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REVIEW

Engineering root microbiomes for healthier crops and soils using beneficial, environmentally safe bacteria

Pilar Martínez-Hidalgo, Maskit Maymon, Flora Pule-Meulenberg, and Ann M. Hirsch

Abstract: The Green Revolution developed new crop varieties, which greatly improved food security worldwide. However, the growth of these plants relied heavily on chemical fertilizers and pesticides, which have led to an overuse of synthetic fertilizers, insecticides, and herbicides with serious environmental consequences and negative effects on human health. Environmentally friendly plant-growth-promoting methods to replace our current reliance on synthetic chemicals and to develop more sustainable agricultural practices to offset the damage caused by many agrochemicals are proposed herein. The increased use of bioinoculants, which consist of microorganisms that establish synergies with target crops and influence production and yield by enhancing plant growth, controlling disease, and providing critical mineral nutrients, is a potential solution. The microorganisms found in bioinoculants are often bacteria or fungi that reside within either external or internal plant microbiomes. However, before they can be used routinely in agriculture, these microbes must be confirmed as nonpathogenic strains that promote plant growth and survival. In this article, besides describing approaches for discovering plant-growth-promoting bacteria in various environments, including phytomicrobiomes and soils, we also discuss methods to evaluate their safety for the environment and for human health.

Key words: biofertilizer, biosafety, PGPR/PGPB, biopesticide, phytomicrobiome.

Résumé : La révolution verte a permis de développer de nouvelles variétés de cultures qui ont grandement amélioré la sécurité alimentaire à travers le monde. Toutefois, la croissance de ces végétaux dépend fortement de fertilisants et de pesticides chimiques, ce qui a conduit à une surutilisation de fertilisants, d'insecticides et d'herbicides synthétiques dont les conséquences environnementales et les effets négatifs sur la santé humaine sont sérieux. Les auteurs proposent ici des méthodes qui favorisent la croissance des végétaux, respectueuses de l'environnement, visant à remplacer notre dépendance actuelle aux produits chimiques synthétiques et développer des pratiques agricoles plus durables afin de compenser le dommage causé par les produits agrochimiques. Une solution potentielle réside dans l'utilisation accrue de bioinoculants qui consistent en microorganismes capables d'établir des synergies avec les cultures cibles et d'influencer la production et le rendement en favorisant la croissance des végétaux, en contrôlant les maladies et en fournissant des nutriments minéraux clés. Les microorganismes trouvés dans les bioinoculants sont souvent des bactéries ou des champignons qui résident soit dans le microbiome externe ou dans le microbiome interne des végétaux. Toutefois, avant qu'ils puissent être utilisés de façon courante en agriculture, on doit confirmer que ces microbes se composent de souches non pathogènes qui favorisent la croissance et la survie des végétaux. Dans cet article, en plus de décrire des approches visant à découvrir des bactéries favorisant la croissance des végétaux à partir de divers environnements, dont les phytomicrobiomes et les sols, les auteurs discutent également de méthodes d'évaluation de leur innocuité pour l'environnement et la santé humaine. [Traduit par la Rédaction]

Mots-clés : biofertilisant, biosécurité, RFCP/BFCP, biopesticide, phytomicrobiome.

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Introduction

Microbes function as biofertilizers, biopesticides, and plant growth promoters and have been utilized to enhance crop growth in numerous countries around the world, but especially in developing and emerging nations (Bashan et al. 2014). For decades, companies worldwide have supplied farmers with nitrogen-fixing inoculants and formulations of plant-growth-promoting (PGP) microbes, both fungi and bacteria, to enhance crop production (Wood 2015). Many microbial products are also used by home gardeners and for organic agriculture, and large-scale commercial farms in China, the United States, and Europe are beginning to adopt biological materials as substitutes for chemical fertilizers and pesticides (Parnell et al. 2016). Replacing chemical fertilizers and pesticides is critical for agricultural sustainability (Kecskés et al. 2016; Menendez and Garcia-Fraile 2017), but there is a huge gap in information about the effectiveness of PGP microbes based on laboratory studies versus their performance in the field. It is not always clear how useful many of the bioinoculants discovered in the laboratory are once they are tried in the field or whether or not they or their products might have untoward effects on non-target organisms, including humans.

This review focuses on certain PGP bacteria (PGPB) and PGP rhizobacteria (PGPR), which are becoming better known for their potential to promote sustainable agriculture. Currently, bioinoculants are available mostly as single entities (Bashan et al. 2014) but are also being formulated as consortia of multiple bacteria and fungi, which have synergistic PGP traits to (*i*) enhance the growth of different crops (Yanni et al. 2001; Laabas et al. 2017), (*ii*) exhibit biocontrol activity (Khan et al. 2012; Bach et al. 2016), (*iii*) prime the plant for more efficient pathogen defense (Aziz et al. 2016), and (or) (*iv*) increase crop nutritional value (Egamberdiyeva 2007). In some cases, PGPB help the plant grow under extreme conditions, such as nutrient deficiency, aridity, salinity, and drought (Vílchez and Manzanera 2011; Wang et al. 2012; Khan et al. 2017; Shinde et al. 2017). To find the bacteria that are the most effective, they first must be isolated from their original sources, their identity determined, and the traits they possess to support plant growth rigorously evaluated. Moreover, their success under both laboratory and natural conditions needs to be determined, and their potential risks to other plants, animals, and humans must be evaluated. Finally, the question of whether natural soil microbiomes are negatively affected by adding foreign microbes must also be addressed.

