, *Burkholderia*, *Cellvibrio*, *Sphingomonas*, *Variovorax* and *Paraburkholderia*[71]. By contrast, that of potato has been defined as *Bradyrhizobium*, *Sphingobium*, *Microvirga*, *Blastococcus* and *SMB53* (ref. 72). The hypothesis behind this strategy is that the core microorganisms are adapted to a given plant species and can serve to benefit the growth of that plant across a variety of habitats. By focusing on species that comprise the core microbiome, it is possible to circumvent the assembly challenges with a greater diversity of microorganisms. While these approaches have been met with success, there are drawbacks related to the fact that there can be minor differences between strains and species of the same genus that can lead to major differences in function, particularly in specialized metabolism. This means that building a SynCom from available isolates defined at the genus (or even species) level can be misleading. One alternative is to use isolates cultivated directly from the soil site of interest for construction of native SynComs to increase the potential for inclusion of site-adapted strains with desired functions.

There are several examples of SynComs that have been built for specific soil environmental applications. A SynCom was developed to provide tomato plants protection against bacterial wilt disease caused by *Ralstonia solanacearum*[73]. The SynCom consisted of four Gram-positive bacteria and helped to confer disease resistance to tomato plants. Another example was the use of a SynCom to help alleviate water-deficit stress in maize[74]. This SynCom consisted of 17 isolates from the sugarcane core microbiome[75]. Application of this SynCom may have helped to stimulate production of plant osmolytes, as suggested by the enrichment of ABC-type transporters for different osmolyte molecules in the genomes of the community members[76]. This is an example of cross-species protection because the same SynCom was beneficial to both sugarcane and maize (Poaceae)[75,76].

While applications of SynComs to soil have led to some benefits, there are also important concerns about applying non-indigenous microbial consortia to a soil ecosystem. In some cases, the applied SynComs can alter the existing microbiome, shifting the abundances

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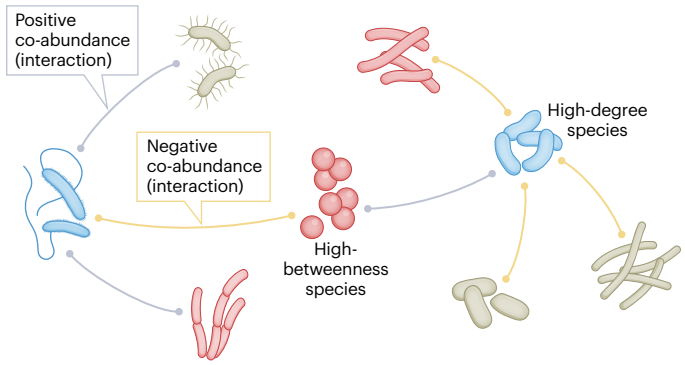

Fig. 4 | Schematic of a co-abundance network for microbial taxa. Microbial taxa are represented with distinct shapes and colors. Potential interactions between species (for example, exchange of metabolites, predation, cross-feeding) are indicated by lines connecting taxa. These lines are drawn based on co-abundances of these species across a range of conditions or samples. Gray lines indicate positive co-abundances and may point to positive interactions, while yellow lines indicate negative co-abundances and putative negative interactions. By using this approach at a community level, taxa that are critical to the community can be identified. These may be species of 'high betweenness' (acting as a bridge point connecting two separate clusters of taxa) or 'high degree' (having a large number of connections to other taxa).

of certain rhizosphere members based on interactions and recruiting new members from the existing rhizosphere community. This was found to be the case with the maize SynComs described above[74,75]. Here, the added SynCom competed with the existing microbiome and displaced it, in the process promoting maize growth. However, these experiments were carried out in a laboratory setting and over a relatively short timeframe (4 weeks). In another case, SynComs directly added to field sites had a positive effect on plant growth[77]. To what extent the members of the added SynCom were still present was not evaluated in this case, but the effect of the SynCom addition was clear even when applied in the context of an existing community[77]. Another study found a similarly positive effect of SynComs in promoting the growth of wheat. The persistence of the SynCom was monitored in the native soil, finding that it dropped dramatically after 2 weeks[78]. These conclusions and other work[79] suggest that some SynComs may be short lived in native soil, and their use may therefore be limited by the need for repeated applications.

