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Research Article

Current Understanding of Bacterial Endophytes, Their Diversity, Colonization and Their Roles in Promoting Plant Growth

Penistacia Mehabo Maela and Mahloro Hope Serepa-Dlamini*

Faculty of Science, Department of Biotechnology and Food Technology, University of Johannesburg, Johannesburg, Gauteng, South Africa

Abstract

Plants are associated with a variation of diverse microorganisms, which occur as either endophytes or epiphytes. Endophytes are found within the plant while epiphytes are attached to the plant surface. Endophytic bacteria use various mechanisms such as chemotaxis and quorum sensing to colonize plants. The study of microbial communities has been revolutionized by the application of post genomic studies such as metagenomics, metaproteomics and metatranscriptomics, which have allowed scientists to analyse endophytes directly from the plant internal environment *in-situ*. This review aims to address the potential of bacterial endophytes and the application of post genomic techniques such as metagenomics, metatranscriptomics and metaproteomics to better understand and identify novel genes which could contribute to understanding the important roles that microbial communities play in plant growth and in the improvement of crop yield.

Keywords: Endophytes; Epiphytes; Colonization; Metagenomics; Metaproteomics; Metatranscriptomics

Introduction

Endophytes have gained scientific and commercial interest due to the association they have with internal tissues of host plants as they have proven to have potential in improving the quality and growth of plants [1]. Endophytes are defined as microorganisms, which colonize living internal tissues of most plants, including the xylem vessels, and grow inside the plant without causing any infection or disease to the host plant [2,3].

Endophytes can be differentiated either as obligate or facultative endophytes. Obligate endophytes are those that are not culturable, and require more specific conditions for their growth, whereas facultative endophytes are those that are able to survive in soil, artificial nutrient medium, plants surface and inside the plants [3,4]. The advantage of facultative endophytes is that they are widely distributed throughout the plant kingdom and that their potential for the development of commercially natural products can be exploited, as they can be isolated easily compared to obligate endophytes [4,5].

The most common endophytes that have been reported have been isolated from wild or cultivated crops of monocotyledonous and dicotyledonous plants [6]. Endophytes may be classified as actinomyces, bacteria and fungi depending on the microorganism, with bacterial and fungal endophytes being the most studied [7,8]. Other microbial forms such as archaebacteria and mycoplasmas exist as endophytes in plants but there is no evidence of them that has been presented [9]. The interactions between endophytes and the host plants are complex and involve mutualism and antagonism. The association can either be obligate or facultative [2]. Endophytic actinomyces are usually found within the inner tissues of non-symptomatic plants [10]. The most isolated actinomyces from different plant parts (stem, leaves and roots) is Streptomyces [11].

Bacterial endophytes include several genera and species and as such cannot be classified as single species [12]. Some of the bacterial endophytes that have been isolated from different plants such as soybean and cotton include various endophytic genera such as *Azoarcus*, *Klebsiella*, *Pantoea* and *Pseudomonas* [13]. Amongst the studied bacterial endophytes, Plant Growth Promoting Bacterial Endophytes (PGPBs) have also been identified which promote plant growth and development [14,15]. Their identification as plant growth promoting bacterial

***Corresponding author:** Mahloro Hope Serepa-Dlamini, Faculty of Science, Department of Biotechnology and Food Technology, University of Johannesburg, Johannesburg, Gauteng, South Africa, E-mail: hopes@uj.ac.za

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endophytes is attributed to their role in enhancing plant growth using various traits such as the production of ammonia, cyanide, indole-3-acetic acid siderophores, nitrogen fixation and phosphate solubilisation [16-18]. Examples of bacterial endophytes that have been reported to promote plant growth include *Rhizobia* spp. and *Frankia* spp. [19].

Fungal endophytes are a group of polyphyletic ascomycetous fungi that are usually associated with a variety of plants such as seed plants, ferns, mosses and lycophytes [10,20]. Furthermore, they are classified into two major groups, which are clavicipitaceous and non-clavicipitaceous fungal endophytes. Clavicipitaceous fungal endophytes belong to the genera *Epichloe* and *Balansia* and they mostly found in grasses while non-clavicipitaceous fungal endophytes belong to the Ascomycota and usually found colonizing the inter and intracellular spaces of plants [21,22]. Examples of ascomycetous fungi that are known as endophytes include *Candida guilliermondii*, *Candida oleophila*, *Candida railenensis* and *Wickerhamomyces anomalus* [23,24].

The symbiotic relationships between endophytes and host plants is advantageous for both parties in that the endophytes benefit from the nutrients made available by the plants while the plants benefit indirectly from the endophytes which increase resistance against pathogens and herbivores [22]. In addition, plants also benefit from endophytes by facilitating nutrient uptake (nitrogen, iron and phosphorus) from the environment, stress tolerance and promotion of plant development and growth [16,24]. Endophytes have also been reported to produce phytohormones (auxin, gibberellin, cytokinin etc), vitamins and various bioactive compounds that may be used in the development of pharmaceutical drugs and enzymes of biotechnological interest [25,26]. The need to exploit endophytes for use in many biotechnology applications is required to improve growth of food, increased tolerance for growth on marginal lands and production of energy crops in higher yields [1]. This review will provide insight into the recent knowledge of endophytes, focusing mainly on bacterial endophytes and the roles they play in plant growth as well as their applications.

Biodiversity of Endophytes

Most endophytic studies, which focused on diversity of endophytic bacteria obtained from the rhizosphere, were based on surface sterilization methods. It has estimated that there are about 300 000 plant species and most of them are associated with endophytes [27,28]. It is very rare to find a plant species which is endophyte free and if a plant is endophyte free it would not be able to cope with pathogen attack and it would be more prone to environmental stress conditions. Depending on the diversity and composition of microbial communities, endophytes are often associated with a variety of plant organs such as the roots, leaves, stems of which many may harbour similar species [29].

Usually the plants' abiotic and biotic factors determine which endophytes will colonize it and the extent of the microbial population depends on the type of tissue being colonized or on the season in which they were isolated [29,30]. The endophytic microbial communities are usually structured based on environmental conditions including the soil, biogeography, host plant and the interactions between the inhabiting microorganisms as well as the microbe-plant interaction [10,31].