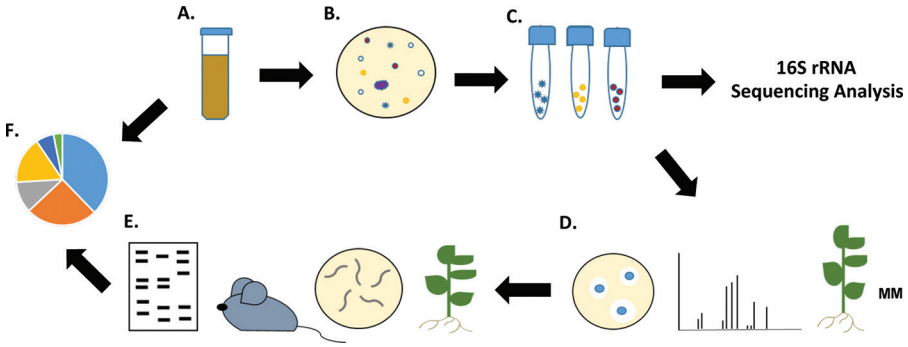
The basics of soil microbe discovery research

Isolating soil microbes (and (or) their DNA) and evaluating their potential as bioinoculants

Both cultivation-dependent and -independent methods are used for constructing inventories of PGPB from their natural habitats, typically soil, roots (rhizoplane or rhizosphere), or internal tissues of plants. For cultivation-dependent analyses, nonselective and selective culture media are traditionally used to find bacteria that readily grow under artificial conditions. The development of inoculants requires that the PGPB multiply in culture, are easily propagated, positively affect plant growth, and are safe for humans and the environment. Many microbes have been cultivated using an enrichment media method whereby a soil sample is mixed with water and the suspension serially diluted onto a nonselective medium such as nutrient agar or any generalized medium that contains a carbon source, amino acids, and salts (Sanders and Miller 2010). A large number of different species of organisms with varied morphologies are likely to grow on nonselective agar plates, so plate washes or individual colonies are subjected to another round of selective media to isolate microbes that grow under more stringent conditions. Subsequent steps often require the use of a culture medium that reveals a particular PGP trait (Menendez and Garcia-Fraile 2017). Once a single species is isolated, it is usually identified by 16S ribosomal RNA (rRNA) gene sequence analysis. Many of these steps are illustrated in Fig. 1.

Although the above steps seem easy to accomplish, cultivation-dependent methods are often problematic because not all bacterial soil isolates can be grown in vitro. Indeed, it has been estimated, based on the discrepancy between the numbers of cells directly counted in an environmental sample versus the number growing in culture medium, that only approximately 1% of environmental microbes are cultivatable (Katz et al. 2016). Nevertheless, the current state-of-the-art is that microbes must be cultured if they are to be used as commercial inoculants, but many possibilities exist as to why certain strains cannot be grown in artificial media. Some bacteria may depend on other microbial species to catabolize a substrate that neither species can break down alone or because two or more bacteria may synthesize a particular metabolite or antibiotic only in the presence of a partner or in a consortium (D'Onofrio et al. 2010; Adnani et al. 2017). Such relationships make it highly unlikely that certain microbes will be cultivated on standard media. However, cultivation techniques continue to be improved, and bacteria missed in previous attempts are being identified. Thus, it is highly likely that new methods and media may help in the search for cultivatable PGPB. In the meantime, elucidating soil and plant-associated bacterial biodiversity without having to go through a culturing step is where advances in molecular biology, genomics, and bioinformatics have not only helped identify new natural products but have also provided insight into the diversity of microbial populations in a variety of environments. These techniques may also help to distinguish pathogenic from nonpathogenic species through a deep analysis of microbial genomes.

Fig. 1. An overview of efficacy and biosafety evaluation methods for plant-growth-promoting bacteria. A soil sample from a natural habitat is diluted (A) and plated on selective and nonselective media (B). After incubation (short- and long-term), individual isolates are plate-purified and identified by 16S rRNA sequencing (C). Identified bacteria are subjected to multiple biochemical assays, such as phosphate solubilization (illustrated), along with quantitative mass spectrophotometry studies to investigate the presence of plant hormones, elicitors, and other compounds and determine their potential as PGP molecules. Isolates are then used as bioinoculants on plants to confirm growth enhancement (D). A thorough biosafety assessment is applied based on phylogenetic, physiological, and molecular testing for the presence or absence of virulence factors. Pathogenicity and toxicity are determined by testing the isolated strains on various model organisms such as mice, nematodes, and plants (E). DNA fingerprinting (also illustrated in E) is often used as a molecular tool to discriminate between pathogenic and nonpathogenic strains. The ecological effects of using the bioinoculants on the endogenous environment are assessed by metagenomic analyses of the soil microbiome pre- and postinoculation (A and F). Artwork is by Maskit Maymon (MM).



