2 Endophytic Actinobacteria: Diversity and Ecology

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Abstract

Actinobacteria are a group of Gram-positive microorganisms with a high G+C content in their DNA and belong to the phylum *Actinobacteria*, one of the largest phyla within bacteria. Some of these actinobacteria have an endophytic lifestyle which occurs abundantly in most plants. The abundance and diversity of endophytic actinobacterial colonisation depend on plant species, type of soils and other associated environmental conditions. *Streptomyces* spp. were reported as the most predominant species, and *Microbispora*, *Micromonospora*, *Nocardioides*, *Nocardia* and *Streptosporangium* are other common genera of endophytic actinobacteria isolated from a diverse range of plant species, including those found in estuarine/mangrove ecosystems and algae and seaweeds of marine ecosystems. Over the years, isolation media have been devised and numerous methods have been standardised for the isolation, identification and characterisation of these endophytic actinobacteria. Recent advances in molecular tools have revealed the 'not yet cultured' diversity within this group. Therefore, a combination of both culture-based and molecular techniques is essential to describe the diversity and ecology of endophytic actinobacteria. The quest for actinobacteria and their metabolic capabilities is ongoing, as they represent the largest ecological resource for secondary metabolites (plant hormones, antibiotics and other bioactive compounds), with potential biotechnological applications in agriculture, industry and medicine.

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1 Introduction

Plants are naturally associated with microorganisms both externally and internally in various ways. On the exterior surface of plants, diverse microbial interactions occur mostly in the root zone (rhizosphere) and on aerial parts, especially the leaves (phyllosphere) (Hiltner 1904; Yang et al. 2001; Lindow and Brandl 2003; Gray and Smith 2005). Some of the rhizosphere- and phyllosphere-derived microorganisms, which are either bacteria or fungi, are able to penetrate the interior of the plant and colonise intercellular spaces and vascular tissues, where they reside at least part of their lives showing beneficial/symbiotic, neutral or pathogenic interactions (Tervet and Hollis 1948; Hallman et al. 1997; Araujo et al. 2002; Rosenblueth and Martínez-Romero 2006). In the well-studied endosymbiotic beneficial interactions, like the root nodule symbiosis of legumes with rhizobia or the formation of arbuscular mycorrhiza with fungi, the formation of organised symbiotic structures is a common phenomenon, where the microsymbionts reside intracellularly surrounded by a host membrane (Fisher and Long 1992; Downie 1994; Wang and Qiu 2006). On the other hand, there are pathogenic interactions, in which bacteria or fungi often produce effector molecules/proteins inside plant host cells that elicit symptoms of plant disease, causing deleterious effects (Montesinos et al. 2002). In contrast to these interactions, another kind of beneficial interaction exists within the interior of the plant, which is poorly understood at the molecular level. The microorganisms involved in these interactions are commonly referred to as 'endophytes' (Wilson 1995). By definition, endophytes are bacteria or fungi that colonise the host tissues internally, sometimes in high numbers, without damaging the host or harming the host through symptoms of plant disease (Wilson 1995; Compant et al. 2005). Unlike endosymbionts, they do not reside inside the host cells or surrounded by a membrane compartment. Endophytes are distributed throughout the host in all plant organs roots, stems, leaves, flowers, fruits and seeds.

Plants are endophytically colonised by a variety of bacteria belonging to different phylogenetic groups (Chelius and Triplett 2001; Reiter and Sessitsch 2006; Berg et al. 2005). Among them, endophytic bacteria are mostly *Proteobacteria*, but also *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (Rosenblueth and Martínez-Romero 2006). However, the structural composition of endophytic bacterial communities depends on the host plant genotype, the plant organ as well as on the vegetative stage, and may be significantly influenced by plant stress (Sturz et al. 1997; Sessitsch et al. 2002; Reiter et al. 2002; Rasche et al. 2006a, b) and soil type (Conn and Franco 2004a). The *Actinobacteria* are of interest as they are a primary source of secondary metabolites which include bioactive compounds with biotechnological significance. The actinobacteria mainly inhabit the soil, and a large number of actinobacteria have already been isolated and described. Recently, the rate of discovery of new actinobacteria isolated from soils has decreased. Therefore, researchers have examined other ecological niches, such as plant surfaces and interior tissues of plants, and also estuarine and marine ecosystems.

The actinobacteria represent a large portion of the rhizosphere microbial community (Lundberg et al. 2012). Early studies have demonstrated that some actinobacteria can form intimate associations with plants, such as the endosymbiotic association of *Frankia* species in nonleguminous plants and the pathogenic association of a narrow range of *Streptomyces* species on potato (Benson and Silvester 1993; Doumbou et al. 1998). Recent studies have revealed a diverse group of endophytic actinobacterial species with different functions from various plant species (Araujo et al. 2002; Coombs and Franco 2003a; Ryan et al. 2008; Bascom-Slack et al. 2009). Some of them can act as biological control agents (Coombs et al. 2004; Cao et al. 2005; Misk and Franco 2011), and some act as plant growth promoters (Igarashi et al. 2002; Hasegawa et al. 2006). However, the genotype, physiological status of the host plants and its surrounding environment (soil type, including its physicochemical properties, microbial load and diversity) have a major impact on species richness and diversity of endophytic actinobacterial

populations and their related functions (Conn and Franco 2004b; Franco et al. 2007). Due to their ability to colonise the interior of plants coupled with their antimicrobial activities, many initial studies tested endophytic actinobacteria for biological control of plant diseases. In recent years, endophytic actinobacterial research has received special attention mainly as a result of their many other plant growth-promoting properties. In addition, actinobacteria cultured from different endophytic habitats are considered as a potential source for many novel secondary metabolites (Guo et al. 2008).