Many bacterial endophytes are usually isolated from the intercellular spaces and the vascular tissues of the host plants and these include a variety of plants such as cotton, sweet corn, pea cultivars, rice and soybean that have been reported [10,13]. The most occurring genera that have been reported to be occurring mostly in agricultural crops are *Bacillus* and *Pseudomonas* [29]. A study on endophytic bacteria discovered that even when bacterial endophytes colonize the entire plant; these get out-numbered by high number of species found in the roots [32]. The most dominant endophytic bacteria belong to the phylum Proteobacteria including the β and γ-Proteobacteria sub-groups which mostly are related to epiphytic species [29,33]. Other classes which are most consistently found as endophytes includes the Firmicutes and Actinobacteria while the Acidobacteria, Bacteroidetes, Planctomycetes and Verrucomicrobia are less commonly found as endophytes [10].

Few studies have been conducted in which different environmental variables such as seasonal influences and phytoplasma infection were analysed for their effects on the diversity of endophytes in grape plants using taxon-specific real time Polymerase Chain Reaction (Real Time PCR). From the study, several bacterial genera were detected and amongst them, some had biocontrol strains that were associated with the phytoplasma infection process and these included *Burkholderia*, *Methylobacterium*, *Sphingomonas*, and *Pantoea* [33].

The diversity of bacterial endophytes has been reported in different plant species, most were described by previous reviews with much interest on the ones with agronomical properties [9,24]. The 16S rRNA gene pyrosequencing technique was used to determine the composition of bacterial endophytes in tomato leaves and the results obtained revealed that the leaves were composed of five phyla which were Proteobacteria, Actinobacteria, Planctomycetes, Verrucomicrobia and Acidobacteria, with Proteobacteria as the dominant phylum and least dominant phylum was Acidobacteria [33]. With the application of modern sequencing technologies, it is expected that many studies undertaken will be based on the diversity of endophytes, expanding the knowledge of the ecological roles that endophytes play in the internal plant microbiome with the hope of using the information to improve crop yield [29,32,33].

Colonization of Endophytes

The colonization process for both bacterial and fungal endophytes are similar however, their mode of colonization are different. Bacterial colonization happens intercellular and they are found in the vascular tissues of plants while fungal colonization is both intercellular and intracellular throughout the plant roots [34-36]. It has been assumed that the colonization of plants by endophytes depends on the physiology and biodiversity of the host plant, microbial prevalence and absorption of soil aggregates [37,38].

There are two routes in which endophytes may be able to penetrate the plant tissues which are the root hairs and the epidermis through the production of pectin degrading enzymes produced by the endophytes [30,39]. The production of pectin degrading enzymes makes it possible to penetrate the plant tissues without causing any harm and can colonize the intracellular spaces or in the vascular tissues of the host plants (xylem or phloem). There are factors that may play a role in the regulation of microbial colonization such as the genotype of the plant, its growth stage, physiological status, soil condition, type of plant organ and agricultural practices [37].

Colonization of plants by bacterial endophytes

The rhizosphere attracts a variety of microorganism due to the presence of rhizo-deposits and the roots exudates which are released during seed germination and root development [36,39]. The rhizospheric bacterial communities develop and are attracted to the rhizosphere due to the carbon that is formed, which is rich in nutrients when the exudates are released. Bacteria get their carbon source from the roots exudates through the breakdown of organic compounds within the root exudates [39].

Colonization of host plants by bacterial endophytes usually involve passive invasion in the root which can be at open root sites (lateral roots emergence) or wounds [34]. Systematic colonization by bacterial endophytes can be achieved even from a single entry into the plant host. Certain bacterial traits known as colonization traits regulate the whole plant colonization process including communication between the bacterial endophytes and the plant and vice versa [40].

The colonization process of plants by bacterial endophytes is quite complex as it includes the host recognition, germination of spores, penetration of endophytes into the host, colonization and the consistency of the endophytes in the host cells [37]. With that being said, endophytic bacteria must be well equipped with cellulolytic enzymes to actively hydrolyse the exodermal cell walls of plant host. Cellulase, which is the main enzyme for hydrolysing cellulose, was detected at the primary sites of entry of *Azoarcus* sp. BH72 [41]. Only a small fraction of bacterial endophytes are able to colonize the upper parts of the shoots, leaf apoplast and reproductive organs (fruits, seeds, and flowers) as there is a limited concentration of nutrients and have been identified using cultivation methods and microscopic visualisation [42]. Colonization of bacterial endophytes in host plants is depended on numerous biotic and abiotic factors which include the characteristics of the host plant (biological or physical), humidity, seasonal fluctuations, temperature and some of cohabiting microorganisms [43].

In addition to using plant roots to enter the plants, other tissues may be used by endophytic bacteria such as entry through aerial tissues that are above the ground (stem, leaves, flowers and fruits) which involve passive or active mechanisms that permit the bacterial endophytes to travel from the rhizoplane into the cortical cell layers [36,40,44]. Within the cortical layer, the endodermis becomes a barrier in which the colonization process continues for those bacteria that were successful in entering the plant tissues which get transported by the xylem vascular system. This results in systematic colonization by endophytic bacteria of internal tissues within the host plants. However, there are factors that may reduce the colonization of plant surfaces by bacteria such as Ultraviolet light (UV), desiccation and the deficiency of nutrients [36].

Bacterial endophytes use different mechanisms to enter the host plants such as motility, chemotaxis and quorum sensing depending on the strain and host species [12,44]. Motility is the movement of bacteria using flagella through the production of polysaccharides to facilitate the colonization process into the root hairs of plant species such as *Alcaligenes faecalis* and *Azospirillum brasilense* [13].