For cultivation-independent methods, successful DNA extraction from soil microbes is required, and often the heterogeneous material that makes up soil, namely, clay particles, organic matter, humic acids, etc., interferes with extracting high-quality material for PCR analysis. Fortunately, the methods and efficacy of pursuing cultivation-independent approaches have improved significantly in recent years and have been embraced by both environmental microbiologists and natural product chemists, albeit with different aims.

Once high-quality environmental DNA (eDNA) is obtained, several strategies may be employed for species identification, with the ultimate goal of examining the collective genomes of all the microorganisms within a community to get a better idea of which microbes can be used as future inoculants. Commonly, the 16S ribosomal RNA gene is chosen for community analysis (Winsley et al. 2012), although other highly conserved genes may be used as well (*gyrB*, *rpoB*). However, the use of certain primers in metagenomic sequencing may lead to problems because the primers are often not as universal as they are expected to be. In addition, some bacterial groups can be overrepresented in an environment, whereas others may be under- or even not represented at all (Wang and Qian 2009; Schloss et al. 2011).

Another approach is the use of whole-genome shotgun sequencing, whereby total eDNA, composed of random short fragments representative of the microbial community, are pooled and assembled with sequence assembly algorithms (Sharpton 2014). This method is said to produce a more comprehensive sample of a complex environment and also sheds additional light on the abundance of various species and the overall diversity within the sample. Furthermore, for plant microbiome analysis, e.g., nodule microbiomes, the plant DNA may need to be subtracted from the total shotgun array of sequences. To bypass this difficulty, alternative isolation methods, using single-cell extractions, can be used and then followed by multiple displacement DNA modification and traditional 16S rDNA screenings (Levy et al. 2018). With the increased availability of sequenced plant genomes, plant versus microbe DNA sorting will become less of a problem (Busby et al. 2017) and allow a more efficient and reliable analysis. The cultivation-independent method reveals the larger scale phylogenetic relationships of bacterial species in a particular environment because it includes those microbes that cannot be cultured (Ellis et al. 2003; Ranchou-Peyruse et al. 2006; Štursa et al. 2009).

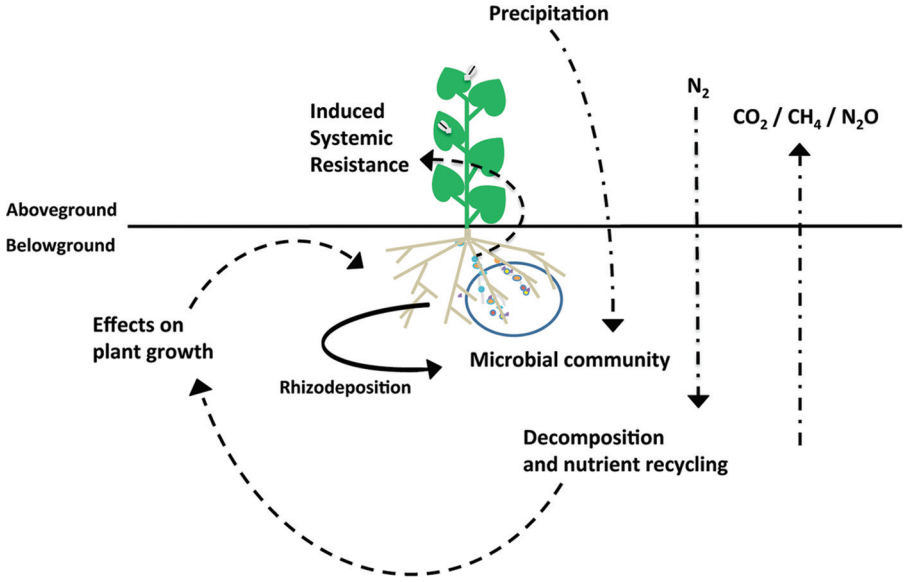
Assays for finding potential PGPB

Traits for promoting plant growth

A number of assays, which might predict the isolates' potential performance as PGPB in planta, are used to select strains for inoculation. Although phenotypic analyses are important for a first screening, in planta studies are absolutely required to ensure that the microbial isolates generate a positive growth effect.

Nitrogen fixation is one of the most important of all PGPB traits, and several methodologies have been employed to measure the nitrogen-fixing capabilities of a strain (e.g., Wertz et al. 2012). However, the ability to grow in an N-limited environment is not a test for nitrogen-fixation ability because the media used are rarely completely depleted of nitrogen, which leads to false positives (Martínez-Hidalgo et al. 2014*a*). For example, bacteria that either effectively scavenge N or have 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity may survive for a time on an N-free medium

Fig. 2. The interactions of plants and their microbial community as well as the abiotic and biotic factors associated with soil that highly influence plant growth. Plants, microbes, and soil chemistry are all linked together by their requirement for water. In addition, the interactions between the microbial community and the various elicitors and volatiles they produce that trigger induced systemic resistance (in this case against white flies), as well as rhizodeposition and decomposition and (or) nutrient recycling brought about by the plant–microbe collaboration all affect plant growth. The various gases diffusing in soil are starting or end points for microbial metabolism. Modified with permission from an unpublished drawing prepared by Drora Kaplan.



