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REVIEW ARTICLE

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Endophytic actinobacteria: Diversity, secondary metabolism and mechanisms to unsilence biosynthetic gene clusters

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ABSTRACT

Endophytic actinobacteria, which reside in the inner tissues of host plants, are gaining serious attention due to their capacity to produce a plethora of secondary metabolites (e.g. antibiotics) possessing a wide variety of biological activity with diverse functions. This review encompasses the recent reports on endophytic actinobacterial species diversity, *in planta* habitats and mechanisms underlying their mode of entry into plants. Besides, their metabolic potential, novel bioactive compounds they produce and mechanisms to unravel their hidden metabolic repertoire by activation of cryptic or silent biosynthetic gene clusters (BGCs) for eliciting novel secondary metabolite production are discussed. The study also reviews the classical conservative techniques (chemical/biological/physical elicitation, co-culturing) as well as modern microbiology tools (e.g. next generation sequencing) that are being gainfully employed to uncover the vast hidden scaffolds for novel secondary metabolites produced by these endophytes, which would subsequently herald a revolution in drug engineering. The potential role of these endophytes in the agro-environment as promising biological candidates for inhibition of phytopathogens and the way forward to thoroughly exploit this unique microbial community by inducing expression of cryptic BGCs for encoding unseen products with novel therapeutic properties are also discussed.

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Introduction

Actinobacteria (previously referred to as Actinomycetes) are prokaryotic, Gram-Positive bacteria widely distributed in nature (Selvakumar et al., 2014), and show a wide morphological gamut ranging from unicellular organisms to branching mycelial filaments (Trujillo et al., 2015). They are typified by the formation of substrate and aerial mycelium on solid media, presence of spores and DNA with a high G + C content, ranging from 51% in some corynebacteria to more than 70% in *Streptomyces* and *Frankia* (Ventura et al., 2007).

They produce numerous extracellular enzymes and thousands of metabolic products, most of which are antibiotics with applications in many therapeutic areas (Bérdy, 2005; Genilloud et al., 2011). In fact, more than half of the bioactive compounds, including immunosuppressive agents, antitumor agents, antibiotics and enzymes known today are of actinobacterial origin (Qin et al., 2011). Although increase in antibiotic resistance to commonly used drugs is becoming a common phenomenon (Adegboye & Babalola, 2012), the actinobacteria isolated from natural environments continue to provide us with a steady flow of novel antimicrobials that have made significant contribution to the societal health and well-being throughout the world. However, the present renewed interest in new biologically active compounds from actinobacteria for designing novel drugs can be attributed to antibiotic resistance developed in bacterial pathogens coupled with marked increase in occurrence of new pathogens and diseases, such as acquired immunodeficiency syndrome (AIDS), severe acute respiratory syndrome, and H1N1 flu virus (Qin et al., 2011).

Actinobacteria are predominantly free living and are found in diverse environments including soil, freshwater habitat, marine habitat, organic matter habitat, rhizosphere, rhizoplane, and within the plants. They are found in a vast majority of cultivated and non-cultivated soils, in tropical and mangrove forests, marine/aquatic ecosystems, natural caves, termite nests, limestone deposits, mines, in the rhizosphere and as endophytes in various plant parts (Brader et al., 2014; Naikpatil & Rathod, 2011; Nimaichand et al., 2015; Selvakumar et al., 2014; Sujada et al., 2014).

In natural habitats, *Streptomyces* is usually a major component of the total actinobacterial population and 75% of biologically active compounds are produced by

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this genus (Bouizgarne & Ait Ben Aouamar, 2014). The non-streptomycetes actinobacteria encompass a plethora of taxa known to synthesize novel antibiotics, as exemplified by the genera *Actinomadura*, *Actinoplanes Actinokineospora, Actinoalloteichus*, *Acrocarpospora, Actinosynnema, Amycolatopsis, Catenuloplanes, Cryptosporangium, Dactylosporangium, Kineosporia, Kutzneria, Microbispora, Microtetraspora, Nonomuraea, Planobispora, Planomonospora, Pseudonocardia, Saccharomonospora, Saccharothrix*, *Spirilliplanes, Streptosporangium*, *Thermobifida, Thermomonospora*, *Verrucosispora,* and *Virgosporangium* (Jose & Jebakumar, 2013).

Another group of actinobacteria latently residing in the internal living plant tissues are called endophytic actinobacteria and the association between such actinobacteria and the host plant are in most cases commensalistic or sometimes symbiotic wherein the endophytes obtain nutrition and shelter from the host plant and in return, they produce biologically active secondary metabolites that enhance the fitness and resilience of the host plants against environmental stress (Qin et al., 2011). Numerous findings on endophytic actinobacterial isolates from several plants including cereals, vegetables, oil seeds, medicinal plants, and even woody tree species, ferns, club mosses and halophytes have been reported and it has been conjectured that endophytic bacteria form more sturdy interactions with plants, compared to rhizospheric or epiphytic bacteria (de Oliveira et al., 2010; Janso & Carter, 2010; Misk & Franco, 2011; Wu et al., 2015). Besides, among the actinobacterial communities, endophytic actinobacteria are gaining genuine attention due to their capacity to produce a range of secondary metabolites possessing a wide variety of biological activity with diverse functions (Hardoim et al., 2015; Palaniyandi et al., 2013; Qin et al., 2011).

The species distribution among the culturable endophytic actinobacteria from these plants is narrow with *Streptomyces* being the most dominant species. *Microbispora*, *Micromonospora*, *Nocardioides*, *Nocardia*, and *Streptosporangium* are the other common genera encountered (Tiwari & Gupta, 2012). These endophytic strains represent a rather untapped source of heretofore unknown reserves of exotic metabolic products with novel therapeutic properties for both human and plant health care. Obviously, mining this unexplored endophytic actinobacterial community for novel secondary metabolites would help in negating the threats posed by antibiotic resistance while simultaneously enhancing crop yields through enhanced plant growth and disease suppression.

Diversity

Though several strains of actinobacteria have been identified as important members of the endophytic bacterial community (Govindasamy et al., 2014; Tiwari & Gupta, 2012), and the degree of diversity among this community may vary between regions and plant species (Qin et al., 2011), ~50% of the endophytic actinobacterial strains are from the genus *Streptomyces* (Shimizu, 2011). Concordant with these reports, Qin et al. (2015b) observed considerable diversity among the 257 endophytic actinobacterial isolates obtained from surface sterilized seeds, stems, roots, and leaves of the oil-seed plant *Jatropha curcas* L. The isolated strains were distributed under seven suborders: *Streptomycineae*, *Pseudonocardineae*, *Corynebacterineae*, *Propionibacterineae*, *Micromonosporineae*, *Streptosporangineae,* and *Micrococcineae*. Among these *Streptomyces* was the most frequently isolated genus (65%). A vast majority of the earlier studies have been based on culture-dependent methods wherein only a small fraction of the actinobacterial strains could be cultivated (Qin et al., 2012). However, studies on diversity using culture-independent molecular approaches (16S rRNA) are fast emerging as reliable tools for confirming the precise genus and species identity of actinobacterial species.

For instance, through preliminary morphological identification, Machavariani et al. (2014) reported that *Streptomyces* was the most abundant genus constituting 65% of the 179 actinobacterial strains isolated from the leaf samples of medicinal plants like *Aloe arborescens*, *Mentha arvensis*, *Lysimachia nummularia*, *Fragaria vesca*, and *Arctium lappa*. Further 16S rRNA gene sequence analysis of unidentifable strains suggested that eight strains belonged to the genus *Nocardiopsis*. Passari et al. (2015a) also reported the relative abundance (50.3–54.2%) of *Streptomyces* spp. among the 42 strains isolated from various tissues of seven medicinal plants collected from two locations of Mizoram, India. Based on 16S rRNA gene analysis they were the first to report the isolation of *Brevibacterium* sp., *Microbacterium* sp., and *Leifsonia xyli* from endophytic environments of medicinal plants like *Mirabilis jalapa* and *Clerodendrum colebrookianum*.