Chemotaxis is a sensing mechanism in which bacterial endophytes use directional motility to the root exudates where there are high concentrations of attractant, which gets activated when there are changes in the soil environment such as temperature, pH, viscosity and osmolarity [43,45]. Chemotaxis may be different for bacterial endophytes and in most cases; it is believed that there are multiple parallel paths that evolve during plant-microbe interactions. The most common chemo-attractants which have been reported include organic acids in *P. fluorescens*-tomato interactions and amino acids which attract *Corynebacterium flavescens* and *Bacillus pumilus* to rice [45]. These interactions may be related to the nutritional requirements of the bacterial endophytes.

Quorum sensing is another communication method in which bacteria especially gram-negative bacteria synthesis autoinducers of the N-acyl-homoserine lactone (AHL) which gets released into the cellular environment in response to the cell density [46]. By releasing these metabolites, bacterial endophytes can sense the quality of the cellular environment and this in turn aids in their adaptation to the given conditions and to the regulation of their gene expression [47]. Autoinducer Quorum Sensing (QS)-molecules have different structures and are widespread among bacterial endophytes however; only cyclic peptides are found in gram-positive bacteria only [46].

The QS compounds may be used by bacterial compounds such as when there are pathogens in the surrounding environment or if there are mutualists interacting with the plant roots. The first specific quorum sensing mechanism was demonstrated in the legumes *Phaseolus vulgaris* and *Medicago truncatulan* [46,48]. The importance of bacterial quorum sensing compounds during the colonization process is supported by a recent study in which a quorum sensing mutant of *Bukholderia phytofirmans* PsJN was unable to colonize and promote the growth of *Arabidopsis thaliana* [13]. There are compounds that may limit the production of QS compounds which have the potential to disrupt the signalling of QS compounds by certain bacteria [46].

Bacterial traits involved in colonization

The importances of certain traits involved in the colonization process have been confirmed by molecular studies such as genomics, metagenomics and transcriptomics with other related studies. These traits are utilized by bacterial endophytes for infecting the plant host and for adaptation within the plants [28]. Some of the traits that ensure the success of the

colonization process include the formation of lipopolysaccharides, cell-wall degrading enzymes, motility and chemotaxis [12,13,28]. A survey, which was conducted using comparative genomics of bacterial strains, hypothesized that plant colonization and the lifestyle of endophytes within the plants are attributed to several genes which are involved in the attachment of endophytes to the roots, motility, and biofilm production.

The bacterial endophytes enter the host plant by first attaching to the plant roots or other opening sites caused by wounds using type IV pili which are important for bacterial adhesion and colonization [49]. The attachment process may be facilitated by the synthesis of Exopolysaccharides (EPS) and lipopolysaccharides by bacterial cells which may also be important in the early stages of colonization [13,50]. The importance of EPS production by the endophytic bacterium *Gluconacetobacter diazotrophicus* Pal5 was reported as an important factor during surface attachment and colonization in rice roots. Moreover, recognition of bacterial endophytes by host plants is regulated by Type III Protein Secretion Systems (TTSS) which help modulate bacterial effectors into plant cells [49].

The success of roots attachment by bacterial endophytes is followed by the formation of biofilms as the bacteria would have multiplied, reaching a population density [49]. Biofilms are communities which have been structured of sessile microbial aggregates, enclosed in polymeric matrix, attached to an abiotic or biotic surface [51]. They are often composed of water and bacterial cells. The development of biofilm formation often requires cell to cell communication between the colonizing bacteria and the host plants. The bacterial communities within biofilms may exhibit cooperative behaviour and may be vulnerable to environmental conditions which may be harsh such as antibiotics, drying and osmotic shock [51,52].

Biofilms have unique properties depending on the type of tissue that has been colonized. Bacterial interactions with plant tissues are facilitated by active motility which initiates surface contact and through adhesions, polysaccharides and surface proteins [52]. By forming biofilms, this also permits microorganisms in the rhizosphere which are non-spore forming to also colonize their surrounding environments [49]. A gene cluster, gumD was reported to be required for biofilm formation and plant colonization in *Gluconacetobacter diazotrophicus* [13].

Flavonoids are known to stimulate the colonization process by regulating bacterial genes such as type III secretion, genes for phytoalexin resistance and genes for synthesising lipopolysaccharides [40,53]. All these genes participate in the interactions between the bacteria and plants and help competent endophytes in occupying suitable and permanent niches on the plant roots and in the rhizosphere [40]. It has been reported that endophytic colonization was improved in *Serratia* spp. rice seedlings by flavonoids and some phytohormones such as auxin which is important for plant growth and development [49,54]. Other types of phytohormones include abscisic acid, cytokinin, ethylene and gibberellin [55].

Isolation and Identification of Endophytes Using Culture Dependent Methods

The most utilized method for isolation of endophytes is surface sterilization of disease-free organs, in which the plants must be free of microbes that are present on the plant surfaces. Different organs of the plant tissues may be used for isolating endophytes such as the leaves, roots, stem and fruits [13,56]. There are two requirements that must be considered when plant organs are sterilized for isolation of endophytes, (1) all the plant surface microorganisms present must be eliminated, and (2), the sterilization procedure should have minimal or no negative effect on the endophytes [57].

The surface sterilization method is usually carried out in the laboratory, under aseptic conditions. Prior to carrying the surface sterilization method, the plant tissues are usually rinsed a few times with running tap water to remove soil particles from the plants. This is then followed by the surface sterilization in which the rinsed plant tissues are treated with 70% ethanol for 1 min, followed by submerging the plant tissues in sodium hypochlorite for 1 to 5 min in which the concentration has been predetermined and finally the plant tissues are rinsed with sterile distilled water several times [1,10]. Other Researchers have utilized other sterilizing agents such as hydrogen peroxide and mercuric chloride with varying concentrations of 0.05-0.2% [58].

Other researchers such as Gohain et al. used 0.1% Tween 20 to rinse plant samples before carrying out sterilization process [24,25]. Coombs et al. used 99% ethanol to sterilize roots, which was followed by washing with 3.125% sodium hypochlorite (NaOCl) and 99% ethanol followed by final rinse with sterile Reverse-Osmosis (RO)-treated water [59,60]. In another study, the plant samples were surface sterilized by immersing in a solution of cycloheximide (50 μg/ml) for 4 h and then washed in 3.15% NaOCl for 15 min, followed by overnight storage in the refrigerator at 4°C [3]. The sterilization procedure is then verified by plating the final wash water onto nutrient medium, in which no growth is expected.