(Schwartz et al. 2013). The apparent Nif+ phenotype on –N media and ethylene synthesis inhibition are linked because ACC, the precursor to ethylene, a plant growth inhibitor, is converted by AcdS to ammonia and α-ketobutyrate. Ammonia serves as a nitrogen source for the bacteria and the reduction in ethylene synthesis further promotes growth. In planta or in vitro analyses with N^{15} provide more accurate measurements for nitrogenase activity and are recommended for verifying nitrogen-fixation activity (Martínez-Hidalgo et al. 2014*b*).

Phosphorous, another essential macronutrient, is usually found as insoluble forms in the soil — organic and inorganic phosphates. Hence, phosphate-solubilizing capabilities are a key PGP trait and are easily detected by plate assays. Numerous in vitro analyses have been described (Peix et al. 2001), and published methods generally take into consideration the existence of acidic, alkaline, and neutral phosphatases. However, they rarely consider phytases (myo-inositol hexakisphosphate phosphohydrolase), which break down organic phosphates. Phytates are serious pollutants in certain agricultural areas (Rodríguez et al. 2006), and a number of bacteria, e.g., *Bacillus* species, have phytase genes. In addition, most Gram-negative bacteria break down mineral phosphate by secreting gluconic acid. Several bacterial mechanisms used to solubilize phosphate have been described (Rodríguez and Fraga 1999; Rodríguez et al. 2006).

Iron is another indispensible nutrient that is difficult for many plants to obtain, especially those that do not produce their own siderophores. Siderophores are "iron-carrier" molecules that can give adaptive advantages to the plant if the sequestered iron is released by the bacteria and used by the plant. Siderophores may also act as plant pathogen defense factors because bacterial siderophores are better at binding Fe^{3+} than fungal pathogens are. Siderophore activity can also be detected through plate assays (Alexander and Zuberer 1991).

Microbes also synthesize plant hormones, including indole acetic acid (IAA), gibberellins, cytokinins, and others (Lugtenberg and Kamilova 2009; Glick 2012). Perhaps the best way to detect phytohormones is through spectrophotometric analyses rather than bioassays such as the Salkowski test for IAA, which often results in false positives. For example, we identified incomplete IAA-synthesis operons in the *Bacillus simplex* genome, but no IAA was detected by LC–MS/MS–MRM (Maymon et al. 2015). However, genes for the synthesis of polyamines, which also regulate plant growth, were found, and peaks for spermine, spermidine, and putrescine were detected (Maymon et al. 2015). Spermidine, also synthesized by *Bacillus subtilis* OKB105, promotes plant growth by inhibiting ethylene synthesis in plant root cells (Xie et al. 2014).

Many beneficial microbes also produce volatiles, such as 2,3-butanediol, and (or) other elicitors, e.g., proteins (flagellin); cell wall or membrane fragments (lipopolysaccharide); antibiotics (2,3-diacetylphoroglucinol, phenazine); and siderophores that trigger induced systemic resistance (ISR) to a broad range of parasites and pathogens (Van Loon and Bakker 2005; Pieterse et al. 2014) (Fig. 2). For example, the actinomycete *Micromonospora*, which was isolated from alfalfa root nodules, generates an ISR

in tomato against *Botrytis cinerea* (Martínez-Hidalgo et al. 2015). Microbial molecules prime the plant and trigger its innate immunity, which results in the expression of genes for the synthesis of endogenous phytohormones, including salicylic acid, jasmonic acid, and ethylene, thereby reducing infection and disease. Volatiles are also reported as being nematicidal or antifungal.

In summary, the number of molecules involved in beneficial microbe–plant communication overall is staggering and a recent review describes many of them (Chagas et al. 2018).

Impact of bioinoculants on soil microbiome

Although microbial fertilizers have been released into the market and the strains used in them studied in depth, the effects of adding large numbers of foreign microorganisms to indigenous soil microbiomes are under-investigated. It is important to initiate studies to creating a data set that gives the microbial baseline of soil prior to inoculation as well as afterwards (Fig. 1). In this way, we can assess the importance of microbiome shifts and how they affect soils and crops (van Elsas et al. 2015). Careless additions could affect soil health negatively, altering soil performance in nutrient cycling and the capability to promote plant growth (van Elsas et al. 2015).

To differentiate the indigenous bacterial communities from inoculants, several methods that aim to obtain more quantitative and statistically robust data have been developed (Kowalchuk et al. 2004). Semenov et al. (2014) described taking measurements on the so-called normal operating range in different soils at undisturbed states at different times. This strategy defined a reference (the normal operating range of a soil) and used a mathematical approach to evaluate how much divergence a soil shows from this unaltered state. Other strategies include the study of soil microbial biomass and enzyme activity, which respond very quickly to changes in soil management (García-Ruiz et al. 2008).