Although a single-strain inoculum has the advantage of ease of growth and formulation compared to a multi-species inoculum, consortia potentially allow for a wider range of desirable traits to be added to soil and take advantage of the inherent benefits of microbial interactions driving emergent functions. However, it must be acknowledged that use of single inoculants or consortia depends on the specific environmental application. A meta-analysis of over 400 experiments showed that single-species inoculations increased crop growth up to 41% more compared[80] to inoculations with consortia of microorganisms. In addition, a combined treatment with three fungal strains was less efficient at promoting strawberry plant growth than a treatment consisting of a single strain[81]. Another study with tomato plants found no synergistic effect of adding two species (*Bacillus subtilis* and *Azospirillum brasilense*) compared to each species individually[82]. It should be noted that many of the studies in these analyses were focused on plants in controlled environments over short timeframes. In natural field soils, combined inoculations may perform better. For example, three strains of bacteria (belonging to *Microbacterium*, *Stenotrophomonas* and *Xanthomonas*) that were specifically recruited in the rhizosphere of *Arabidopsis thaliana* plants provided control of the downy mildew plant pathogen when they were inoculated together in soil, but the individual-strain inoculants were not able to do so[83]. Because multi-species communities have been used for far less time than single-strain inoculants, a direct comparison of the two approaches does not provide equal weight to the scientific history of each approach. Another approach to consider is inoculation with fully complex communities. This approach relied on soil translocation to successfully to restore degraded soils[84]. Ultimately, the use of single species or communities comes down to the specifics of the plant being targeted, the environmental conditions and the desired output.

Strategies for genetic manipulation of microorganisms

New synthetic biology tools are continually being developed for genetic transformation, strain optimization and biocontainment of soil microorganisms[85] that are designed to address current environmental and sustainability challenges (Fig. 5). Synthetic biology is a field of science that incorporates aspects of both molecular biology and genetic engineering[86]. For example, advances in synthetic biology and genetic engineering tools are being used to discover and enhance native microbial functions[87,88], introduce new traits[89], develop biological sensors[90], eliminate bottlenecks that limit critical pathways[91] and combine multiple beneficial traits into a single organism[92]. Additionally, the environmental persistence of engineered functions can be controlled through containment measures at the species and community levels. To meet growing needs for synthetic biology studies on environmental isolates[93], considerable advances have been made in the following areas: (1) developing genetic tools for new soil microbial isolates, (2) high-throughput genome-scale gene editing, (3) in situ microbial community editing using conjugation and CRISPR–Cas technologies and (4) platforms to reproducibly interrogate plant–microbe interactions via microscopy and multiomics. An important ethical consideration for engineered microbes in soil environments is the potential ecological and biosecurity impacts of their environmental proliferation. Despite substantial advances in the field, challenges remain in predicting soil microbiome functions and interactions from available data and algorithms and in optimizing engineered microbiome functions in the field.

Most synthetic biology to date has been performed on model laboratory isolates that are used as an experimental chassis for DNA circuit and metabolic pathway design. For soil applications, the bacterium *P. putida* has been used extensively as a model soil microbial chassis[94]. Among other relevant applications for soil, *P. putida* has been used as a model strain for bioremediation of environmental pollutants[3]. The explosion of interest and momentum in studying soil isolates and communities are likely to add hundreds of new microbial hosts compatible with synthetic biology practices. To date, a maize-associated, nitrogen-fixing strain of *Klebsiella variicola* was engineered to increase fixation of atmospheric nitrogen in the field by engineering the genome to relieve gene-regulatory barriers[91]. Furthermore, root-associated *Ralstonia* and *Pseudomonas* strains have been engineered to increase bioavailable phosphate for plant growth by breaking down phytate[89].