The aim of this chapter is to describe the recent taxonomy, ecology and diversity of endophytic actinobacteria and to summarise recent findings on isolation of novel endophytic actinobacteria from cultivated crops and also other unexplored plant sources from different ecosystems. Recent advances in the methods to study uncultured/not yet cultured endophytic actinobacterial diversity will also be covered.

2 Taxonomy and Molecular Phylogeny of Endophytic Actinobacteria

Taxonomically the endophytic actinobacteria are a group of Gram-positive bacteria belonging to the phylum *Actinobacteria.* With 6 classes, 25 orders, 52 families and 232 genera (Table 2.1), the phylum *Actinobacteria* represents one of the largest taxonomic units among the 18 major lineages currently recognised within the domain *Bacteria*, including 5 subclasses and 14 suborders (Stackebrandt and Schumann 2000). The phylum *Actinobacteria* comprises Gram stain-positive bacteria with a high G+C content in their DNA.

The species that constitute the *Actinobacteria* have morphologies that include a range of cell types, i.e. coccoid, rod-coccoid and hyphae, that fragment or are highly differentiated. In some genera the spores are formed from aerial mycelia, and may be motile, or may be contained in sporangia or other unusual spore-bearing structures. They have a diverse range of physiological properties and are sought after because of their production of extracellular enzymes but primarily for the production of secondary metabolites and increasingly for applications in agriculture.

Notably, many such secondary metabolites are antibiotics of medical importance (Lechevalier and Lechevalier 1967; Schrempf 2001). *Actinobacteria* play a crucial role in the recycling of biomaterials by organic matter decomposition and humus formation (Goodfellow and Williams 1983; Schrempf 2001; Stach and Bull 2005). This phylum includes human pathogens, e.g. *Mycobacterium* spp., *Nocardia* spp., *Tropheryma* spp., *Corynebacterium* spp. and *Propionibacterium* spp.; plant commensals, e.g. *Leifsonia* spp.; nitrogen-fixing plant symbionts, e.g. *Frankia* spp.; plant endophytes (many genera); plant pathogens, e.g. *Streptomyces* spp.; and inhabitants of the human gastrointestinal tract, e.g. *Bifidobacterium* spp.

Although *Actinobacteria* form a distinct cluster in the 16S rRNA phylogenetic trees, the only 'shared derived character' is a homologous insertion of ~100 nucleotides between helices 54 and 55 of the 23S rRNA gene (Ventura et al. 2007). Recent analysis has identified conserved indels and proteins that can be used to distinguish this important group of bacteria (Gao and Gupta 2005; Gao et al. 2006; Ventura et al. 2007; Hayward et al. 2009).

The initial genome sequencing results confirmed that, unlike most bacterial genomes, many *Streptomyces* genomes are linear (Dyson 2011) and so too are genomes of *Rhodococcus* spp., but the other genera have circular genomes (Bentley et al. 2002) with sizes ranging from 7.7 to 9.7 Mb (Redenbach et al. 2000) for the filamentous actinobacteria. In addition, large 'linear plasmids' typically possessing short inverted repeats at their termini and protein-bound 5′ends, are also reported to be present in the various genera of *Actinobacteria* (Kalkus et al. 1998; Redenbach et al. 2000). The first actinobacterial genome to be sequenced was that of the human tuberculosis agent, *M. tuberculosis* H37Rv (Cole et al. 1998). In the last few years, genomes of different *Actinobacteria* (including plant beneficial *Frankia*, *Leifsonia* and *Streptomyces* species) have been sequenced to completion

Table 2.1 Taxonomy of the phylum *Actinobacteria* and genera with endophytic life style as per *Bergey's Manual of Systematic Bacteriology* (Volume 5, Part A; 2nd edition, 2012) and 'List of Prokaryotic Names with Standing in Nomenclature' (Euzeby http://www.bacterio.cict.fr/)