Experiments with turmeric (*Curcuma longa*), a crop that exhausts the soil, revealed that the use of *Azospirillum lipoferum* and *Bacillus megaterium* combined with organic manure could increase microbial biomass compared with adding chemical fertilizers. Also improved was the amount of total N mineralized as well as oxidoreductase enzyme production, an indicator of microbiological activity. In this study, the soil quality was improved by the use of inoculants (Dinesh et al. 2010).

Another indirect form of microbial activity measurement is cellulolytic activity. A study by Zhao et al. (2005) showed the differences in cellulolytic activities in soil depending on the biofertilizer used. For biofertilizers that contain a mixture of different bacteria, results show that certain inoculants cause a shift in cellulose degradation in PGPB-inoculated fields versus uninoculated fields, suggesting that the type of biofertilizers or inoculants used could affect the microbial community structure.

A more direct way of monitoring changes in soil over time in response to an inoculum is through metagenomic sequencing (Fig. 1). The same strategy of isolating eDNA via the cultivation-independent method described earlier needs to be followed, but it will be essential to have samples taken prior to the addition of both biological and abiotic amendments. Such an approach is a research area that will need a great deal of development over the next decade.

Biosafety concerns

Mutualists or pathogens?

Greater use of PGPB as supplemental inoculants or total replacements for chemical pesticides and herbicides is key to sustainability, but questions of efficacy and biosafety must be addressed. For example, a number of bacterial species potentially harmful to mammals, including humans, have been isolated from plant rhizospheres. Many bacteria with PGP traits belong to the genera *Burkholderia*, *Enterobacter*, *Ochrobactrum*, *Pseudomonas*, *Serratia*, *Klebsiella*, and *Ralstonia*, and are phylogenetically related to species that are virulent or are opportunistic human pathogens (Berg et al. 2005). Such relationships are not to be taken lightly because the possibility exists that some of these bacteria might cause nosocomial infections and disease in immunocompromised patients (Baldwin et al. 2007; LiPuma 2010). Strains with PGPB activity that are related to the *Burkholderia cepacia* or *Burkholderia cenocepacia* lineages are commonly isolated from soil and also from root nodules (Martínez-Hidalgo and Hirsch 2017).

The genus *Pseudomonas* has for many years been used in commercial inoculants, but a member of that genus, *P. aeruginosa*, is a dangerous human pathogen. It is a common cause of respiratory tract infection in people with cystic fibrosis, a serious hereditary lung disease. Other commonly found pseudomonads in clinical samples and in soil include *P. fluorescens*, *P. putida*, *P. pseudoalcaligenes*, *P. stutzeri*, and *P. putrefaciens* (Ortega-Calvo and Saiz-Jimenez 1998; Baum et al. 2009). Commercial phenotypic tests are not always able to differentiate among the different species. In a study in which Spilker et al. (2004) tested 66 pseudomonads from sputum from various laboratories across the world, the results showed that 38 of the pseudomonads were initially misidentified using phenotypic traits. Using genus- and species-specific PCR assays and 16S rDNA sequencing, those authors reported that many of the strains were identified as *P. aeruginosa*. Among the isolates, they also detected *P. fluorescens*, *P. lundensis*, *P. pseudoalcaligenes*, *P. stutzeri*, and *P. synxantha*. Thus, where molecular techniques are not available, the identification of pseudomonads can be challenging, strongly suggesting that the probability of human infection could increase.

Table 1. Important plant-growth-promoting rhizobacteria (PGPR) and their potential health risks.

Bacteria	Uses as PGPR	Health risk
Acinetobacter baumannii	Rokhbakhsh-Zamin et al. 2011	Peleg et al. 2008†
Achromobacter xylosoxidans	Dawwam et al. 2013	Duggan et al. 1996†
Bacillus cereus group	Guttmann and Ellar 2000; Liu et al. 2015	Kotiranta et al. 2000‡
Bacillus simplex	Schwartz et al. 2013	Angus et al. 2014*
Burkholderia cepacia	Dinesh et al. 2014, 2015	da Costa Capizzani et al. 2017‡; Mali et al. 2017‡
Burkholderia cenocepacia	Ho et al. 2015	Scoffone et al. 2017†
Enterobacter cloacae	Nelson 1988; Singh et al. 2017	Sanders and Sanders 1997†; Davin-Regli and Pagès 2015†
Enterobacter sp.	Selvakumar et al. 2014 and references therein	Selvakumar et al. 2014 and references therein†
Klebsiella pneumoniae	Pramanik et al. 2017	Clegg and Murphy 2016‡
Micromonospora sp.	Martínez-Hidalgo et al. 2014*a*, 2014*b*, 2015	ND
Ochrobactrum anthropi ribotype B	Chakraborty et al. 2009	Teyssier et al. 2005†
Ochrobactrum intermedium ribotype A	Paulucci et al. 2015	Teyssier et al. 2005†
Other *Ochrobactrum* ribotype C	Hahm et al. 2012	Teyssier et al. 2005*
Pantoea agglomerans	Mishra et al. 2011	Dutkiewicz et al. 2016†
Phyllobacterium spp.	Flores-Félix et al. 2015	Swings et al. 2006*
Pseudomonas putida	Patten and Glick 2002	Fernández et al. 2015†
Pseudomonas stutzeri	Lim et al. 1991; Yan et al. 2008; Islam et al. 2016	Shalabi et al. 2017†
Ralstonia mannitolilytica	Grönemeyer et al. 2012	Ryan and Adley 2014†
Ralstonia pickettii	Paul et al. 2013	Ryan and Adley 2014†
Serratia marcescens	Lavania et al. 2006	Marin et al. 2017†
Stenotrophomonas maltophila	Islam et al. 2016	Ribbeck-Busch et al. 2005†; Berg and Martinez 2015†
Stenotrophomonas rhizophila	Alavi et al. 2013	Berg and Martinez 2015*
Streptomyces somaliensis/sudanensis	Qin et al. 2015	McNeil and Brown 1994‡; Quintana et al. 2008‡