Genetic transformation of new hosts

Synthetic biology approaches have advanced considerably in the last decade to enable targeted optimization and design of microorganisms, including soil isolates, for specific sensing or metabolic functions. Gene editing is a fundamental tool of synthetic biology that has undergone major recent advances for non-model hosts. Directed gene edits are primarily carried out through native recombination of homologous DNA, enhanced by approaches that increase recombination efficiency[92] or allow the selection of gene edits without antibiotic markers[95]. Precision gene editing requires whole-genome sequence data and methods for genetic transfer into the cell.

Genetic transformation efficiency can be highly variable across microbial taxa, even at the genus level, and remains a technical challenge for environmental isolates. Optimization of intracellular

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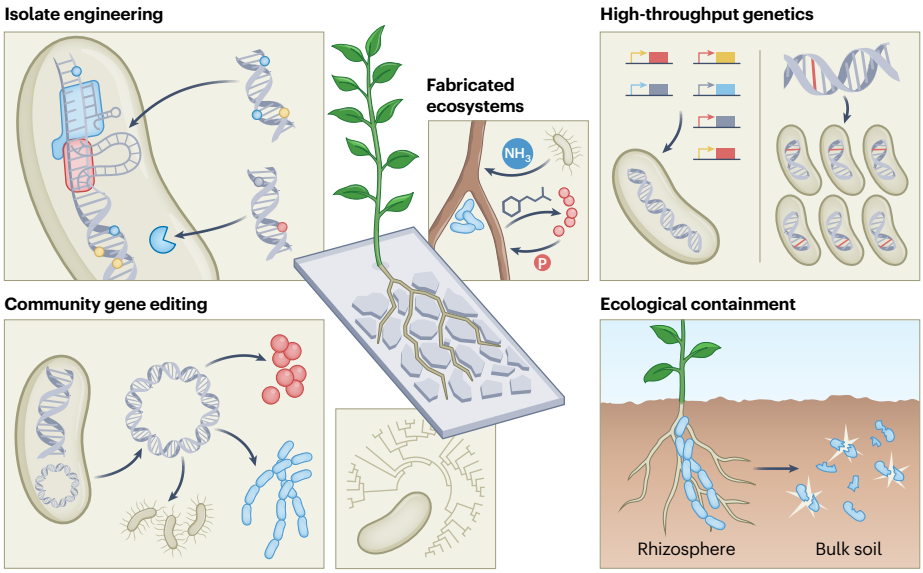

Fig. 5 | Platforms to edit and evaluate genome modifications of soil microbiome isolates. Platforms and approaches in the image, starting clockwise from the top left: (1) isolate engineering: establishing genetic tools for soil microbiome isolates must overcome transformation barriers, such as host-specific methylation patterns (colored dots on DNA strands), and use genetic parts for gene editing and gene expression that are compatible with the target host. (2) Fabricated ecosystems: the design, build, test and learn cycle for soil microbiome engineering will be substantially accelerated by field-simulant fabricated ecosystems that reveal plant–microbe interactions and can be reproducibly deployed across research laboratories. (3) High-throughput genetics: when employed at the genome scale, high-throughput genetic tools can indicate microbial function in the context of microbial communities and accelerate the development of engineered functions. Community contexts provide insight into strategies to control the environmental persistence of engineered functions. (4) Ecological containment: specific strains can be engineered to carry out a desired function at the target site, for example, the rhizosphere. In this example, either engineering metabolic dependency on the plant or introduction of genes for which the products kill the cells when they leave the rhizosphere can be effective biocontainment options. (5) Community gene engineering: this approach relies on plasmids to introduce functional genes to other members of the soil community. Plants and bacteria are not drawn to scale.

DNA-delivery efficiency has been realized through advances in electroporation methods[96,97]. Low-volume microfluidic electroporation enabled the transformation of combinatorial libraries[97], whereas large-volume, continuous-flow microfluidic electroporation enabled the transformation of large mutant libraries at scale for strains with lower transformation efficiencies[96]. A major barrier to DNA stability after entry in the cell is cellular defense mechanisms, such as the degradation of introduced nucleic acids via restriction endonucleases. Restriction endonucleases degrade intracellular DNA that does not contain the host's native methylation patterns. To overcome this barrier, heterologous expression of methyltransferases from a target host in a surrogate strain, such as *Escherichia coli*, enabled efficient transformation of plasmid DNA by mimicking the methylation patterns of the target host[98]. Native methylation patterns have been decoded through single-molecule pore sequencing and custom workflows[99], which are likely to extend genetics to new soil isolates by creating surrogate methylation strains that generate host-compatible DNA.