Similarly, Kim et al. (2012) isolated 61 actinobacterial strains using culture-dependent approach from surface sterilized root samples of several Korean native herbaceous plants and through 16S rRNA analysis they found that the genus-wise dominance was in the order: *Streptomyces* (45.9%) > *Micromonospora* (18.8%) > *Rhodococcus* (6.6%) > *Microbispora* (4.9%) > *Micrococcus* (4.9%). Other minor constituents included members of

Microbacterium, *Streptacidiphilus*, *Arthrobacter*, *Dietzia*, *Kitasatospora*, *Herbiconiux*, *Mycobacterium*, *Nocardia*, *Rathayibacter*, and *Tsukamurella*. Likewise, using amplified 16S rRNA gene restriction analysis and limited sequencing, Kaewkla & Franco (2013) also reported the preponderance of *Streptomyces* spp. among the 576 actinobacterial isolates from root leaf and stem of four Australian endemic trees— native pine tree (*Callitris preissii*), red gum tree (*Eucalyptus camaldulensis*), Grey Box tree (*Eucalyptus microcarpa*) and native apricot tree (*Pittosporum phylliraeoides*). Consistent with these reports, El-Shatoury et al. (2013) also recovered 131 endophytic actinobacteria belonging to 14 genera from 10 Compositae plant species collected from South Sinai in Egypt. Using 16S rRNA gene analysis, they confirmed that the dominant genera were *Streptomyces* sp., *Kitasatospora* sp. and *Nocardioides* sp. Rare genera, such as *Microtetraspora* and *Intrasporangium*, which have heretofore not been reported to be endophytic, were also isolated. Likewise, Goudjal et al. (2014) recovered 34 endophytic actinobacteria from eight native Algerian Saharan plants (*Aristida pungens, Cleome arabica, Solanum nigrum, Panicum turgidu, Astragallus armatus, Peganum harmala, Hammada scopari,* and *Euphorbia helioscopia*) of which majority of the isolates (29 isolates) belonged to the genus *Streptomyces* and Bhosale & Kadam (2015) reported that all the endophytes isolated from the roots of sugar cane (*Saccharum officianarum*) belonged to *Streptomyces*. Further, the DGGE profile based on 16S rRNA gene showed that *Streptomyces* species in the roots of rice plants were more varied than other genera, with a dominance of *S. alboniger* and *S. acidiscabies* in almost all the samples (Mahyarudin & Lestari, 2015). In accordance with these reports, a more recent study by Kampapongsa & Kaewkla (2016) indicated that among the 116 actinobacteria isolates obtained for roots, stems, leaves, and leaf sheaths of rice (*Oryza sativa* L.), 63 were *Streptomyces* isolates (54.3%), 50 were *Microbispora* isolates (43.0%), and 3 were *Kineococcus* isolates (2.0%)

Conversely, actinomycetes-specific PCR and 16S rRNA gene analysis indicated the abundance of genera *Nocardia*, *Nonomuraea*, *Pseudonocardia,* and *Actinomadura* within the roots of Eaglewood (*Aquilaria crassna* Pierre ex Lec) (Nimnoi et al., 2010). More recently, the 16S rRNA gene sequence of 59 actinobacterial strains isolated from the stems of a medicinal plant (*Gynura cusimbua*) had higher similar to the closest type strain and belonged to *Microbacterium*, *Arthrobacter*, *Micrococcus*, *Curtobacterium*, *Okibacterium*, *Quadrisphaera,* and *Kineo*coccus (Zhang et al., 2016). Besides these, several novel endophytic actinobacterial strains are being reported from different plant species (Table 1) suggesting that several new actinobacterial assemblages are yet to be isolated, identified and characterized for their economic traits. While Streptomyces continues to be the dominant actinobacterial species, the observation by Masand et al (2015) that the reported literature on endophytic actinobacteria mostly encompasses the non-streptomyces species (78%) indicates the proclivity of researchers towards rare endophytic actinobacterial communities for isolation of novel compounds

In planta colonization

Endophytic colonization by actinobacteria could happen in any living plant niches like leaves (phylloplane), stem (caulosphere), flowers (anthosphere), fruits (carposphere), roots (rhizoplane) and even seeds. Recently, Passari et al. (2015b) isolated 42 endophytic actinobacteria from 560 tissues of seven medicinal plants and found that out of 42 isolates, the majority (52.38%) were isolated from roots followed by stem (21.42%), leaf (14.28%), flower (7.14%), and petiole (4.76%). Similarly, Kaur et al. (2013) reported that out of 62 actinobacterial strains isolated from nine plants (potato, tomato, mustard, wheat, rice, basil, turmeric, cabbage, and radish), 50% of the isolates were from roots, 29.03% from stems and 20.97% from leaves. These reports confirmed that roots are the most preferred niche for colonization by actinobacteria (El-Tarabily et al. 2009; Gangwar et al., 2014; Golinska et al., 2015; Zin et al., 2010).

In slight contrast, Qin et al. (2015b) found that among the 257 actinobacterial endophytes isolated from the interior tissues of *Jatropha curcas* L. plants, the majority, 110 (42.8%) were isolated from stems, 73 (28.4%) from roots, 30 (11.7%) from leaves, and 44 (17.1%) from seeds. Earlier, Tian et al. (2007) found that in rice, strains similar to *Streptomyces cyaneus*, *S. aurantiacus* and *S. paresii* resided in both roots and stems, whereas *Nocardioides thermolilacinus*, *S. exfoliates*, *S. glauciniger* and *S. kathirae* were found only in the roots while *S. caviscabies* and *S. scabies* were found only in the stems. These findings indicated a more diverse actinobacterial community in the roots and stems, with the diversity being pronounced in the roots than even the soil (Mahyarudin & Lestari, 2015). This would probably explain the successful isolation and identification of endophytic actinomycete from plants roots in a number of studies. Apparently, the relatively larger population and broader diversity of endophytic actinobacteria in the roots indicates that the soil-inhabitant actinobacteria readily move to the rhizosphere via chemotaxis or chemical signaling and it is through the roots that the

Table 1. Some newly reported actinobacteria from plant tissues (2012–2015).

Actinobacteria	Host plant	Plant part	Reference
Micromonospora lycii (NEAU-gq11^T)	Wolfberry (*Lycium chinense* Mill.)	Root	Zhao et al. (2015a)
Brevibacterium sp. (KJ584877))	Four o'clock flower (*Mirabilis jalapa* L.)	Leaves, stems, roots, flowers, and petioles	Passari et al. (2015b)
Leifsonia xyli [KJ584866]	East Indian Glory Bower (*Clerodendrum colebrookianum* Walp.)	Leaves, stems, roots, flowers, and petioles	Passari et al. (2015b)
Micromonospora costi CS1-12^T	Crêpe ginger (*Costus speciosus* (Koen.) Sm.	Leaf	Thawai (2015)
Micromonospora endophytica DCWR9-8-2^T	Rice (*Oryza sativa* L.)	Leaf	Thanaboripat et al. (2015)
Paenibacillus dauci JCM30283^T	Carrot (*Daucus carota* subsp. *Sativus*)	Rhizome	Wu et al. (2015)
Phytoactinopolyspora endophytica EGI 60009^T	Chinese licorice (*Glycyrrhiza uralensis* F.)	Root	Li et al. (2015a)
Novosphingobium endophyticum EGI 60015^T	Chinese licorice (*Glycyrrhiza uralensis* F.)	Root	Li et al. (2015b)
Arthrobacter endophyticus EGI 6500322^T	Saltwort (*Salsola affinis* C. A. Mey.)	Root	Wang et al. (2015a)
Frigoribacterium endophyticum EGI 6500707^T	Halophyte (*Anabasis elatior* (C. A. Mey.) Schischk)	Root	Wang et al. (2015b)
Labedella endophytica 6500705^T	Halophyte (*Anabasis elatior* C. A. Mey.) Schischk.)	Stem	Wang et al. (2015c)
Okibacterium endophyticum EGI 650022^T	Saltwort (*Salsola affinis* C. A. Mey.)	Root	Wang et al. (2015d)
Actinotalea suaedae EGI 60002^T	Halophyte (*Suaeda physophora* Pall.)	Stem and root	Zhao et al. (2015b)
Cellulomonas pakistanensis NCCP-11^T	Rice (*Oryza sativa* L.)	Grain	Ahmed et al. (2014)
Sphaerisporangium rufum R10-82^T	Rice (*Oryza sativa* L.)	Root	Mingma et al. (2014a)
Glycomyces phytohabitans KLBMP 1483^T	Chrysanthemum *(Dendranthema indicum* (L.) Des Moul.)	Stem	Xing et al. (2014)
Micromonospora zeae NEAU-gq9^T	Corn (*Zea mays* L.)	Root	Shen et al. (2014)
Micromonospora violae NEAU-zh8^T	Chinese violet (*Viola philippica* Car.)	Root	Zhang et al. (2014b)
Actinoplanes hulinensis NEAU-M9^T	Soybean (*Glycine max* (L.) Merr.)	Root	Shen et al. (2013)
Williamsia sterculiae sp. nov. CPCC 203464^T	Malva nut tree (*Sterculia lychnophora* Hance.)	Stem	Fang et al. (2013)
Jatrophihabitans endophyticus S9-650^T	Barbados nut (*Jatropha curcas* L.)	Stem	Madhaiyan et al. (2013)
Streptomyces halophytocola KLBMP 1284^T	Chinese tamarisk (*Tamarix chinensis* Lour.)	Stem	Qin et al. (2013a)
Modestobacter roseus KLBMP 1279^T	Glasswort (*Salicornia europaea* L.)	Root	Qin et al. (2013b)
Amycolatopsis jiangsuensis KLBMP 1262^T	Chrysanthemum *(Dendranthema indicum* (L.) Des Moul.)	Stem	Xing et al. (2013)
Blastococcus endophyticus YIM 68236^T	Happy tree (*Camptotheca acuminata* Decne.)	Leaf	Zhu et al. (2013)
Promicromonospora endophytica EUM 273^T	Grey box (*Eucalyptus microcarpa* Maiden)	Root	Kaewkla & Franco (2012)
Kineococcus endophytica KLBMP 1274^T	Halophyte (*Limonium sinense* (Girard) Kuntze)	Leaf	Bian et al. (2012a)
Streptomyces phytohabitans KLBMP 4601^T	Medicinal plant *(Curcuma phaeocaulis* Valeton.)	Root	Bian et al. (2012b)
Kibdelosporangium phytohabitans KLBMP 1111^T	Barbados nut (*Jatropha curcas* L.)	Root	Xing et al. (2012a)
Pseudonocardia nantongensis KLBMP 1282^T	Chinese tamarisk (*Tamarix chinensis* Lour.)	Leaf	Xing et al. (2012b)
Nocardioides panzhihuaensis KLBMP 1050^T	Barbados nut (*Jatropha curcas* L.)	Stem	Qin et al. (2012)