Note: ND, none determined. No pathogenic representatives have been found in the literature.
*No pathogenic strains have been found for the species based on some of the tests described herein.
†Opportunistic strains have been isolated from diseased humans.
‡Pathogen of importance for human health.

The rhizosphere and some plant parts such as leaves have been shown to also house opportunistic human pathogens, including *P. aeruginosa* (Berg et al. 2005). A study by Kumar et al. (2013) using *recN* sequencing, multilocus sequence typing, and comparative genome hybridization showed that a *P. aeruginosa* strain isolated from black pepper in India initially did not cluster with *P. aeruginosa* strains that originated from clinical isolates. However, the same strain later proved to be resistant to many antibiotics, grew at high temperatures, and was toxic to mammalian cells. Other researchers have published on additional *P. aeruginosa* strains. Together, these studies highlight the need for putting any potential PGPR through a rigorous biosafety protocol. Interestingly, while regulatory frameworks for biosafety are said to be in place, most government publications on the topic are unclear on the topic of inoculants or do not define what a bioinoculant is or regulate them using outdated lists of genera that do not represent current knowledge on biosafety, if they are mentioned at all. The latter is a concern because the use of molecular methods to better differentiate bacterial taxa has resulted in genus name changes for many species, e.g., *Pseudomonas cepacia* is now *Burkholderia cepacia*, *Pseudomonas maltophilia* is *Stenotrophomonas maltophilia*, and others. The name changes and related evidence are not readily disseminated to many government agencies. Thus, similar to dietary supplements, where the risk of toxicity or contamination from an unwanted source is possible because of the lack of standardized quality control (Coutinho Moraes et al. 2015), "agricultural amendments" also require rigorous testing for safety as well as efficacy.

Although for the majority of the rhizosphere microbiomes investigated, information on how they impact plant growth is incomplete, many studies (Glick et al. 1997; Rodríguez and Fraga 1999; Bloemberg and Lugtenberg 2001; Vessey 2003) reported a positive impact of pseudomonads on plant growth. The mechanisms of plant growth by these organisms are well researched and documented (Compant et al. 2005, 2010; Glick 2012).

Table 1 shows several examples of genera that have both pathogenic and mutualistic representatives. Because of their potential health risk, the use of the *Burkholderia cepacia* complex (Bcc) in the field has been restricted (US Environmental Protection Agency 2003; see also Chiarini et al. 2006 for a description of the Bcc).

Eberl and Vandamme (2016) discussed these topics in great depth with reference to *Burkholderia*, and efforts are being made to separate the pathogenic *Burkholderia* species from the beneficial ones (see Estrada-de los Santos et al. 2016, 2018) based not only on phylogeny but also on physiology and the absence or presence of factors associated with virulence. Earlier, Gyaneshwar et al. (2011) showed that the plant-associated and nodulating *Burkholderia* (now *Paraburkholderia*, Sawana et al. 2014) has a lower G + C content than the pathogenic species. Whether this is coincidental or meaningful is difficult to evaluate at this time.

The genus *Ochrobactrum* is similar to *Burkholderia* in that several strains induce nitrogen-fixing nodules on legume roots (Willems 2006), whereas others appear to have PGP capabilities but lack nodulation ability (Tariq et al. 2014). However, the symbionts *O. lupini* and *O. cytisi* are closely related to the opportunistic human pathogen *O. anthropi* (Trujillo et al. 2005; Zurdo-Piñeiro et al. 2007). Other studies show that human pathogenic strains of *O. anthropi* form a subpopulation that differs from the plant-associated strains (Romano et al. 2009), but more testing is needed.

Rhodococcus is another example of a genus in which some species can be either plant pathogens or PGPB. Some members of the genetically diverse genus *Rhodococcus* are pathogenic and cause fasciations and hyperplasias when certain virulence genes are expressed (Putnam and Miller 2007; Creason et al. 2014). An example of a PGPB strain is *Rhodococcus erythropolis*, which promotes pea growth especially at low temperatures and in heavy-metal-contaminated soils (Trivedi et al. 2007). This *Rhodococcus* species, isolated from *Hedera helix*, is important for phytoremediation (Stevens et al. 2017) as well as plant-growth promotion via plant hormones (Francis et al. 2010). However, some studies show that this same species contains strains that can cause septicemia or encephalitis in immunocompromised patients (Park et al. 2011; Bagdure et al. 2012). Efforts are being made towards an effective way of distinguishing the plant pathogens from beneficial bacteria. Given that molecular methods can potentially be used to distinguish pathogenic strains from beneficial ones (Savory et al. 2018), one goal would be to employ such methods routinely to address this issue in the future.