High-throughput strain optimization

Certain technologies allow for high-throughput and tailored optimization of microbial strain phenotypes (Fig. 6). High-throughput approaches for genome integration and transformation enable optimization of strain function and genetic stability in genetically tractable hosts. These approaches include the creation and evaluation of gene expression libraries across taxonomically diverse microbes[100,101], engineered protein libraries[102] and combinatorial pathway-expression libraries[103]. These approaches overcome uncertainties regarding sequence–function relationships by creating and screening massive collections of functional variants. The advent of deep-learning approaches applied to synthetic biology[104,105] could rapidly accelerate the realization of designer functions in soil microorganisms through genome engineering.

Multiple genome-editing approaches have been applied to engineer genetically stable bacterial strains that could be used for soil microbiome engineering. Chromosomally integrated DNA is more genetically stable and does not require antibiotics that are generally necessary to maintain plasmid DNA and impractical for deployment in soil environments. Recombineering is a short-homology recombination method designed to integrate single-stranded oligonucleotides or double-stranded DNA into microbial genomes and can be accomplished efficiently using homologous sequences as short as 35 bp[106]. This approach was adapted to create CRISPR–Cas9 gene-edited mutant libraries that are genetically barcoded to map functions to individual genes in a high-throughput, genome-wide manner[107]. Another successful approach to genetic transformation is the construction of an engineered or minimal integrative and conjugative element (mini-ICE) in *B. subtilis* to specifically engineer the genomes of target hosts[108]. The mini-ICE system removes fragments that enable jumping from organism to organism, resulting in 'one-hop' modifications of microbial genomes, with efficacy in Gram-positive bacilli, including in soil environments.

Phage recombinase-based methods have emerged as powerful techniques for engineering large gene clusters and creating high-throughput genome variant libraries. Recombinase-assisted approaches are more universally effective than recombineering strategies, which have primarily found success in model hosts such as *E. coli*[92]. By contrast, chassis-independent recombinase-assisted genome engineering (CRAGE)[109] demonstrated the integration of large biosynthetic gene clusters in dozens of proteobacteria hosts using Cre–*lox* recombination and was used to show that engineered bacteria can release phosphate to plants[89]. Using a collection of up to ten independent integration sites and their cognate recombinases, serine recombinase-assisted genome engineering (SAGE)[101] has been demonstrated to integrate heterologous DNA constructs in

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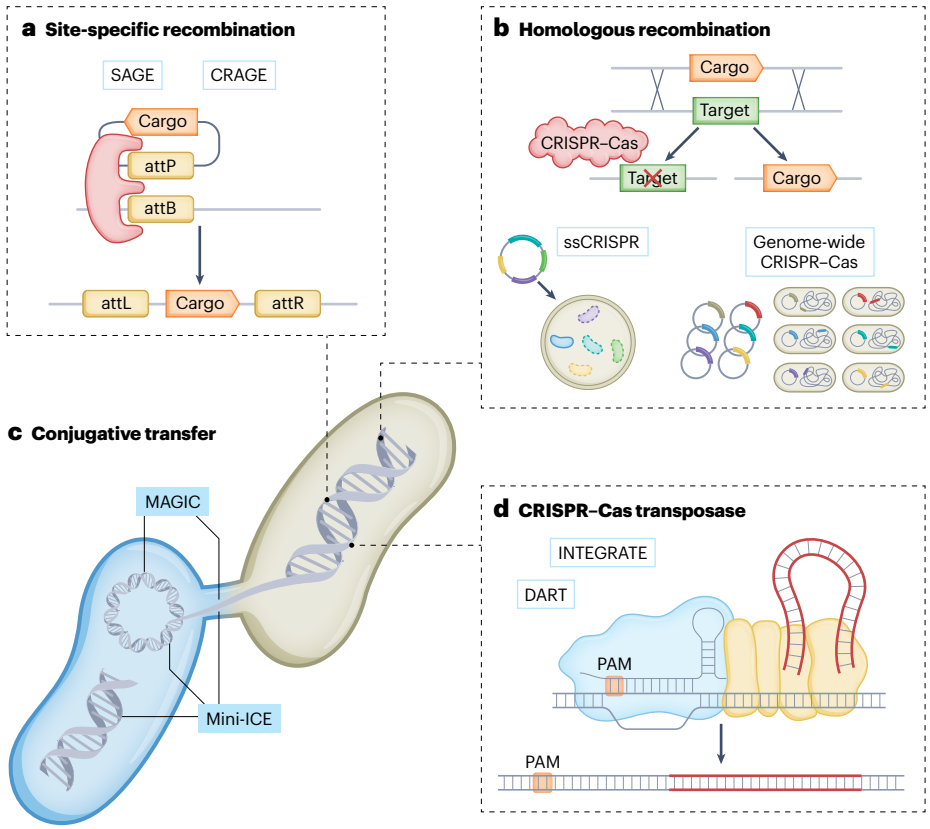