The isolation efficiency may be influenced by the nutrient medium used; this will depend on the species of interest and the research goal [1]. There are different types of media that may be used during the isolation of endophytes that are minimal, rich and complex media. Minimal media contains specific amounts of nutrients and complex media contains undetermined nutrients in high quantities. The type of nutrient medium chosen may affect the number and the diversity of endophytes isolated from a specific plant tissue and the cultivability of some endophytic bacteria [1,58].

The classical approach that is used in traditional microbiology for isolating and culturing microorganisms from an environment usually involves growing the microorganism on different nutrient media under different growth conditions to obtain pure colonies, this however results in other novel microorganisms not being studied as they are difficult to grow using culture-dependent techniques [61]. The use of culture dependent methods to isolate bacterial endophytes are only limited on the endophytes ability to grow on nutrient media, while obligate endophytes is unable to grow and cannot be

recovered from media [62]. It has been estimated that more than 99% of prokaryotes found in the environment cannot be cultivated and only 5% of bacterial species have recently been documented [63,64].

Functional characterization of endophytes

There are different microbial communities, which are very diverse that inhabit plants ranging from the rhizosphere, phyllospheres to the endosphere [65]. Majority of these microbes produce a wide range of substances, which in turn regulate plant growth. Among the substances produced by different microbial communities, bacteria produce phytohormones, which regulate plant growth by enhancing nutrient and water uptake through the modification of the root systems [66]. The properties of plant growth promoting bacteria may vary and as such, it is important to study their properties from microbial communities associated with plants that are economically important [67]. Some of the properties that are exhibited by endophytes include synthesis of 1-aminocyclopropane-1-carboxylate deaminase (ACCD), indole-3-acetic acid (IAA), siderophore production, phosphate solubilisation and production of antimicrobial metabolites.

1-Aminocyclopropane-1-Carboxylate Deaminase (ACCD): The synthesis of ACCD is produced by various bacterial and fungal species; it is a pyridoxal phosphate independent enzyme which promotes plant growth and development under abiotic and biotic conditions by reducing the levels of ethylene which inhibits plant growth [68,69]. Ethylene levels are reduced when ACCD hydrolyses 1-aminocyclopropane-1-carboxylic acid (ACC) which is an immediate precursor of ethylene. The precursor ACC is hydrolysed into ammonia and α-ketobutyrate, which gets metabolized further by bacteria, in doing so, reducing the effects of ethylene and promoting plant growth [66].

The activity of ACC deaminase portrait by plant growth promoting bacteria is one of the common traits of plant development and it is widely known to have biocontrol activity against plant pathogens, delay senescence, protection from deleterious effects of environmental conditions [68]. The importance of ACCD synthesis by various bacterial communities has been reported in various studies including phytoremediation, rhizodegradation and detoxification of heavy metals. Rodriquez et al. conducted a study on tobacco plants in which their growth was enhanced by *Pseudomonas putida* HS-2, in which the ACC enzyme was purified [20,68,70].

Indole-3-acetic acid (IAA): Several microorganisms that are found in the soil, synthesis phytohormones especially auxin, which is essential for plant growth and development [69]. Amongst the synthesized phytohormones, IAA has been found as essential phytohormones due to its role in plants. It contains a carboxyl group, which is attached to the third carbon of the indole group [71]. The important roles that IAA plays in plant growth development includes cell elongation, cell division and cell differentiation in plants, which in turn affect plant nutrition and development [67].

The production of IAA is widespread amongst many roots associated microorganisms such as *Enterobacter* sp., *Pseudomonas* sp., and *Azospirillium* sp. in both pure culture and in soil [72]. Higher levels of IAA have been reported to be produced by microorganisms, which have been isolated from the rhizosphere and rhizoplane. It has been reported that production of IAA is thought to be a direct mechanism by plant growth promoting bacteria to enhance plant growth and yield [73]. However, high levels of IAA production by bacteria can cause plant abnormalities during development, stimulating the formation of adventitious roots while low levels of IAA promote root elongation [69].

As the production of IAA is an important factor in plant growth, it is important to screen microbial strains for their potential to synthesis IAA to select effective plant growth promoting bacteria. A study was done in which five bacterial strains from the genera *Bacillus*, *Escherichia*, *Micrococcus*, *Pseudomonas* and *Staphylococcus* with wild herbaceous flora were tested for their potential to increase IAA levels and growth of *Triticum aestivum* var. Inqalab-91, using Gas Chromatography and Mass Spectrometry (GC-MS) and the results showed that the bacterial strains enhanced root length and seed weight by 16% and 70%; respectively [73]. Another study was done using high liquid chromatography in which two bacterial strains *Klebsiella* sp. (PnB 10) and *Enterobacter* sp. (PnB 11) isolated from *Piper nigrum* were found to have high plant promoting properties in *Vigna radiata* seedlings [67].

Siderophore production: Siderophores are low weight secondary metabolites (400-1 000 Daltons) that have a high iron chelating affinity which are produced by various fungal and bacterial species [72,74]. Iron is an important nutrient for all forms of life and it usually exists as Fe^{3+}, forming insoluble hydroxides and oxyhydroxides that cannot be utilized by microorganisms [75]. Iron is made available by bacteria through the productions of siderophores, which solubilize it from mineral or organic compound by binding ferric ions and transporting it into cells [76]. The iron can also be made available by the expression of specific proteins by siderophore producing microbes. Siderophores are classified into four groups based on their structure, functional group and the type of ligand which are carboxylates, catecholates, hydroxamates and mixed types [77].