Another feature used to distinguish nonpathogenic from pathogenic strains is that the latter grow at human body temperature and the former do not (Berg and Martinez 2015; Eberl and Vandamme 2016). Growth of a strain at 37 °C is a definite concern and, accordingly, such isolates should not ever be employed as PGPBs. The risk of opportunistic infections is far too great, especially for immunocompromised patients, and points to the need for exclusion of certain taxa from consideration as bioinoculants. Further study is warranted.

Given these examples, it is clear that a deeper understanding of the molecular, physiological, and biochemical characteristics of PGPB is needed. Microorganisms are currently classified into different risk groups based on their safety of use to avoid human health risks. Only microbial strains included in Risk Group 1 (Europe) or Biosafety Level (BSL) 1 (USA) are regarded as safe and utilizable as bioinoculants. However, this classification should not be considered as the only valid reference to determine the potential risk of a novel or established microorganism.

In addition, mutualistic as well as potential pathogenic characteristics of a microbial strain are often clustered into pathogenicity or symbiotic islands, the genes of which are responsible for either the synthesis of virulence factors or the mutualistic interaction of the strain with a particular host (Dobrindt et al. 2004). Hence, a good first start is to sequence the genomes of potential PGPB microbes and determine whether pathogenicity islands or genes are present. This strategy is extremely helpful in determining the potential risks of a microbial inoculant. Furthermore, whole-genome comparisons between potentially pathogenic and mutualistic members of a single genus, as described for *Rhodococcus*, will provide critical information about the potential avirulence or virulence of a strain. Also, studies testing whether pathogens and commensals and (or) mutualists have the ability to take up and, more importantly, maintain genes conferring each other's behaviors may also need to be performed. Cases where there are definite blocks to gene exchange between the different strains and species might be a benchmark for using a particular strain in agriculture.

Species of *Micromonospora* follow many of the trends indicated by Levy et al. (2018), such as increased genome size in root and nodule-associated species (data not shown). However, this correlation is not strict because non-ecto- and endo-rhizospheric species (sensu Carro et al. 2018), such as *M. pallida* DSM 45599^T and *M. carbonacea* DSM 43148^T (7 762 816 and 7 941 928 bp, respectively), have larger genomes than either *M. coriariae* DSM 44875^T (6 929 687 bp) or *M. lupini* Lupac 08 (7 321 224 bp), which are nodule isolates. However, similar to the findings of Levy et al. (2018), the genomes of *Micromonospora* spp. are replete with a large number of genes involved in carbohydrate metabolism (Carro et al. 2018).

Biosafety tests for bioinoculants

Before the use of bioinoculants can expand further into routine field applications, concrete regulation and testing with a system of assessment of the biosafety of PGPB strains with respect to humans, animals, other plant life, and the environment is needed (Berg 2009; Selvakumar et al. 2014). As mentioned earlier, thorough strain characterization is essential along with tests for pathogenicity and toxicity to eliminate strains that pose even a minimal risk. In the lab, *Caenorhabditis elegans* has

been used as a model organism to obtain insight into whether certain bacterial strains of *Burkholderia*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas* were or were not harmful to the nematodes (Aballay and Ausubel 2002; Zachow et al. 2009; Angus et al. 2014). Additional tests include the use of insect and other animal hosts as well as plants (Fig. 1) (Vílchez et al. 2016). Plant tests are usually performed with the host of a known pathogen. In the case of plant-pathogenic *Burkholderia*, *Allium cepa* bulb scales have been used to screen for *Burkholderia cepacia* strains that cause disease (Jacobs et al. 2008). Tests to determine the disease potential of various microbes have also been carried out on the non-host *Nicotiana benthamiana* (Wei et al. 2007; Savory et al. 2018).

Ecological toxicity must also be considered because a wide range of micro- and macroscopic organisms could be affected by inoculating novel PGP strains (Stephens and Rask 2000; Köhler and Triebskorn 2013). Vílchez et al. (2016) proposed the Environmental and Human Safety Index (EHSI) that assesses the biosafety of the bacterial strains used as bioinoculants. The EHSI is based on a panel of assays on model organisms for all trophic levels and has two primary advantages: (*i*) it avoids the high economic cost of testing the environmental impact of bioinoculants and (*ii*) does not employ assays on vertebrates. This economic factor helps primarily small industries that cannot afford the large-scale series of tests that large multinational companies undertake. Nevertheless, some vertebrate testing may be required depending on the potential risks of the species in question (Fig. 1).