Fig. 6 | Genome-editing approaches for soil microbiome engineering. a, Site-specific recombination uses serine recombinases (SAGE, shown in graphic) or tyrosine recombinases (CRAGE) to integrate engineered DNA into specific sites on bacterial chromosomes, including those of soil isolates, with high efficiency. **b**, Engineered homologous recombination, or recombineering, relies on native or engineered proteins to create deletions, insertions or directed mutations in DNA using single-stranded or double-stranded substrates with homology for the target site. Recombineering methods enhanced by CRISPR–Cas counterselection ease introduction of mutation without selection markers and can be employed to selectively promote or kill strains in microbial communities (ssCRISPR) or to create genome-wide pooled mutant libraries. **c**, Direct community editing has been demonstrated using conjugative transfer, using *an E. coli* host (MAGIC) to transfer a replicative or integrative plasmid to the mouse microbiome or a *Bacillus* host expressing a mini-ICE to Gram-positive bacteria in soil communities. **d**, Engineered CRISPR–Cas transposase complexes can create directed gene edits without homologous recombination (INTEGRATE) and have been demonstrated to engineer members of microbial consortia (DART). PAM, protospacer-adjacent motif.

five taxonomically diverse hosts as efficiently as plasmid transformation, which conventionally transforms orders of magnitude more efficiently. SAGE enables construction of combinatorial strain libraries through iterative integration of DNA constructs and was used in multiple plant-derived soil isolates to evaluate a panel of hundreds of promoters from taxonomically diverse species as tools for genetic circuits or metabolic pathways.

Engineering microbial communities in situ

Recent developments show promise for in situ control of microbial community composition, host-agnostic community function and host-specific genome edits, including those that originate from soil (Fig. 6). Strain-specific CRISPR (ssCRISPR) was developed to edit the membership of a microbiome community and selectively purified or eliminated members of a synthetic collection of model hosts[110]. The approach uses CRISPR–Cas guide RNA species unique to a target host to elicit strain-specific DNA cleavage and cell death or to introduce an antibiotic-resistance marker gene to selectively enrich a target strain. Metagenomic alteration of the gut microbiome by in situ conjugation (MAGIC) was developed to transfer a function to the community via conjugation without specific knowledge of the metagenome or the genetic tractability of community members[111]. In MAGIC, a replicative or integrative plasmid was transferred by *an E. coli* donor to a complex community to confer a fluorescence or antibiotic-resistance phenotype that, under some circumstances, persisted in a mouse host for at least 11 days. Although MAGIC was demonstrated in the mouse gut, similar approaches could be extended to transform a soil microbiome with an engineered function in a host-agnostic manner.