actinobacteria mostly gain access to various niches in the plant. These findings are consistent with the reports that the majority of the actinobacterial endophytes that colonize various plant organs are mostly derived from the rhizosphere (Bouizgarne & Ait Ben Aouamar, 2014) and distinct actinobacterial communities are found in various plant organs such as roots, stem, leaves, flowers as well as fruits and seeds or even during plant development indicating differential capacities of actinobacterial strains to colonize various plant compartments. This is supported by the findings of Cheng et al. (2014) that the strain *Streptomyces felleus* YJ1 could colonize chronically in the soil and then the root from where the strain moved to colonize in the stem and leaves of oil seed rape. There are also instances wherein endophytic isolates belonging to the genera *Microbacterium, Micrococcus* and *Kocuria* were obtained from apoplastic sap of the medullary parenchyma of the stem of healthy sugarcane plants (Velázquez et al., 2008), while 136 different orange-pigmented actinobacterial colonies belonging to the genus *Micromonospora* were isolated from surface-sterilized N fixing nodules of both leguminous and actinorhizal plants (Carro et al., 2013; Trujillo et al., 2010). Therefore, most of the studies suggest that significant variation exists in the community structure of both soils and host plant, and the rhizosphere soils serve as a potential microbial seed bank that is differentially recruited by the plant thereby

shaping the microbial assemblages of the aboveground organs, which subsequently influences the characteristics of host plants (Zarraonaindia et al., 2015). Nevertheless, the fact remains that there is not a single plant species devoid of actinobacteria, though they occur at lower population densities than those in the rhizosphere, rhizoplane, and soil *per se*.

Factors influencing endophytic colonization

Reports suggest that endophytic actinobacterial community structures are dependent on the rhizosphere microbial community, as well as the presence or absence of phytopathogens, indicating the complex interactions with microbes found immediately surrounding the root (rhizosphere) and inside of plant tissues (Shakya et al., 2013). More importantly, the soil type and soil physico-chemical characteristics are important players that shape the endophytic actinobacterial community especially soil organic C (Zarraonaindia et al., 2015) including moisture regime (Hardoim et al., 2012).

Also, the variation in endophytic colonization and species-specificity may depend on physiology and chemistry of plant tissues as well as the abilities of endophytes to adjust themselves and utilize the substrates in plant tissues (Nimnoi et al., 2010). Evidently, type of plant tissue also seems to influence the community and diversity of endophytes within the host plants (Prakamhang et al., 2009). Also, the species diversity, richness and relative abundances within the plant is dynamic and is influenced by abiotic and biotic factors such as plant species, microbe–microbe interactions and plant–microbe interactions, both at local and larger scales (Gaiero et al., 2013).

Other factors include age and type of tissue and habitat distribution of the host plants (Golinska et al., 2015). Besides, the species specific community differences have been found to vary with seasons (Shakya et al., 2013; Shen & Fulthorpe, 2015). Reported data further suggest that geography plays a greater role in regulating actinobacterial community structure with the greatest diversity occurring in the tropical and temperate regions. However, in terms of richness and diversity, the tropical rainforests seem to have the potential to offer exotic and promising strains that have, heretofore, not been reported from other regions (Qin et al., 2011). For instance, Janso & Carter (2010) isolated 123 endophytic actinobacteria representing 17 different genera from tropical plants collected from several locations in Papua New Guinea and Mborokua Island, Solomon Islands. Among these, rare genera such as *Sphaerisporangium* and *Planotetraspora*, were reported as endophytic for the first time. Moreover, genetic modification of plants and exogenous application of fertilizers, herbicide and pesticide during crop production can also cause variation of the diversity, structure and richness of the endophytic microbial community (da Silva et al., 2014). It is therefore obvious that myriad factors influence the composition and structure of endophytic actinobacterial communities, and also that their distribution among the plant organs is influenced by highly localized biogeographic factors and shows interannual variation even at a small spatial scale (Zarraonaindia et al., 2015).

Among the endophytic actinobacterial community, it has been repeatedly demonstrated that irrespective of soil type, tissue type, chemistry, physiology and geographical location, agronomic management etc., *Streptomyces* species are the most frequently isolated actinobacteria from plants followed by *Microbispora* species and among Streptomyces, *S. aureus*, *S. galilaeus*, *S. caviscabies*, *S. setonii*, *S. cyaneus,* and *S. thermocarboxydus* are reported to be the most commonly encountered species (Govindasamy et al., 2014; Selvakumar et al., 2014; Shimizu, 2011), suggesting that these species could have a higher adaptability to a wide range of plants and environment.

Portals of entry

To gain entry into suitable niches within the plants, the actinobacteria need to migrate to the rhizosphere and subsequently to the rhizoplane of their hosts. Like other rhizosphere microorganisms, this migration is mostly mediated by chemotaxis, which involves deposition of root exudates with a surfeit of organic nutrients (organic acids, phytosiderophores, sugars, vitamins, amino acids, nucleosides, mucilage) by plants into their direct surroundings i.e. spermosphere, phyllosphere, rhizosphere, and mycorrhizosphere (Berendsen et al., 2012; Vorholt 2012). Apparently, the root exudates constitute the main signals that initiate recognition between the root and microorganisms and their composition may vary depending on the nature of the interaction. Besides these nutrient rich root exudates, plant roots produce signals to initiate cross-talk with soil microorganisms, which in turn respond by producing signals that aid in rhizosphere colonization (De-la-Pena et al., 2008; Badri et al., 2013). It is also possible that, like other endophytic bacteria, the actinobacteria avoid plant defenses by stopping elicitor production, which would otherwise lead to activation of effector-triggered immunity by the plant. Once the elicitor production is subverted, the actinobacteria can rapidly colonize the root. However, regardless of whether the approaching microorganism in the rhizosphere is a friend or foe, the

host plant treats the invasion with caution and mounts a broad range of defense responses, including the synthesis of reactive oxygen species (ROS). Nonetheless, in order to survive in this oxidative environment in the rhizosphere and reach the rhizoplane and subsequently the internal organs of the plant, the actinobacteria need specialized mechanisms like the one in *Micromonspora lupine* strain Lupac 08, whose genome revealed several genes (sod genes, a catalase HPII katE, a catalase peroxidase katG, a catalase hydroperoxidase katA, four hydroperoxide reductases and a thiol peroxidase) encoding protein to neutralize oxidative stress (Trujillo et al., 2014).

Once they reach the rhizoplane, the details on precise mechanisms by which actinobacteria enter the plants and colonize various organs are still murky. Hence, investigations are still underway to fully comprehend the machinations underlying entry into the plant because unlike fungi which possess penetration pegs (Toruño et al., 2016), bacteria are unable to enter the plant organs through forced entry. Therefore, entry could be *via* stomata, wounds, lenticels, projecting areas of lateral roots, and broken trichomes by forming clump of their cells (Shimizu, 2011). In most cases, the actinobacteria allow their branching hyphae to spread across plant surfaces and enter them through natural openings or opening caused by mechanical wounds and those caused by microbes or nematodes or through the stomata found in leaves (Shimizu, 2011).

While Shimizu et al. (2009) were unable to resolve the inter- and intracellular growth of *Streptomyces* sp. (MBCu-56) in the epidermis of cucumber leaves, they were successful in determining that the inoculum developed dense mycelial mass at the epidermal cell junctions of leaf surfaces, which penetrated the cuticle layer and expanded into the underlying epidermal cells. Other ingress points easily accessed by endophytic bacteria could apply for endophytic actinobacteria as well. Like for instance, emergence points of lateral roots and to some extent through the zone of differentiation and elongation near the root tip, where slightly disrupted or not completely differentiated tissues may facilitate penetration (Reinhold-Hurek & Hurek, 2011), cracks formed at points of emergence of lateral roots and root hairs *per se* (Prieto et al., 2011;), active endocytosis occurring at the tip of growing hairs and subsequent trafficking of the endocytosed membranes (Mercado-Blanco & Prieto, 2012) etc may provide a way for active/passive entry of actinobacteria into the root hair from where they quickly migrate to the inter- and intra-cellular spaces within the various plant parts.