Many microbes are already viewed as nonpathogenic (Risk Group 1/BSL1), including most species of *Rhizobium* and allied genera as well as *Azospirillum* and *Azotobacter* species, which fix nitrogen and also exhibit numerous PGPB traits. Rhizobial species and *Azospirillum* are well represented among the commercial inoculants such as Monsanto BioAg (Monsanto BioAg 2015*a*) or Seedland (Seedland 2013). Moreover, many *Bacillus*, *Paenibacillus*, and *Brevibacillus* species are commonly employed for their PGP ability and may also be used as biocontrol agents. *Bacillus* species are frequent PGPB partners with rhizobia or mycorrhizal fungi to establish effective tripartite symbioses with plants (Francis et al. 2010; Schwartz et al. 2013). A number of *Bacillus* species are already available as bioinoculants (Monsanto BioAg 2015*b*). Nonetheless, some *Bacillus*, *Paenibacillus*, and *Brevibacillus* species are animal pathogens, namely *Bacillus anthracis*, *Paenibacillus larvae*, and *Brevibacillus laterosporus* (Francis et al. 2010; Grady et al. 2016; Marche et al. 2017) and, hence, should be avoided in any consideration of their use as PGPB.

Actinobacteria, such as the genus *Micromonospora*, some *Streptomyces* species, and *Frankia*, which is a nitrogen-fixing genus that nodulates certain non-legume trees and shrubs, e.g., *Casuarina*, *Alnus*, and *Ceonothus* (Froussart et al. 2016), are good candidates for bioinoculants. Although *Micromonospora* strains are not associated with biological nitrogen fixation (Martínez-Hidalgo et al. 2014*b*), they are common inhabitants of both legume and actinorhizal nodules, and frequently in high numbers (Trujillo et al. 2015). In addition, *Micromonospora* strains are important agents for biocontrol and plant growth promotion (Martínez-Hidalgo et al. 2014*a*, 2015). Like the *Firmicutes*, *Actinobacteria* have been co-inoculated with nitrogen-fixing rhizobia onto legumes to enhance the mutualistic interaction (Solans et al. 2009; Benito et al. 2017). So far, no human disease-causing isolates have been detected in the genus *Micromonospora* nor have any plant pathogens been described, which strongly suggests that this BSL1 genus consists of biologically safe microorganisms. Interestingly, only 1% of *Streptomyces* species are plant pathogens (Wanner and Kirk 2015), and to our knowledge, only one human pathogen, *Streptomyces somaliensis*, has been described in the literature; its genome has been sequenced (Kirby et al. 2012).

Rigorous studies of the efficacy as well as the potential risks of novel microbes in sustainable agriculture must be pursued, especially in light of the effort to replace chemical pesticides and fertilizers. Nevertheless, another problem may surface when an inoculant reaches the market, and this is related to whether the farmer is willing to buy the product. Besides the lack of communication between academic scientists and farmers, there are several issues that may keep farmers from using inoculants. The most pressing is that chemical fertilizers provide almost instantaneous positive results, whereas inoculants need more time and are not always consistent in their beneficial effects in the field (Parnell et al. 2016). Also, the knowledge level farmers need to correctly apply bioinoculants is higher than with chemical fertilizers. The procedures for application are new and the benefits from that application may be unclear or different from the readily visible effect of chemicals, a risk that some farmers may not be willing to take (Tabassum et al. 2017).

Future prospects

A new era in plant–microbe interactions has begun. In the past, the one plant – one microbe model was very effective in understanding the complicated genetic interactions that occurred between two organisms. Now it is important to elucidate how diverse microbes in either small clusters or large consortia interact with their plant hosts, their environment, and the indigenous microbial communities. Also, we need to learn more about what differentiates a beneficial microbe from a pathogen.

A recent large-scale genomic comparison of plant-associated bacteria (comprising endophytes and root-adhered (rhizoplane or rhizosphere), soil, and non-plant-associated (NPA) microbes) has given us clues as to the characters that will help in this en-

deavor. Levy et al. (2018) found that the dominant bacteria associated with plants are *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, all of which had been suggested as dominant phyla by earlier studies. Interestingly, bacteria in these phylogenetic groups have much larger genomes than the NPA microbes, as well as more genes coding for enzymes involved in carbohydrate metabolism (Levy et al. 2018). Another intriguing difference was that the NPA microbe genomes had more mobile genetic elements (phages and transposons) than the plant-associated group, even though the NPA microbes had smaller genomes. Analyses of these and other differences between the plant-associated bacteria and the NPA, especially in the genera that consist of both beneficial and pathogenic species, may not only prove to be useful in designing agricultural microbiomes but may also result in a better delineation of the differences between pathogenic and beneficial microbes.

Culturing new varieties of PGPB is also an extremely important priority for the future because unless the bacteria can be grown and developed into commercial inoculants, new players in plant–microbe interactions will remain small in number. Efforts are being made in this direction, but it will take a dedicated, as well as well-funded effort, to bring more scientists into pursuing this goal. Building on data obtained from basic studies in plant–microbe interactions, scientists now need to develop a diverse toolset not only for preserving soil health but also for growing crops sustainably. It will take a dedicated assemblage of scientists to develop microbial consortia as critical inputs into agriculture to ensure that our environment remains not only productive but also healthy.

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