Direct genome edits within complex communities have been enabled by engineering advances in high-efficiency CRISPR–Cas systems. The insertion of transposable elements by guide RNA-assisted targeting (INTEGRATE), an engineered version of recently discovered CRISPR–transposon systems, enabled high-efficiency genome integration of large genetic constructs without selection markers[95]. DNA-editing all-in-one RNA-guided CRISPR–Cas transposase (DART), a similar CRISPR–transposase system, was exploited to establish host-specific genome editing within a nine-member, synthetic soil microbial community and extended to infant gut microbiome samples[112]. Efficient DART targeting was enabled by environmental transformation sequencing of the microbial community, a method to identify genetically transformable members of a mixed community by mapping genome-integration events of a transposable element by high-throughput sequencing. Genome editing in microbial communities enabled by environmental transformation sequencing could take advantage of accessibility of metagenome-assembled genomes (MAGs) from soil metagenomes, although rare species might not be represented. While these advances represent unprecedented access to genome editing within a microbial community, substantial hurdles

remain to assess the efficacy of these in situ editing approaches in non-laboratory environments.

Containment of engineered microbes

When deploying microbial systems to promote soil and plant health, biosecurity is often a critical consideration. In this context, 'biosecurity' refers to preserving the resilience of native ecosystems and restricting the proliferation of engineered functions beyond the expected spatiotemporal operating bounds. Developing effective containment approaches for engineered microbial functions could help minimize regulatory hurdles and public hesitation regarding engineered microbial systems being used in the environment. Risk assessment involves several steps that need to be evaluated before field application. These include extensive identification and evaluation of potential adverse consequences[113]. Therefore, despite the original promise and excitement about the potential applications of genetically modified microorganisms, there have been few recent examples of field releases. The majority have focused on non-food applications, such as bioremediation of heavy metals and xenobiotic compounds and/or biostimulation of the rate of microbial degradation of pollutants[113–115]. Currently, it is difficult to gauge how readily future applications that target crop productivity and climate-change mitigation will be approved.

Traditionally, containment measures for soil microorganisms have relied on introduction of amino acid auxotrophies, environmentally triggered toxins or combined use of these approaches[116]. For example, a strain of *P. putida* was engineered to be metabolically dependent on phosphite, a rare compound in nature[117]. The strain was engineered both to assimilate phosphite and disable its ability to transport and grow on phosphate. Synthetic biology has introduced other new approaches, including the integration of multiple signals, and inherent redundancy to suppress escape via individual mutations. These approaches include introduction of non-canonical amino acids[118] and environmentally sensitive kill switches that incorporate logic gates[119]. More recently, increased genetic stability of kill switches has been demonstrated by introducing redundancy[120]. Despite early interest in biocontainment of genetically engineered microorganisms designed for soil applications, there are only a few recent examples[85]. One example is the control of the persistence of *Bacillus thuringiensis* spores by introducing a genetic circuit that prevented sporulation[121]. An important consideration for biocontainment is to avoid the use of antibiotic-resistance genes that could potentially spread and contribute to the growing health concern of antibiotic-resistant pathogens[122]. While these technical advances provide new tools to control the function of engineered microbial systems in the environment, efficacy in field environments remains a frontier for biosecurity research related to the release of engineered microbial functions.

Soil microbiome-testing platforms

A challenge is scaling results from the laboratory and the greenhouse to the field. We acknowledge that laboratory studies are invaluable for gaining detailed knowledge regarding species interactions, how they can be harnessed and modified, what the keystone species are and the development of tools to change these species to gain more from the soil. However, laboratory studies are not 'the field', and care must be taken that the information gained is physiologically and ecologically relevant to field processes. Although direct analysis of soil microorganisms in the field, for example, using multiomic analyses, is progressing, it remains a daunting challenge due to the complexity of the natural environment.