Siderophores are mainly produced by growth promoting bacteria as they produce siderophores under extreme environmental conditions such as when there is a scarcity of nutrients or in the presence of heavy metals [77]. Production of siderophores makes it possible for microorganisms such as endophytes as they may help in the associations between plants and bacteria and in the colonization process of plants (roots, leaves and stems) [78]. The production of siderophores is also beneficial to plants as they inhibit the growth of plant pathogens by limiting iron [67,72,74]. In addition to siderophores being important for promoting plant growth and development, they may also be used in bioremediation by binding to heavy metals which are toxic such as Chromium (Cr), Aluminium (Al), Lead (Pb) and Mercury (Hg) [75,79]. Furthermore, they can also be used in various ways such as biosensors for sustainable agriculture and in medicine.

Siderophore production by microorganisms is determined using the Chrome Azurol Sulphonate (CAS) assay which was first used by Schwyn and Neilands [76,77]. This method can be used to estimate siderophore production either quantitatively using supernatants of microbial cultures or qualitatively using solid CAS agar media. During the CAS assay there is competition of iron uptake between the siderophore and Fe^{3+} complex of the CAS dye and as the siderophore chelates the iron from the iron-dye complex, there is a colour change from blue to orange as the dye becomes free in the media [80]. There are currently 500 different siderophores which have been identified which differ in their structure and functional group [75].

Two endophytic bacterial strains *Bacillus subtilis* (KDRE01) and *Bacillus megaterium* (KCRE25) were identified using the CAS assay method which had positive reactions for siderophore production under iron limiting conditions [81]. Several bacterial and fungal species were identified to produce siderophores and were also found to inhibit pathogenic fungi in rice plants using BOX-PCR fingerprinting. The study identified the genus *Burkholderia* and *Pseudomonas* as good antagonists of *Azospirillium brasilense* and *Herbaspirillum seropedicae* [72].

Phosphate solubilization: The second most essential nutrient required by plants following nitrogen is phosphorus which is usually found in soil (400-1200 mg/kg of soil) and exists as mineral salts or organic compounds which are insoluble [82,83]. Most of the phosphorus found in soil is unable to support plant growth as it is insoluble and has a poor mobility, which is mainly due to the reactivity of phosphate ions with other soil constituents [84]. Microorganisms secrete various organic acids (acetic, citric, succinic and oxalic acid) and phosphates, thereby making phosphorus be available to plants by converting insoluble phosphate into soluble mono- and di- basic ions [85]. The process of phosphate solubilisation may be complex and can be affected by environmental factors such as pH, oxygen concentration, humidity and temperature.

Many research studies have shown that Phosphate-Solubilizing Bacteria (PBS) are able to transform insoluble phosphate to soluble forms using various mechanisms such as acidification, chelation, and exchange reactions and by formation of polymeric substances [85]. The solubilisation and mineralization of insoluble P by PBS is also important in plant growth promoting fungi such as mycorrhizae [19]. Several rhizosphere bacteria from the genera *Pseudomonas*, *Bacillus* and fungi from the genera *Penicillium* and *Aspergillus* have been identified to have the ability of transforming insoluble phosphate into soluble form through the secretion of organic acids [82].

A study in which endophytic bacteria were isolated from surface sterilized leaf and stem of various medicinal plants such as *Azadirachta indica* and *Zingiber officinale* Rosc using different nutrient media. The isolates were screened for plant promoting traits such as phosphate solubilisation, IAA and siderophore production using morphological, biochemical and molecular ribotyping; five isolates were characterized as *Bacillus tequilensis* (AAU K1), *Bacillus endophyticus* (AAU K2), *Beijerinckia fluminensis* (AAU K3), *Bacillus safensis* (AAU K4) and *Pseudomonas aeruginosa* (AAU K5) [83]. A phosphate solubilising fungus *Penicillium radicum* was isolated from wheat roots, which showed to have plant promoting traits through phosphate solubilisation using *in vitro* studies [86]. Phosphate solubilising bacteria have key role to play in agriculture as the demand to increase phosphate fertilizers is on the rise. Moreover, more understanding is needed on the solubilisation process by PBS and their application [85].

The study of microbial ecology is limited due to variation of diversity of endophytic life, but culture-depended methods have allowed for the isolation, identification and characterisation of important genes involved in beneficial interactions between the endophytes and host plant [87]. Due to the biases related with nutrient medium for isolation of endophytes and their culturing conditions, this has resulted in the inability to fully study the endophytic diversity of plants and as such, the introduction of culture-independent methods were needed [61,88].

Current Culture-Independent Techniques for Studying Bacterial Endophytes

There are approximately 99% of microorganisms that cannot be cultivated and as such a wide variety of molecular techniques have been developed over the last few decades which have proven to be valuable tools of studying the diversity and function of endophytic bacteria in plant host [89]. However, certain concerns need to be taken into consideration when applying molecular techniques that need to be evaluated such as the biasness of each technique. This then led to the improvement of molecular techniques, which have been previously used to study bacterial endophytes to provide more accurate information on the composition and functions of microbial communities [89,90]. The improved techniques such as metagenomics, metaproteomics and metatranscriptomics will aid in answering questions about the microbial communities on the roles and functions they play in the rhizosphere as well as their interactions with plant host [87,91,92].

Metagenomics

There are two main methods that are used for studying the structure and function of the microbiome population using high-throughput sequencing which are marker-gene studies and whole-genome-shotgun metagenomics. Marker gene studies involves the amplification a gene of interest from all the genomes present in the sample through PCR and the amplified gene gets sequenced and clustered into Operational Taxonomic Units (OTUs) which are compared across samples [93,94]. The advantages of marker gene studies include cheap costs and rapid methods; this method however has a disadvantage in that it does not reveal other information about the other genes which are encoded in the metagenomes that are not sequenced.

Metagenomics on the other hand is a complementary method that can be used as an alternative for marker-gene studies. Metagenomics is the application of modern genomics that is used to analyse the sequences of genomes obtained directly from various ecological communities to gain access to the physiology and genetics of un-cultivatable microorganisms [87,94,95].

Metagenomics has become a common method for the study of the microbiome population as it does not only focus on a single

marker gene, but rather focuses on sequencing the genomes of all the organisms to provide information on the organization, structure and functions of the genes [94]. Furthermore, it also aids in the identification of novel genes and biocatalysts, community structure as well as evolutionary relationships within the microbiome population.