Also, actinobacteria may deploy a suite of specialized compounds to facilitate entry into host plants, albeit absence of specialized penetration pegs. Foremost among them is the array of enzymes they possess such as cellulases, xylanases, and pectinases that would play a role in internal plant colonization by degrading plant cell walls (Jog et al., 2016). But unlike the plant pathogenic bacteria and fungi, which gain access by actively degrading plant cell wall compounds using glycoside hydrolases including cellulases and endoglucanases, the nonpathogenic endophytic microorganisms have only a reduced set of cell-wall degrading enzymes (Trujillo et al., 2014). By genome sequencing of endophytic actinobacterium *M. lupini* Lupac 08, they revealed 79 genes with the potential to hydrolyze plant polymers, 14 genes which presumably bind to and interact with carbohydrates, 46 genes with a hydrolytic or binding function towards cellulose, 12 putative genes related to the metabolism of xylan including several related to starch and pectin degradation. Besides, these hydrolytic enzymes, the entry may also be facilitated by the presence of bacterial expansins, which are plant proteins that loosen cell walls via a non-enzymatic mechanism that induces slippage of cellulose microfibrils in the plant cell wall. These expansins are acquired by bacteria through multiple horizontal gene transfers from plants and reports suggest that genetic deletion of expansin from bacteria cripples their ability to colonize plant tissues (Georgelis et al., 2015).

Once they gain entry into the root hair the actinobacteria are capable of colonizing rapidly by establishing populations both inter- and intra-cellularly. Among the inter-cellular spaces, the major sites of colonization could be the peri-space between the cell wall and the plasma membrane as in the case of other endophytes (Thomas & Reddy, 2013). In leaves of *Rhododendron, S. galbus* hyphae were observed to be embedded in an amorphous, mucilage-like material individually or in colonies in intercellular spaces between epidermal and mesophyll cells but not inside those cells (Shimizu et al., 2009). Ergo, it is surmised that endophytic actinobacteria adhere firmly to the host plant surfaces and acquire the nutrition by using extracellular polymers containing adhesive compounds and hydrolytic enzymes, similar to that of fungi (Bouizgarne & Ait Ben Aouamar, 2014).

Once they gain entry, the actinobacteria can enter the xylem cells and vascular tissue to a lesser extent though entering the latter could make them all-pervasive and enable them to systemically spread to other niches in the plant. This is evident from several studies that have reported that endophytic actinobacteria, following rhizosphere soil colonization, can be detected inside the endorhiza, in stems, leaves as well as inside

plant reproductive organs of different host plants (Qin et al., 2011; Shimizu, 2011) Their presence of endophytic actinobacteria in different plant organs and the rhizosphere suggests that they can migrate from seeds to plant roots and other tissues and from stem to roots and may even be able to exit the plant and colonize the rhizosphere, which suggests a seamless continuity between the plant-, root-, and rhizosphere-microbiomes. This is consistent with the recent report of Bonaldi et al. (2015) that *Streptomyces* sp. (ZEA17I strain) was able to colonize soil, developing roots, and rhizosphere of lettuce, making it both rhizospheric and endophytic.

Bioactive metabolites from endophytic actinobacteria

Of the 20,000 biologically active compounds sourced from microorganisms (Demain & Sanchez, 2009), the majority are derived from actinobacteria which account for 45% of all antibiotics currently in use (Bérdy, 2005). Actinobacteria synthesize functionally diverse class of bioactive compounds, often through non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) pathways, most of which have already found widespread application in the pharmaceutical industry (Miller et al., 2012; Weber et al., 2015) as immunosuppressants (e.g. rapamycin), antibiotics (e.g. erythromycin), anticholesterol drugs (e.g. lovastatin), and anticancer drugs (e.g. epothilone B).

Among the actinobacteria, most of the biologically functional secondary metabolites owe its origin to the genus *Streptomyces*. It has been repeatedly reported that out of 10,100 secondary metabolites reported to be produced from actinobacteria, 7630 are from *Streptomycetes* and 2470 are from non-streptomyces actinobacteria (Solecka et al., 2012). While it is not surprising that this microbial genus is the most thoroughly investigated microorganism for secondary metabolite production (Brader et al., 2014), the potential of more "exotic" and "rare" actinobacterial taxa is less established and the representation in terms of metabolite production in the 20th century was only 5% (Bérdy, 2005). Nonetheless, non-streptomyces actinobacteria like *Micromonospora*, *Actinomadura*, *Streptoverticillium*, *Actinoplanes*, *Nocardia*, *Saccharopolyspora,* and *Streptosporangium* spp. are increasingly playing a significant role in the production of a wide spectrum of antimicrobial metabolites and antibiotics (Jose & Jebakumar, 2013). Further, it has recently become evident that there is a lull in the discovery of new compounds from the existing genera of actinobacteria obtained from the soil. Hence, it is imperative that recovery and pursuance of novel biologically active compounds be made from actinobacteria that reside in new habitats like those within plant tissues.

After gaining residence in the plant tissues, the actinobacteria establish a mutually benefitting relationship with its host. In return for nutrition and protection they obtain from the host plant, they provide certain functional metabolites (such as auxins, cytokinins, and gibberellins, essential nutrients, enhanced 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity etc) which promote growth of the host plants besides conferring plant protection against pathogens by induction of plant defense mechanisms, pathogen-antagonistic substances or through competition for colonization sites and nutrients (Weyens et al., 2009). The endophytic actinobacteria have been implied in the production of metabolites that induce direct physiological and biochemical changes in the host plants (Yandigeri et al., 2012). Besides such direct interventions on plant growth, a significant revelation has been that the endophytic actinobacteria have the potential to markedly enhance the metabolic potential of plants to produce useful compounds. For instance, Li et al. (2012) found that the endophytic actinobacterium, *Pseudonocardia* sp. strain YIM 63111, enhanced the production of the antimalarial compound artemisinin in its host plant *Artemisia annua* due to the up-regulation of the CYP71AV1 and CPR genes. Elegant reviews that focus on secondary metabolites from a range of actinobacteria isolated from terrestrial/marine ecosystems as well as those that are endophytic have been made (Brader et al., 2014; Govindasamy et al., 2014; Qin et al., 2011; Solecka et al., 2012; Xu et al., 2014). Nevertheless, some newly discovered bioactive compounds produced by endophytic actinobacteria that hold great promise in formulating and designing drugs for both human and plant health care are mentioned in Table 2.

A vast majority of such studies on production and identification of secondary metabolites by actinobacteria have relied on traditional chemical/bioactivity-based screening techniques that do not allow successful culturing of the entire collection of endophytes. Hence, to the chagrin of researchers worldwide, these techniques have led to either rediscovering known metabolites or production at such low levels that they go undetected or are difficult to purify using traditional methods. This has always resulted in underexploitation of their vast potential, underlining the fact that large reserves of secondary metabolites produced by endophytic actinobacteria are yet to be discovered.

Nevertheless, difficulties in successful culturing of many of the novel endophytes can be overcome by

Table 2. Recent reports on bioactive metabolites from endophytic actinobacteria and their antimicrobial activity (2013–2015).

			Metabolite			
Plant host	Habitat	Endophyte	Name	Type	Activity	Reference
D. stellata	Tropics	Streptomyces sp. YIM67086	2-amino-3,4-dihydroxy-5-methoxybenzamide	Benzamide	Antibacterial against *Escherichia coli*	Yang et al. (2015)
Alpinia oxyphylla	Tropics	*Streptomyces* sp. YIM66017	2,6-dimethoxy terephthalic acid, yangjinhualine A, ahydroxyacetovanillone and cyclo(Gly-Trp)	Alkaloid	Cytotoxic to MCF-7	Zhou et al. (2014a)
Inula cappa (Buch.-Ham. ex D. Don) DC.	Tropics	*Streptomyces* sp. YIM67005	Cyclo(l-Tyr-l-Pro-l-Phe-*trans*-4-hydroxy-l-Pro), Cyclo(l-Phe-*trans*-4-hydroxy-l-Pro) Cyclo(l-Val-l-Tyr),	Cyclic tetrapeptide Cyclodipeptides	Not cytotoxic	Zhou et al. (2014b)
Xylocarpus granatum	Mangrove	*Jishengella endophytica* 161111	2-(furan-2-yl)-6-(2*S*,3*S*,4-trihydroxybutyl)pyrazine (1) and 12 known compounds (2-13)	Pyrazine derivative	Compound 9 (1-hydroxy-β-carboline) found promising against H1N1	Wang et al. (2014)
Soybean	Tropics	*Streptomyces* sp. neau-D50	3-Acetonylidene-7-prenylindolin-2-one (isoprenoids, 7-isoprenylindole-3-carboxylic acid, 3-cyano-methyl-6-prenylindole, 6-isoprenylindole-3-carboxylic acid and 7,40 - dihydroxy-5-methoxy-8-(g,g-dimethylallyl)- flavanone)	Prenylated indole derivative	Cytotoxic against human lung adenocarcinoma cell line A549	Zhang et al. (2014a)
Orchid	Tropics	*Polymorphospora rubra* K07-0510	Trehangelins A, B and C	Trehalose	Trehangelin A and C showed potent inhibitory activity against hemolysis of red blood cell	Nakashima et al (2013)
Clinacanthus siamensis Bremek.)	Tropics	*Microbispora* sp. GMKU 363	Linfuranone A	Polyketide	No appreciable activity in antimicrobial and cytotoxic assays	Indananda et al. (2013)