The development of new testing platforms allows for a detailed study of complex, field-relevant systems in a laboratory setting. As these tools become more common, they can be used to generate specific hypotheses that can be tested in a native field system, bridging the gap between laboratory and field for a more detailed but translational understanding of soil microbial systems. For example, rhizotrons and other specialized chamber systems have been developed to study plant–microbe interactions[123]. Testing systems are also available to determine the fate and efficacy of engineered and native soil microorganisms for environmental applications. For example, fabricated ecosystems (ecoFABs) provide standardized platforms for plant–microbiome studies across laboratories that promote reproducible findings (Fig. 5)[124]. Another platform is the 'rhizochip', which uses structured polymer molds to mimic irregular soil particles and leads to root growth and exudation profiles that mimic soil growth[125]. Rhizochip growth assays are compatible with light microscopy and spatial mass spectrometry. To enable spatially resolved investigations of plant–microbe interactions, three-dimensional-printed 'rhizogrid' scaffolds allow integration of X-ray computed tomography, high-throughput taxonomic profiling and metabolomics data from segmented soil and rhizosphere samples at multiple depths[126]. Currently, an opportunity exists to increase the field relevance of these platforms by integrating synthetic, reproducible soil systems to emulate the geochemistry of regional soils using laboratory reagents and resources[127].

Engineering the rhizosphere of the plant host

Another approach for engineering the soil microbiome is to engineer the plant to produce specific compounds in the rhizosphere that select for beneficial members of the community. This approach relies on the ability of plant root exudates to shape the composition of soil microorganisms associated with their roots. For example, root exudation of sucrose promoted colonization of the beneficial soil microorganism *B. subtilis* on the roots of *A. thaliana* plants[128]. Differences in root exudation of specific flavonoid compounds also influenced the ability of arbuscular mycorrhiza to colonize the invasive plant *Triadica sebifera*[128]. Production of benzoxazinoids that are defensive metabolites produced in roots resulted in alteration of the rhizosphere microbiomes of maize plants[129]. Barley engineered to produce rhizopine[130] coupled with a synthetic nitrogen-fixation phenotype regulated by rhizopine in the bacterium *Azorhizobium caulinodans* enabled activation of nitrogen fixation by the microbe only in the presence of the modified barley[131,132]. These studies suggest that plants may be engineered to produce specific root-exudate compounds for targeted recruitment of beneficial microbes, natural or engineered. A related approach is to stimulate growth of beneficial microbes by conditioning soil with specific root exudates that are known to recruit beneficial microbes. For example, combinations of fatty acids and amino acids enriched different rhizosphere microbes when compared to combinations of organic acids and sugars. Several bacterial isolates were isolated from the soil enrichments, and some of them protected the host from a foliar pathogen infection[91]. Identification of plant quantitative trait loci that are responsive to specific beneficial microorganisms and/or environmental conditions can also be used for plant selection[132].

Remaining gaps and opportunities for soil microbiome engineering

Although this Review has summarized the current state of the science, several hurdles remain before wide-scale implementation of soil microorganisms can be applied for tackling pressing issues in the face of climate change, an increasing global human population and a decline in wildlife habitat and biodiversity. Soil microorganisms have the potential to alleviate issues associated with these challenges, but we are still largely working in context with several unknowns with respect to understanding the functional potential of soil microorganisms, how they work together as a community and how they can best be collected and formulated to provide the desired beneficial outcome. Furthermore, with respect to working with genetically engineered strains, regulatory hurdles remain that limit their widespread application.

The largely untapped potential of soil microorganisms presents a tremendous opportunity for mitigating many of the challenges facing the environment. These opportunities include using natural or

engineered soil inoculants and consortia for remediation of polluted and otherwise degraded soils, maintaining and improving crop performance and mitigating certain negative consequences of climate change. For example, soil microorganisms may help to reduce levels of greenhouse gases and sequester carbon in soil[14]. Advances in genetic engineering of soil microorganisms and associated knowledge of biocontainment of released soil microorganisms can also be valuable for containment of unintended releases or cases of bioterrorism. Lastly, knowledge obtained on how best to manipulate soil microorganisms for desired functions may be used at a 'systems level' by soil managers for specific crops by adding soil amendments that optimize the chances for natural soil microorganisms to grow and thrive. This last situation may turn out to be one of the most valuable for taking advantage of the inherent beneficial properties that are carried out by interacting members of the soil microbiome. Ultimately, the combination of several strategies is necessary to meet challenges that we are facing with climate change and a growing human population, including genetic engineering of microorganisms and plants and use of best practices that promote ecosystem sustainability[133].

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Author contributions

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Additional information

Correspondence and requests for materials should be addressed to Janet K. Jansson.

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