Prior to sequencing, genomic DNA is extracted directly from an environmental sample [95]. There are currently two approaches that are used to sequence metagenome samples, which are cloning or by using one of the next generations sequencing techniques such as pyrosequencing. Cloning involves the use of small plasmids or Bacterial Artificial Chromosomes (BACs), and the sequences are determined using dideoxy chain termination sequencing (Sanger sequencing) while pyrosequencing the DNA is sequenced without the use of cloning [96].

Both the Sanger sequencing and pyrosequencing have advantages and disadvantages such as that Sanger sequencing creates longer sequence reads but due to cloning it has inherent bias while pyrosequencing has a higher throughput and a lower error rate per base when compared to Sanger sequencing [96,97]. After the sequencing data is generated it is then followed by the analysis of the metagenome sequence where it is compared against known sequences. This involves the use of bioinformatics analysis pipelines that involves the construction of contiguous sequences (contigs and scaffolds) by assembling the sequenced data followed by the prediction of genes and putative genes and the prediction of functions, pathways and domains for the putative proteins [94].

Several researchers have applied the use of metagenomics to study the diversity of microbial communities as well as their distribution across different environments such as in grasslands, soil and sea sediments [94,98]. A study was done by Mashiane et al. using PCR-DGGE and high throughput sequencing were done to determine the bacterial endophytes associated with Bt maize genotypes (Mon810). The study revealed that the Proteobacteria phyla were the most dominant followed by the Gamma-proteobacteria in the maize phyllospheres. Furthermore, it was found that the Alphaproteobacteria and the Actinobacteria were only dominant in non-Bt maize [99-101]. In addition, endophytes with beneficial traits were identified between the cultivars and these included *Acidovorax*, *Burkerholderia*, *Bracgybacterium*, *Enterobacter* and *Rhodococcus*. Another study was done to detect microbes with Antibiotic Resistant Genes (ARGs) in the gut with the aim of detecting methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococcus* and multi-drug resistant *Enterobacteriae* by Anderson et al. using metagenomics [101,102]. The study consisted of detecting the AGRs in three groups which were low risk outpatients, high risk inpatients and controls and it was found that there was a higher number of AGRs in patients as compared to the controls.

Metagenomics studies mainly depend on the aim of the project that is being conducted to select an optimal sequencing strategy such as Illumina HiSeq sequencing which allows the sequencing of less abundant microorganisms that may be involved in ecosystem functioning at a lower cost [101]. Metagenomic studies are important as they aid in the understanding of the interactions between plants and endophytes and help in discovering the potential of uncultivable microbial communities as information beyond the genomic information of individual taxa is revealed when using the metagenomics approach [87].

Metaproteomics

Metaproteomics is defined as the study of all the proteins that are present in an environmental sample such as the rhizosphere to get insight on roles that microorganisms play in the ecosystem such as in biochemical, degradation and bioremediation processes [89,102]. Proteins are important macromolecules as they carry out most of the cellular activities encoded by a genome and as a result they are responsible for most of the functions and process within the endosphere and rhizosphere communities [103]. The main goal of metaproteomics studies are to the physiology, ecology and evolution of microbial communities in complex environments to understand the ecological interactions and to characterize the metabolic activities that occur within a community [29,87,104].

Metaproteomic analysis may also be used for pure cultures in which the sample of interest is prepared and then this is followed by extracting the proteins which will get separated into two dimensions and eventually the separated proteins will be identified using Mass Spectrometry (MS) [105,106]. The use of tandem MS has become a key leader in proteomic studies in diverse microenvironments revealing expressed functions of microbial communities [87,107]. Metaproteomics takes advantage of MS as it has high power performance when characterizing the complete set of proteins expressed by microorganisms even for those microorganisms found in environments of low diversity (waste-water sludge, acid-mine drainage etc.) and those in anaerobic reductive dechlorinating communities [102,108,109].

Several metaproteomic studies have been used to reveal the diversity of proteins which are expressed between endophytes and host plants. A study done by Lery et al. used a sugarcane-Gluconacetobacter interaction model to determine the number of proteins using Mass-spectrometry which revealed 78 different expressed proteins [88,89]. Another study done by Lin et al. was done in which a metaproteomic profile of ratoon sugarcane in the rhizosphere and the plant sugarcane were compared and the results showed that 24.77% of the proteins found in the soil were derived from bacteria and that majority of the up-regulated expression of their proteins were involved in membrane transportation and signal transduction [81,82,89]. The study also revealed that the ratoon sugarcane induced major fluctuations in soil enzyme activity, the breakdown of microbial populations and in the level of proteins expressed that originated from the plants, microorganisms and fauna.

Metaproteomics has become an important tool for analysing the link between taxonomic diversity and the functional profiles of microbial communities. However, it is still a challenge to analyse the proteomes in mixed communities which are complex and only a small percentage (approximately 1%) of the metaproteome may be determined [103,105].

The setback involved in metaproteomics studies are that without genetic information, studies based on proteomes of microbial communities remains incomplete. Furthermore, there are difficulties when it comes to extracting proteins and sample preparation due to presence of substances causing interference such as alkaloids, organic acids, polysaccharides, polyphenols, lipids and secondary metabolites [87,110]. For this technique to be more effective, more information on the metagenomes of microbial communities from different microenvironments is needed to be able to characterize endophytic microbial communities [102,105,107].

Metatranscriptomics

Metatranscriptomics is the study of messenger RNA (mRNA), by characterizing all the mRNA's (transcripts) produced by all the cells of the bacterium associated with different plants [87,89]. Metatranscriptomics is also known or referred to as environmental transcriptomes, microbial community gene expression profiles, microbial community RNAs and whole community transcripts [110]. This molecular technique aims at identifying active microbial communities in which genes are transcribed and identifying the metabolic processes of these communities [111].