isolating microbial DNA directly from endophytic samples (eDNA) and capturing complete small molecule biosynthetic gene clusters (BGCs) in individual or a small number of overlapping clones in a eDNA library through complementation or insertional mutagenesis of mutants. Other strategies include antibody screening of cDNA expression library, differential display reverse transcriptase PCR, suppression substractive hybridization PCR etc (Cacho et al., 2014). Advances in biotechnology has resulted in high throughput sequencing of DNA, which has in turn revolutionized the potential of examining the genetic complexity of organisms and an enormous database of genomic sequences has been generated. Genomic approaches, like metagenomics, are cultivation independent methods that help in analysis of DNA from a mixed population of organisms and initially involve cloning of either total or enriched DNA directly from the environment into a host that can be easily cultivated (Miao & Davies, 2009). Metagenomics is particularly appealing because BGCs are typically clustered on bacterial chromosomes. With the advent of cutting edge tools like next generation sequencing (NGS) that allow isolated eDNA to be sequenced directly from environmental samples (Shokralla et al., 2012) followed by analyses using a variety of functional or bioinformatic screening methods, novel biocatalysts and small molecule biosynthetic genes are being identified at an increasing pace.

A significant advancement with genome sequencing is its innate ability to reveal the coding capacity of BGCs for secondary metabolites (Chiang et al., 2011). A classic example is the genome sequencing of *Streptomyces coelicolor* A3 (2), a genetic workhorse known to make only five secondary metabolites, revealed 18 additional putative BGCs responsible for production of individual secondary metabolites (Bentley et al., 2002). Likewise, genome sequencing of *Streptomyces griseus* (the producer of streptomycin) revealed 34 clusters, while it was previously reported to have only six biosynthetic pathways (Ohnishi et al., 2008). Similarly, the complete genome sequencing of *S. ambofaciens* ATCC 23877 (Aigle et al., 2014) highlighted the presence of 14 secondary metabolite gene clusters which included a type II PKS gene cluster with the potential to synthesize aromatic polyketide belonging to the angucyclinone family, besides spiramycin and congocidine. This also allowed access to the first complete kanamycin gene cluster thereby providing the opportunity to generate new derivatives with improved antitumour activity by genetic manipulation. The study further revealed a large type I PKS gene cluster supposedly involved in the production of a novel macrolide and macrolides which are very important drugs used in human therapy, for example as antibacterial agents (e.g. erythromycin or spiramycin), as immunosuppressors (e.g. rapamycin) or as antifungal agents (e.g. nystatin). More recently, genome mining and sequencing of the actinobacterium strain A23, identified as *Streptomyces wadayamensis* isolated from the plant tissue of *Citrus reticulate* (tangerine) helped in predicting the presence of 32 gene clusters codifying for secondary metabolites biosynthetic systems as terpene, nrps, bacteriocin-terpene, t1-pks, t3-pks, bacteriocin, nrps-t1pks, tiopeptide-lantipeptide, lantipeptide-nrps-t1-pks, siderophore, ectoine, lassopeptide, lantipeptide, and other clusters (Angolini et al., 2016).

Recently, draft genomic analysis data of strain *Paenibacillus dauci* sp. nov., a novel endophytic actinobacteria from carrot revealed 36 open reading frames (ORFs) related to antibiotic metabolic process, 10 ORFs related to the antimicrobial peptide transport system, 4 ORFs related to plant growth promotion, 12 ORFs related to trehalose, and 19 ORFs related to vitamin B12 production and vitamin B6 metabolism (Wu et al., 2015). Similarly, the BGC predicted to encode the synthesis of divergolides (a group of structurally unprecedented ansamacrolactam antibiotics with antibacterial and antitumor activities) was cloned and sequenced from endophytic *Streptomyces* sp. W112 (Li et al., 2014), paving the way for rational engineering of new divergolide analogs. Furthermore, genome sequencing of the endophytic actinobacteria strain *Micromonospora lupini* Lupac 08 revealed 15 clusters involved in the biosynthesis of secondary metabolites including siderophores, terpenes, butyrolactones, polyketides (PKS), nonribosomal peptides (NRPS), chalcone synthases and bacteriocins (Trujillo et al., 2014; Trujillo et al., 2015). They also identified various polyketide biosynthetic and non-ribosomal peptide synthase pathways specifically as PKI, PKII (2 clusters), PKIII types, NRPS (2 clusters) and hybrid PKS/NRPS clusters (2 clusters), which suggested that *M. lupini* is capable of producing a vast diversity of secondary metabolites such as the antitumor anthraquinone derivative lupinacidins (Igarashi et al., 2007; Igarashi et al., 2011). Likewise, Yang et al. (2014) have reported the draft genome sequence of *Streptomyces* sp. strain PRh5 (China Center for Type Culture Collection [CCTCC] number 2013487), which is used to produce nigericin and nocardamine, which exhibit anti-HIV activity. More recently, draft genome sequencing, genomic library screening, and gene disruption have allowed the characterization of the BGC for maklamicin, a spirotetronate-class antibiotic produced by the endophytic actinobacterium *Micromonospora* sp. NBRC 110955 (Daduang et al., 2015). Besides, the complete genome sequencing of *Kibdelosporangium phytohabitans*

KLBMP 1111(T), a plant growth promoting endophytic actinomycete isolated from the oil-seed plant *Jatropha curcas* L. has allowed the identification of gene clusters responsible for polyketide and nonribosomal peptide synthesis of natural products, and genes related to the plant growth promotion, such as zeatin, 1-aminocyclopropane-1-carboxylate deaminase (ACCD) and siderophore (Qin et al., 2015a).

The secondary metabolite production in endophytes is primarily controlled at the level of transcription. Thus, potentially interesting BGCs encoding useful metabolites with high therapeutic and bioactive potential can remain silent in the unnatural setting of a laboratory due to lack of direct or indirect activation signals that upregulate the expression of specialized metabolite BGCs (Rutledge & Challis, 2015). This suggests that the BGCs are well hidden genetic entities which await arousal by induction (Haferburg & Kothe, 2013). There are several cultivation dependent or molecular techniques approaches to the exploration of products of the cryptic metabolic pathways. Small changes in cultivation conditions, media composition, pH, temperature or aeration can completely shift the metabolic profile of various microorganisms. Cultivation dependent approaches are either biotic or abiotic and abiotic approaches can entail either physical or chemical means. Biotic approaches using entire organisms i.e. co culturing to stimulate natural product synthesis is extensively used in many systems (Abdelmohsen et al., 2015). Hence, investigators are now focusing on developing new approaches to trigger the otherwise hardwired BGCs of actinobacteria for eliciting novel secondary metabolite production. This is done by challenging the actinobacterial cells with external signals or "elicitors" to synthesize new altered metabolites, though in some cases unaltered metabolite profiles or even repression of the sought-after metabolites may occur (Marmann et al., 2014).

A rational and simple approach to induce a change in the expression of specialized metabolite BGCs is to alter the growing conditions of the potential metabolite producing actinobacteria because it has been demonstrated that the production of secondary metabolites in microorganisms is dependent on growth substrate and environment (Gram, 2015). In this context, the OSMAC (one strain/many compounds) method is a simple and effective approach for activating metabolic pathways by altering cultivation parameters, co-cultivation, addition of enzyme inhibitors, etc with the presumption that these alterations may allow expression of cryptic clusters (Haferburg & Kothe, 2013). Polyene ECO-02301, a novel antifungal agent in *Streptomyces aizunensis* (McAlpine et al., 2005) and three new compounds of a rare class of 22-membered macrolides in *Streptomyces* sp. strain C34 (Rateb et al., 2011) were discovered through OSMAC. Hence, by changing screening parameters like media composition, aeration, culture vessel and addition of enzyme inhibitors up to 20 different metabolites belonging to diverse product families could be detected from a single producer strain, thereby yielding novel metabolites as well as more quantities of known metabolites. Though useful, this method can be strenuous and does not guarantee that the altered conditions can simulate the synthesis of all the targeted compounds that the organisms can potentially produce (Chiang et al., 2011).

Activation of BGCs has also been successfully attempted by imposing physical stress through signals like heat shock or ethanol shock (Rebets et al., 2014; Yoon & Nodwell, 2014), or through antibiotics (Derewacz et al., 2013; Imai et al., 2015; Seyedsayamdost, 2014), or low concentrations of rare elements like scandium and lanthanum (Kawai et al., 2007; Tanaka et al., 2010). Also, the silent BGCs can be activated by culturing the potential actinobacteria under conditions akin to their natural environment (Rutledge & Challis, 2015). Therefore, addition of inducers including butyrolactones, metals or other stressors or new media compositions supplemented by molecular approaches that target regulatory networks has been suggested (Haferburg & Kothe, 2013).

Besides chemical signals (Murphy et al., 2011), elicitation of BGCs can also occur through biological means wherein a resident actinobacterium encounters another microbe which might turn on the cryptic gene clusters, thereby triggering the production of new metabolites in the resident bacteria. Interestingly, this actinobacteria-microbe interaction leads to the production of hitherto unknown secondary metabolites which otherwise would be impossible when either of the partners exist in isolation (Traxler et al., 2013). Further, Goodman (2014) stated that endophytes that co-existed in the same tissues provide the opportunity for them to have coevolved, therefore potentially improving their abilities to use quorum sensing to inhibit pathogens. Even though the exact mechanisms underpinning the elicitation process by the inducer strain and consequent production of secondary metabolites by the producer strain is yet to be fully comprehended (Marmann et al., 2014), it is hypothesized that the elicitation could occur through physical cell-cell interactions, production of small molecules (auto-regulator/quorum sensing molecules, siderophores, etc.) or production of enzymes that activate the metabolite precursor produced by the producer strain or horizontal gene transfer (HGT) that may likely affect metabolite activation or repression

(Abdelmohsen et al., 2015). Also, physical interactions have also been found to contribute to the communication among microorganisms and induction of silent BGCs (Schroeckh et al., 2009).

Once the actinobacterium becomes an endophyte it establishes a very close relationship with the host plant and any such cohabitation will favor occasional gene transfer between the microbe and the plant in both directions (Arber, 2014; Janso & Carter, 2010). This horizontal gene transfer (HGT) may introduce genetic novelties to recipient actinobacteria, thus resulting in the production of plant-derived compounds; for e.g. production of paclitaxel by *Kitasatospora* sp. isolated from *Taxus baccata* (Caruso et al., 2000). This *quid pro quo* and shuffling of genetic material between the actinobacterium and host plant offers the exciting possibility of isolation and characterization of novel secondary metabolites for future drug development. A range of novel metabolites synthesized by activation of BGCs through co-culturing a potential actinobacterial strain with another actinobacterial/bacterial/fungal strain is provided by Abdelmohsen et al. (2015). Selective manipulation of epigenetic targets using small molecule inhibitors of histone deacetylase and DNA methyltransferase that are enzymes involved in chromatin remodeling can lead to enhanced expression of BGCs and production of new secondary metabolites (Pettit, 2011; Kumar et al., 2016).

With the advent of versatile tools like NGS, it is imperative to devise a combined approach that includes the transcription activation of key genes and metabolism remodeling. It is possible to genetically modify the producer microorganism through ribosome engineering by altering transcription and translation machineries and over express the regulator proteins to activate the silent BGCs. This can be achieved by replacing the promoter of the transcription factor with an inducible promoter, which in turn can lead to induction of all genes of the cluster leading to a novel metabolite (Chiang et al., 2011). For instance, antibiotic production can be markedly increased by modulating ribosomal components (ribosomal proteins or rRNA), i.e. by introducing mutations conferring drug resistance, as many antibiotics target the ribosome (Tanaka et al., 2009). Besides, this technique allows screening for drug resistance by simple selection on drug containing plates, selection for mutations without prior genetic information and even if the mutation frequency is extremely low ($<10^{-10}$) (Tanaka et al., 2013). They also demonstrated that these *rpoB* mutations that arise in the RNA polymerase (RNAP) β-subunit not only enhance antibiotic production but are also effective in arousing the silent BGCs, eventually leading to discovery of novel antibiotics. Other methods that have been developed to activate silent biosynthetic pathways include manipulation of nucleoid structure, addition of *N*-acetylglucosamine to the medium or deletion of the *dasR* gene, which encodes an *N*-acetylglucosamine-responsive regulatory protein; the constitutive over expression of a pathway-specific LAL regulatory gene; metabolic remodeling; and cell-to-cell interaction (Tanaka et al., 2013). Several other approaches like histone modifications can be also employed for regulating gene transcription. However, efforts to engineer the biosynthetic genes for the production of unnatural variants face a high failure rate in many systems. In this context, it is imperative to study the way in which natural evolution of biosynthetic genes occurs to give unnatural compounds (Medema et al., 2014; Medema & Fischbach, 2015).

Another approach being pursued is to clone these BGCs for expressing in heterologous hosts. In this approach, expression of foreign biosynthetic genes is accomplished in a host that can be cultured more easily and is amenable to genetic manipulation. In principle, it is required that the biosynthetic, resistance and regulatory genes reside on a contiguous stretch of DNA and the host has an appropriate metabolic and genetic background. For instance, Ikeda et al (2014) have examined the expression of more than 20 exogenous BGCs in the large-deletion mutants of *Streptomyces avermitilis* and these large-deletion mutants have been shown to be versatile and effective hosts for the expression of heterologous gene clusters governing the production of a variety of secondary metabolites, including aminoglycosides, nucleosides, ribosomal and non-ribosomal peptides, shikimate-derived metabolites, and terpenes. Another promising approach involves modification of gene expression in the natural producer organism such as *Streptomyes coelicolor* wherein expression of silent gene clusters has been demonstrated using histone deacetylation inhibitors as elicitors (Moore et al., 2012). Heterologous expression can be especially useful when the production of a metabolite by a microbial strain is not sufficient enough to permit investigation of the biosynthetic pathway (Gomez-Escribano & Bibb, 2014). Presently, a combination of several approaches and technologies involving manipulation of culture conditions like substrates and associated microbiota with chemicals coupled with genome and metagenome based analyses to enhance the discovery rate of novel molecules are being adopted. For e.g. Doroghazi et al. (2014) reported a method for classification of gene clusters into families (GCFs), analyzed the biosynthetic potential of 830 sequenced actinobacteria, and found that the GCF network, comprised of 11,422 gene

clusters grouped into 4122 GCFs. This method allows linking previously unassigned GCFs to known natural products. It is, therefore, apparent that, similar to other endophytic systems, arousing the sleeping BGCs holds great potential in opening the black box of the yet unknown reserves of secondary metabolites produced by the endophytic actinobacteria.

Crop growth promotion and biocontrol

Actinobacteria have gained prominence not only in human health care but also play a significant and perceptible role in the agro-environment due to their ability to produce an array of biologically active metabolites that promote plant growth and at the same time suppress a wide range of plant pathogens. Therefore, similar to plant growth promoting rhizobacteria (PGPR), actinobacteria also play a marked role in agriculture due to their immense potential to reduce the rate of agro-chemical usage for sustaining crop yields. Nevertheless, the *in situ* role of endophytic actinobacteria to the plant life is relative poorly understood because of the complexities underlying the investigations associated with the interplay between endophytic actinobacterial communities and the host plant (Bouizgarne & Ait Ben Aouamar, 2014). Conversely, from their potential to produce novel compounds (Table 2) it is evident that they are a potential source of several bioactive metabolites and hence should be considered as promising biological candidates for plant growth promotion and inhibition of phytopathogens.

Suppression of phytopathogens by endophytic actinobacteria is both by direct and indirect antagonisms. Direct antagonism is through production of antibiotics, growth hormones, lytic enzymes or hyperparasitism, while indirect mechanisms most commonly involve competition through production of iron chelating compounds such as siderophores (Palaniyandi et al., 2013; Rungin et al., 2012; Swarnalakshmi et al., 2016). Production of growth hormones like cytokinins, gibberellins, indole-3-acetic acid (IAA), indole-3-pyruvic acid (IPYA) positively regulates plant growth (Nimaichand et al., 2016), and among these IAA enhances the resilience of the host plant to environmental stress including acidic pH, osmotic and matrix stress, carbon limitation and water deficit (Yandigeri et al., 2012). Besides, endophytes also produce the enzyme ACC deaminase, which converts ACC, the precursor of ethylene in plants, into ammonia and a-ketobutyrate, thereby lowering plant ethylene levels and enhancing plant growth. Consequently, under salt stress conditions, it improves seed germination and seedlings growth, besides enhancing host flavonoids production suggesting that endophytes can also enhance the tolerance of crops to abiotic stresses (Qin et al., 2014). Furthermore, plants with endophytic actinobacterial strains belonging to the genus *Streptomyces* and *Nocardioides* were found to demonstrate a higher abundance of defense gene expression thereby activating systemic acquired resistance (SAR) or the jasmonate/ethylene (JA/ET) pathway (Conn et al., 2008).