The ribosomal RNA (rRNA) is the most dominant and it is present in over 90% of RNA species in complex microbial communities and even on pure culture [112]. This plays a huge role in metatranscriptomics studies as the amount of rRNA in a microbe can correlates to the growth activity of the microorganism that is being studied [113]. To study the transcriptomes of microbial communities using high-throughput sequencing technologies, the bacteria should be rich in messenger RNA (mRNA) [112]. The total mRNA content of bacteria can be estimated based on the total RNA recovered from a known number of bacterial cells [114]. Studies based on mRNA may be limited as mRNA degrades quickly and they are important in studying the response of microbial communities.

The application of metatranscriptomics has not been fully used to analyse the rhizosphere community due to the mRNA being unstable and that extracting it from complex ecosystems is quite challenging [89]. Another challenging factor facing metatranscriptomics studies is the separation of mRNA from the other types of RNA which are tRNA, rRNA and miRNA and that there are humic compounds that co-exist with nucleic acids that may cause interference [112]. There are currently 200 bacterial species that have been sequenced by the Genomic Encyclopedia of Bacteria and Archaea Project (GEBA) which was initiated in 2007 and is led by the department of energy in the United States of America [89]. The project aims in improving the identification of protein families, novel genes and organisms that have not yet been described to understand the genomes of microbial communities and to characterize bacterial phylogeny.

Various genes have been identified using the metatranscriptomic approach in some species such as *Eichhornia crassipes* and *Fusarium verticillioidies* associated with strawberry plants to define fungal communities [110]. A transcriptional profiling was done to assess the symbiosis of wheat roots colonized by *Azospirillum brasilense* using dual RNA-seq technology and the results showed that there was an up regulation of nutrient uptake and cell cycle genes [87]. Another study done on soybean plants revealed several small RNA sequences that were unrelated to the plant genome. The disadvantages of using RNA-seq technology are that it expensive and it is difficult to store data and to analyse it, hence the use of microarrays is still commonly used [115].

Metatranscriptomics studies are more effective when they related with metagenomic studies in that metagenomics are based on counting genes which are present and those that are absent in culturable and non-culturable bacteria while metatranscriptomics focuses on the comparing transcriptomes of related bacterial species which aids in understanding how microbial communities respond to changes in the environment [111]. Both techniques are essential to understanding the endophytic bacteria in the ecosystem and may also help in discovering genes and their functions [89].

The advantages of using metatranscriptomics are that there are neither probes nor primers needed, so sequencing of the microbial transcripts is done with little bias [116]. Another advantage is that information based on the expression of non-coding genes and small RNA is provided when using the metatranscriptomics approach. The use of metatranscriptomics to profile bacterial endophytes by direct sequencing will continue to add more knowledge to the growing microbial community databases to further address anticipated questions [112]. These studies, however, may be limited due to bacterial and archaeal mRNA's not being polyA tailed and this in turn results in low yields of expressed mRNAs when they get extracted.

Genomics of Endophytic Bacteria

Research based on genomic sequencing, comparative genomics has increased rapidly due to the rapid developments in next-generation sequencing, and as such this has hugely influenced the understanding on the potential of endophytes in terms of their genomes, ecology and evolution [12,45]. Furthermore, NGS has greatly contributed to research based on plant host interactions with over a hundred bacterial genomes being sequenced, making it possible to compare genomes of closely relate taxa from other microenvironments [117]. Some of the important insights that were revealed from genomic sequencing include the genes for motility, colonization and synergistic interaction between endophytes and the host plant [45].

Currently, very few endophytic bacteria have been sequenced including the complete genomes of plant promoting bacteria. Other bacterial endophytes which are still being investigated include *Enterobacter* sp. 638, *Stenotrophomonas maltophilia* R551-3, *Pseudomonas putida* W619, *Serratia proteamaculans* 568 and *Methylobacterium populi* BJ001 [30]. Amongst the few of the sequenced bacterial endophytes that play a role in growth and development of plants include the nitrogen fixing species such as *Azoarcus* sp. BH72, *Azospirillum* sp. B510, *Herbaspirillum seropedicae* SmR1, *Klebsiella pneumoniae* 342 and *Pseudomonas stutzeri* A1501 [12,117].

The complete genome of *Azoarcus* sp. BH72 was matched with related microorganisms in the rhizosphere [12,34]. Important factors which are related to plant interactions were discovered which were encoded by the *Azoarcus* sp. BH72 which included flagella which produced chemotaxis proteins, ferric siderophore systems, type IV surface polysaccharides on pili and protein secretion systems (type I and type II) [30]. The discovery of these novel genes indicated that there are many mechanisms which rhizosphere microorganisms employ to enter the plant host for them to become endophytes [118].

A variety of genes which are essential for plant growth promotion have been discovered in the sequenced genomes of some bacterial endophytes such as genes responsible for antimicrobial compounds; nitrogen fixation, ACC deaminase activity, acetoin and siderophore production [34]. Furthermore, species that are responsible for regulation phytohormones were identified in some species including as *Burkholderia phytofirmans* PsJN and *Variovorax paradoxus* S110 [117]. Other species which were found to have traits in relation to plant growth were *Pseudomonas putida* W619, *Pseudomonas stuzeri* A1501, *Serratia proteamaculans* 568 and *Stenotrophomonas maltophilia* R551-3 [117,119].

Genomes based on comparative analysis are widespread amongst the mentioned microbial species which have been successful in colonizing different plants hosts such as adhesion, chemotaxis, metabolic versatility, motility and detoxification of ROS (oxygen reactive species) and degradation of plant polymers [12]. However, the colonization process is not the same for all endophytes, making it a challenge when it comes to determining the important traits required for the adaptation and existence of endophytes [118,120].

Plant Growth Promoting Bacteria and their Applications

The mechanisms of plant-growth promoting bacteria has been studied in most plants to date however, their complete understanding has remained a bit vague, making it a challenge to fully understand the complex interactions between bacterial endophytes and their host plants [34,121]. Plant growth-promoting bacteria reside within the internal tissues of plants and participate in the biological activities of the host plant, promoting the growth and health of the plant [40].