With regard to biocontrol, actinobacterial involvement is through (1) antibiosis, (2) competition, (3) induced resistance, and (4) direct parasitism (Jacob & Sudini, 2016). Among these, antibiosis is speculated to be the most common mechanism through which endophytic actinobacteria suppresses fungal pathogens (Govindasamy et al., 2014). Though research on the role of endophytic actinobacteria in crop production is still in its infancy, the existing literature (Govindasamy et al., 2014; Golinska et al., 2015; Jacob & Sudini, 2016; Nimaichand et al., 2016) suggests that endophytic actinobacteria may directly and indirectly promote plant growth and yield by suppressing pathogens, removing contaminants, solubilizing nutrients, or contributing assimilable N to plants. In general, suppression of phytopathogens is through a suite of mechanisms involving production of antibiotics (e.g. geldanamycin, tubercidin, phenazine, pyrrolnitrin, and zwittermicin A etc), cell wall degrading enzymes (e.g. chitinase, cellulase, glucanase, protease, and phospholipase), oxygenated fatty acids (e.g. oxylipins) and iron chelating compounds viz., siderophores (Golinska et al., 2015; Govindasamy et al., 2014; Jacob & Sudini, 2016; Palaniyandi et al., 2013). Interestingly, they have also been found to produce the quorum quenching enzyme homoserine lactone (HSL)-acylase which degrades the N-acyl-L- HSL quorum sensing (QS) system thereby helping in attenuating the virulence of a broad range of bacterial pathogens with different QS signal molecules (Chankhamhaengdecha et al., 2013). While earlier findings on growth promotion and biocontrol by endophytic actinobacteria have been reviewed by Bouizgarne & Ait Ben Aouamar (2014), Govindasamy et al., (2014) and Shimizu (2011), some of the recent reports are presented in Table 3.

Future outlook

The large untapped reserve of secondary metabolites produced by actinobacteria, if plausibly exploited is expected to yield 5000 to more than four times this number (Bérdy, 2005) of novel antibiotics and other biologically active compounds that can markedly affect

Table 3. Published reports on the role of endophytic actinobacteria in plant disease suppression and their mode of action (2013–2015).

Actinobacteria	Source	Plant tissue	Causative pathogen	Disease	Mode of action	Reference
Streptomyces sp.	*Solanum lycopersicum*	Leaf Stem Root	*Fusarium proliferatum*, *F. graminearum*, *F. oxysporum*, *A. flavus*, *Colletotrichum Capsici*	Fusarium wilt Anthracnose	Antibiotic, IAA and kinetin production	Passari et al. (2016)
Microbacterium sp. *Leifsonia xyli* *Streptomyces* sp.	*Eupatorium odoratum*, *Musa superb*, *Mirabilis jalapa*, *Curcuma longa*, *Clerodendrum colebrookianum*, *Alstonia scholaris*, *Centella asiatica*	Root Stem Leaf Flower	*Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *ciceri*, *Fusarium proliferatum*, *Fusarium oxysporum*, *Fusarium graminearum*, *Colletotrichum capsici*	Collar rot Root rot Damping off Fusarium wilt Anthracnose Leaf blight	Production of cell wall degrading enzymes (Chitinase), HCN production	Passari et al. (2015a)
Microbispora spp	*Solanum tuberosum* L.	Stem	*Streptomyces scabies*	Common scab	Quorum sensing	Goodman (2014)
Streptomyces sp.	Leguminous plants	Root	*Xanthomonas campestris* pv. *glycine*	Bacterial pustule disease	Antimicrobial production	Mingma et al. (2014b)
Nocardiopsis sp. *Streptomyces violaceorubidus Streptomyces* sp	*Elaeis guineensis* Jacq.	Empty fruit bunch	*Ganoderma boninense*	Stem rot	Hydrolytic and lignolytic activity	Ting et al. (2014)
Streptomyces mutabilis *Streptomyces cyaneofuscatus*	*Aristida pungens* *Cleome arabica* *Solanum nigrum* *Panicum turgidum, Astragallus armatus Peganum harmala Hammada scoparia Euphorbia helioscopia*	Root	*Rhizoctonia solani*	Damping off	Antibiosis Chitinase/siderophore production	Goudjal et al. (2014)
Streptomyces felleus	Rape seed *Brassica napus*	Root Stem Leaf	*Sclerotinia sclerotiorum*	Stem rot	Activation of superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) etc.]	Cheng et al. (2014)
Streptomyces sp.	*Garuga pinnata* *Gmelina arborea* *Stephania venosa* *Melastoma malabathricum* *Merremia vitifolia*	Leaf Fruit Seed Stem	*Pectobacterium carotovorum* ssp. *carotovorum*	Soft rot	Degradation of *N*-acyl-L-homoserine lactone (HSL) by HSL-acylase activity	Chankhamhaengdecha et al. (2013)
Streptomyces sp.	*Zea mays*	Root Stem Leaf	*P. aphanidermatum*	Root and crown rot	Antibiosis, Chitinase	Costa et al. (2013)
Streptomyces thermodiastaticus	Tomato *Solanum lycopersicum* Mill.	–	*Ralstonia solanacearum*	Bacterial wilt	–	Sreeja & Surendra Gopal (2013)

human, plant and environmental health. Considering that most of the infectious bacterial and fungal strains are acquiring multiple antibiotic resistances with possible chances of lateral spread, it is imperative that the potential of endophytic actinobacterial communities to produce novel metabolites that can be directed at new targets in the bacterial and fungal cell is fully exploited. While the actinobacterial strains belonging to the genus *Streptomyces* continue to rule the roost, the non streptomycete actinobacterial communities are also undoubtedly important sources of novel secondary metabolites and therefore have to be intensively screened for the production of previously unknown metabolites as well as large quantities of known metabolites. Research on endophytic actinobacteria is still a fledgling field and hence unusual/exciting discoveries are yet to be reported. However, to help put things into perspective, we elaborate here on certain avenues of research that will likely open up new insights into the various facets of the endophytic actinobacterial community.

The mechanisms involved in the interplay between a host plant and a resident actinobacteria is still fuzzy and hence investigations should focus on the *in planta* conditions and factors that govern the production of these energetically expensive biological compounds. Prior to this it is imperative to deduce why actinobacteria tend to produce secondary metabolites, though different hypotheses like "ballast for the cells" to "waste detoxification" or "ecological fitness" (Haferburg & Kothe, 2013) are doing the rounds. We also need to comprehend the patterns of gene expression regulating the timing and levels of secondary metabolite produced and determine whether a BGC remains cryptic in all actinobacterial strains. This will provide better insight into the complex role played by the secondary metabolites on the ecological fitness of the actinobacterial strain vis-à-vis host plant. Also, screening of endophytic actinobacteria for secondary metabolism has mostly been done by isolating the actinobacterial strain from the plants and culturing *in vitro*. However, techniques that allow *in situ* analysis under natural conditions will help in detection and characterization of metabolites produced exclusively during plant-actinobacteria interaction (Brader et al., 2014). It is also possible to monitor the *in situ* expression of antibiotic biosynthetic genes by fusing the antibiotic structural genes(s) to a reporter gene for monitoring its expression. For this purpose, the Glucuronidase Reporter System has proved to be unparalleled in plant molecular biology (Myronovskyi et al., 2011) and undoubtedly will also facilitate a wide variety of studies of actinobacterial genetics.

Future strategies for identification of new biomolecules with improved properties and range of activity will be mostly centered on the eDNA libraries and metagenomic sequencing strategies. Both expression dependent (functional) and expression independent (homology) based screening strategies can be adopted for identifying the BGCs. However, the major challenge presently faced in the metagenomic approach is the effective processing of the massive output of sequencing data and difficulties in functional dissection of BGCs due to the shared sequences in genes that direct the synthesis of different products and rearranged gene clusters that direct synthesis of the same product. Further, genetic loci in genome are grouped as those involved in primary product synthesis and those involved in secondary metabolism. This implies that not all genes are expressed under all conditions, which makes it difficult to fully explore the biosynthetic potential by harvesting cells at a particular stage. It is also not possible to scoop out the plethora of genes by gene based screening (degenerate PCR) due to the intertwined nature of the gene clusters of multiple pathways. To circumvent these hurdles, targeted metagenomic screening technique such as those employed in uncultured marine bacteria (Trindade et al., 2015) could be employed to increase the "novelty" hit rate, besides identifying entirely new classes of genes for both known and novel functions (Sharma & Vakhlu, 2014). An added advantage with this targeted approach is that prior knowledge of the gene sequence for the target activity of interest is not required (Trindade et al., 2015). Single cell genomics is also considered to be a useful tool to reduce complexity of metagenomic DNA. The genome of the single cell is amplified by multiple displacement amplification to generate PCR amplifiable DNA and screened by NGS. Also, sequencing of genes and promoters of cryptic pathways can induce expression of BGCs by constitutive expression of activators or genetic depression of repressors. In this context, whole genome sequencing (WGS) is considered to be the best approach to genome mining due to its ability to reveal the inventory of all BGCs in an organism. The recently evolved strategies of NGS have dramatically lowered the cost of DNA sequencing when compared to the earlier strategies of cumbersome library construction and chromosome walking.

However, NGS platforms that generate enormous amounts of sequencing reads within hours is unfortunately challenging due to short read lengths associated with most of the platforms, especially when assembly of highly conserved and repetitively organized pathways like PKS and NRPS are considered.

However, recent strategies like paired-end libraries, deeper sequencing and enrichment methods that have been reported to be successful in marine organisms can be adopted in solving these problems to a great extent. Another plausible challenge here is identifying suitable vectors capable of cloning gene clusters or devising strategies for multiple clones encompassing the gene clusters. NGS strategy is advantageous in that it allows direct link between BGCs and the taxonomic information uncovered from the genome, leading to selection of a suitable host for heterologous expression. Once the potential BGC is identified and characterized, synthetic biotechnology is another emerging field for the improved production of useful molecules (Daduang et al., 2015). Here combinatorial biosynthesis can go hand in hand with heterologous gene expression to identify and obtain several hypothetical products for their judicious exploitation. The BGCs with proven commercial value, which range from 10 to 200 kb may be synthesized and even fragments of these clusters can be used for various combinatorial processes to produce useful molecules in a cost-effective way. Contemporary natural product research programs are thus focusing on establishing a relationship with the molecule and the BGCs, which eventually shed light on biosynthetic pathways involved, to help in analog creation and combinatorial biosynthesis, metabolic engineering and heterologous expression as mentioned above.

The expression of BGCs in heterologous hosts, though promising is laced with difficulties arising in introducing large size gene clusters (e.g. PKS or NRPS-containing gene clusters) into appropriate heterologous host organisms and the requirement of a strong and inducible promoter to express the genes of interest (Chiang et al., 2013). To attenuate these hurdles, the use of cosmid and fosmid vectors for library construction, that can accept fragments from 35 to 45 kb and can be easily transfected to heterologous hosts to produce millions of clones and use of larger insert libraries using bacterial artificial chromosomes can be explored. Additional challenges to heterologous expression, like codon bias, promoter recognition, host toxicity, product yield, and host versatility also need to be considered in future.

Another new concept is proteome mining which is based on using fluctuating growth conditions that ensure differential biosynthesis of the bioactivity of interest. Metaproteomic approaches like Orthogonal Active Site Identification System (OASIS) and Proteomic Interrogation of Secondary Metabolism (PrISM) which are being extensively used for natural product research can also be employed in endophytic actinobacterial systems. In the former, chemical probes that target the active sites of PKS and NRPS enzymes are employed leading to the enrichment of complex proteomic samples before chemical analysis. In the latter, significant size of the above enzymes and unique 4′ Phosphopantetheine associated ions are considered to profile novel BGCs. A significantly improved approach involving carrier protein peptides was utilized to improve the effectiveness of PrISM method and natural products can be discovered (Evans et al., 2011).

Subsequent combination of metabolomics and quantitative proteomics establishes correlations between abundance of natural products and concomitant changes in the protein pool, which allows identification of the relevant BGC. Gubbens et al. (2014) have used this approach to elucidate gene clusters for different natural products in *Bacillus* and *Streptomyces*, including a novel juglomycin-type antibiotic.

Despite the fact that studies on actinobacterial endophytes are limited or are in progress, knowledge from some model or natural product producing strains from other systems will shed light on the key methods and approaches to be adopted for mining BGCs and novel metabolites from actinobacterial endophytes. Besides, the availability of genomic sequences of novel strains of endophytic actinobacteria in the near future will provide gainful insights into a slew of novel bioactive compounds that will be crucial in the design and development of effective stratagems for disease control in human and plant systems. A schematic illustration of the pathway that can be employed to identify and isolate novel metabolites from endophytic actinobacteria for industrial, agricultural and pharmaceutical applications is given in Figure 1. Thus, a plethora of information is available on the enzymatic pathways and specialized metabolites in microbes, encoded in BGCs. The fruitful exploitation of these data is only possible if it is compiled and made available in a consistent and systematic fashion through authentic databases. It is also important for researchers to develop new bioinformatic tools, pipelines, and softwares for the annotation and genomic mining of these BGCs and connecting these to the natural molecules for bioprospecting these novel molecules.

A very recent technique addressing the expression states of genes *vis a vis* environment is gaining popularity under the name “Metatranscriptomics”. Here, overall gene expression profiles are analyzed for rRNA and mRNA, revealing both the community structure and function. Here, the challenge faced is the low amount of gene expression that could be analyzed. Hence, strategies to enhance such *in situ* gene expression levels need to be explored in future.

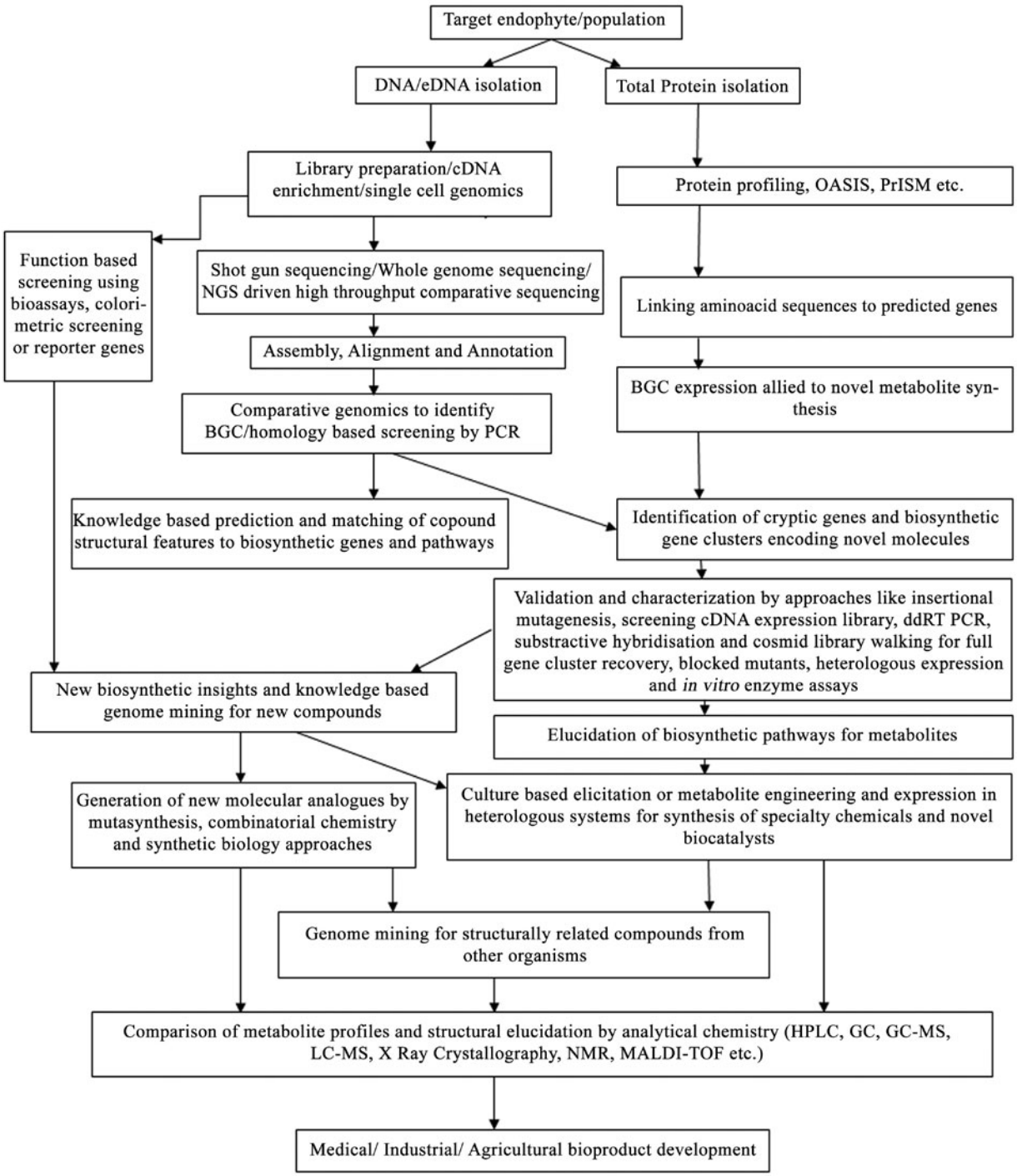

Figure 1. Schematic representation of the proposed pathway to identify and isolate novel metabolites from endophytic actinobacteria for industrial, agricultural and pharmaceutical applications.

Disclosure statement

The authors report no declarations of interest.

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