Currently, research conducted on plant growth promoting bacteria indicate that these bacteria may affect the growth of plants either directly or indirectly [40]. Direct plant growth promotion usually occurs when certain hormones get produced such as auxin, cytokinin and ethylene, availability of nutrients and by the acquisition of environmental resources such as iron, nitrogen and phosphorus. Indirect plant growth promotion occurs when PGPB prevents or limits harm by various pathogenic agents (bacteria, fungi and nematodes), stress conditions and pollutants [34,58]. Common mechanisms used by PGPB to indirectly promote the growth of plants have been reported including antibiotic production, cell-wall degrading enzymes, lowering plant hormones (ethylene), decreasing the availability of iron to pathogens, systemic resistance and production of volatile compounds which inhibit pathogens [33,122].

Biocontrol activity

Some of the plant PGPB act as biocontrol agents by inhibiting the growth of pathogenic microorganisms through the production of siderophores which is commonly produced by bacterial species including *Pseudomonas* and *Enterobacter cloacae* [30,40]. The mechanism of secreting siderophores occurs when there are low levels of iron in the soil inhibiting phytopathogenic fungi such as *Fusarium* sp. which is dependent on iron absorption Pathogens may also be inhibited by the hyperparasitic activity possessed by PGPB which produce hydrolases to inhibit pathogens by degrading their cell walls [39]. Only a few bacterial species have been studied which were found to inhibit various plant pathogens such as *Pseudomonas*, *Bacillus*, *Xanthomonas* and *Erwinia* [30,39].

Plant growth promotion

There are some endophytes which facilitate in nutrient uptake and minerals which contribute to the growth of plants such as nitrogen and phosphorus fixation which are essential macronutrients required for the biological growth and development of plants [123]. The PGPB contribute to plant growth by solubilizing inorganic phosphorus which is insoluble and making it available to plants. This trait is common amongst many microbial communities' associate with various crop plants including wheat, rice, maize and legumes [124]. Several PGPB have been reported to have phosphate solubilizing properties such as members of the *Burkholderia*, *Enterobacter*, *Halolamina*, *Pantoea*, *Pseudomonas*, *Citrobacter* and *Azotobacter* [10].

Other metabolites that are produced by endophytes also contribute to the growth and development of plants such as IAA, indole acetonitrile, gibberellin and cytokine [30]. Other endophytes also produce antibiotics which inhibit the growth of pathogenic microorganisms or by antagonistic material. Furthermore, the ability of host plants to absorb nutrients to promote plant growth may be enhanced by endophytes. With the above information mention, endophytes have the potential to be commercialised industrially based on their roles in promoting plant growth and health [58,123].

Agriculture

The agricultural sector is mainly dependent on the certain factors such as fertile soil and stable climate conditions. Through the recent years, the agricultural sector has become the major source of environmental pollution due to the techniques used and economic framework conditions, which have major influences on the quality of water and soil, ecological balance and preservation of biological diversity [124,125]. The use of PGPB in agricultural practices may be a solution to the challenges faced by this sector.

Furthermore, the development of the agricultural sector may be restricted by plant diseases and insects which result in a great loss of agricultural products and ecosystem instability globally [10,124]. Although the use agrochemicals to decrease plant pathogens, these have major implications of threatening the environment and human health. Moreover, the use of chemical fertilizers to increase yield in agriculture are not only expensive

but also cause harm to the environment by depleting non-renewable energy which have major side effects including water pollution, leaching out and killing microorganism [122].

Endophytes may be used as substitutes for chemical pesticides such as entomopathogenic microorganisms to reduce plant disease and insect pets [126]. A study which was done on banana plants to see if endophytic bacteria and rhizobacteria could improve the resistant of the plant by infecting it with banana bunchy top virus and the results showed that the infection was reduced by 60% [127,128]. The use of endophytes as bio fertilizers could boost the agricultural sector as they may improve the growth and development of plants by providing the plants with important nutrients including nitrogen, phosphorus and ferric ions [58].

Conclusion and Future Perspectives

Currently, there are approximately 500 000 plant species of which many have not yet been exploited for their potential in agriculture, medicinal and industrial sectors. As much as the plant microbiome has been identified as a treasure trove of endophytic bacteria, there is still a deeper understanding of their interactions with different plant species to realise the importance of endophytes as plant probiotics. Plant-microbe interaction needs to be studied extensively to add more knowledge of the influences that endophytes may have on the physiology of plants, biochemistry and their adaptation to different environments.

With the world population increasing rapidly, the agriculture sector is also facing challenges with a need to sustain the agricultural sector while catering for the increasing population globally. Research based on plant-microbe symbiosis may aid in recognizing effective ways of sustaining the agricultural sector, ensuring human and animal production with little disturbance to the environment. However, certain questions need to be addressed when undertaking studies based on endophytes such as the whether it is possible to enhance the productivity of agricultural plants through the exploitation of endophytes, and if their specific traits for secondary metabolite production and for plant development can be exploited also. These key questions can only be answered through information generated from post genomic techniques such as metagenomics along with other system biology techniques.

The discovery of novel plant endophytes could bring light to the agricultural sector in terms of the utilization of microorganisms to develop bio fertilizers, stress protection products and for biocontrol. The study of the plant microbiome also has potential for plant breeding and plant biotechnology. Furthermore, a better understanding of the interactions between plants and endophytes will be important in preventing outbreak of plant diseases or critical human pathogens associated with plants.

There are still missing links in terms of understanding of what causes a rhizospheric bacterium into becoming a plant endophyte. However, clues have been given regarding the endophytic lifestyle such by the characterization of endophytic genes. It has been found that the mechanisms of plant promoting for rhizosphere bacteria and endophytes are quite similar even though most of the research has been mainly focused on rhizobacteria rather than endophytes. The application of NGS techniques will further give more insight into the phylogenetic and functions to better understand the endophytic community. Moreover, the complex networks amongst microbial communities need more attention, as there is little understanding about them. As more information about endophytes is being unfolded, it is expected that through basic research that it will bring more insights into understanding the plant microbiome and possibly engineer bacterial endophytes to exploit their potential in improving plant growth and development.

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