Systematic position/taxonomic hierarchy	Orders	No. of families	No. of genera	Key genera reported to contain endophytes
Phylum XXVI. *Actinobacteria*				
Class I. *Actinobacteria*	Order I. *Actinomycetales*	1	5	*Actinomyces*
	Order II. *Actinopolysporales*	1	1	*Actinopolyspora*[c]
	Order III. *Bifidobacteriales*	1	7	ND
	Order IV. *Catenulisporales*	2	2	ND
	Order V. *Corynebacteriales*	6	13	*Corynebacterium*
				Dietzia[c]
				Gordonia[c]
				Mycobacterium
				Nocardia
				Rhodococcus
				Tsukamurella[c]
				Williamsia[c]
	Order VI. *Frankiales*	6	11[b]	*Blastococcus*[c]
				Frankia
				Jatrophihabitans[a]
				Modestobacter[c]
	Order VII. *Glycomycetales*	1	2	*Glycomyces*[c]
	Order VIII. *Jiangellales*	1	2	*Jiangella*[c]
	Order IX. *Kineosporiales*	1	3	*Kineococcus*[c]
	Order X. *Micrococcales*	15	84	*Arthrobacter*
				Brachybacterium[c]
				Citricoccus[c]
				Herbiconiux[a]
				Janibacter[c]
				Kocuria[c]
				Koreibacter[a]
				Leifsonia
				Microbacterium
				Micrococcus
				Oerskovia[c]
				Promicromonospora[c]
				Rathayibacter[c]
	Order XI. *Micromonosporales*	1	23	*Actinoplanes*[c]
				Dactylosporangium
				Jishengella[a]
				Micromonospora
				Phytohabitans[a]
				Phytomonospora[a]
				Planosporangium[c]
				Plantactinospora[c]
				Polymorphospora[c]
	Order XII. *Propionibacteriales*	2	18	*Actinopolymorpha*[c]
				Flindersiella[a]
				Kribbella[c]
				Nocardioides
	Order XIII. *Pseudonocardiales*	1	22	*Actinomycetospora*[c]
				Actinophytocola[a]
				Amycolatopsis[c]
				Kibdelosporangium[c]
				Pseudonocardia
				Saccharomonospora
				Saccharopolyspora[c]
				Saccharothrix

(continued)

Table 2.1 (continued)

Systematic position/taxonomic hierarchy	Orders	No. of families	No. of genera	Key genera reported to contain endophytes
	Order XIV. *Streptomycetales*	1	3[b]	*Kitasatospora*[c]
				Streptacidiphilus[c]
				Streptomyces
	Order XV. *Streptosporangiales*	3	22[b]	*Actinoallomurus*[c]
				Actinocorallia[c]
				Actinomadura[c]
				Allonocardiopsis[a]
				Microbispora
				Nocardiopsis
				Nonomuraea[c]
				Planotetraspora
				Streptomonospora
				Streptosporangium
	Order *Incertae sedis*[b]	0	1[b]	ND
Class II. Acidimicrobiia	Order I. *Acidimicrobiales*	2	5	ND
Class III. Coriobacteriia	Order I. *Coriobacteriales*	1	13	ND
Class IV. Nitriliruptoria	Order I. *Nitriliruptorales*	1	1	ND
	Order II. *Euzebyales*	1	1	ND
Class V. Rubrobacteria	Order I. *Rubrobacterales*	1	1	ND
Class VI. Thermoleophilia	Order I. *Thermoleophilales*	1	1	ND
	Order II. *Solirubrobacterales*	3	3	ND

[a] New genus discovered as an endophyte
[b] Includes genus *Incertae sedis*
[c] Contains recently identified/discovered endophytic species (after 2010); ND—no type strain identified as an endophyte

(Bentley et al. 2002; Monteiro-Vitorello et al. 2004; Normand et al. 2007), while sequencing of genomes from representatives of 43 or more actinobacteria are still in progress (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

In the recently published 2nd edition of *Bergey's Manual of Systematic Bacteriology* (Whitman et al. 2012), the polyphasic approach was followed for actinobacterial systematics. This taxonomic characterisation is inferred from many parameters, namely, its branching pattern in the 16S rRNA phylogenetic tree (Garrity and Holt 2001; Ludwig and Klenk 2005), taxon-specific 16S rRNA gene sequence signatures (Zhi et al. 2009), as well as chemotaxonomical, physiological and biochemical properties. The separation of this phylum from other bacterial taxa is supported by conserved indels in some proteins (e.g. cytochrome *c* oxidase subunit 1, CTP synthetase and glutamyl-tRNA synthetase), by the presence of a large insert in the 23S rRNA gene (Gao and Gupta 2005; Gao et al. 2006) and by distinctive gene arrangements (Kunisawa 2007).

3 Recent Advances in the Isolation and Characterisation of Endophytic Actinobacterial Diversity

3.1 Culture-Based Approaches

The method of isolation is one of the most crucial steps in obtaining pure cultures of endophytes; therefore, consideration should be given to the implementation of a plant-specific isolation protocol. Some detailed isolation methods and procedures, including plant sampling, surface sterilisation and media relevant for endophytic actinobacteria, were assessed by Hallmann et al. (2006), Qin et al. (2009) and recently by Kaewkla and Franco (2013a).

3.1.1 Plant Sampling, Surface Sterilisation and Processing

After the choice of host plant is made, the next decision is the age of the sample and the plant organ.

In most studies, sampling is a one-off event and the description of the endophytes obtained is provided with little or no acknowledgement of the possibility that the diversity can change with plant age and season or soil type (Conn and Franco 2004a). Very few studies are hypothesis driven, especially if the aim is to maximise the number and diversity of actinobacteria isolated. Sampling decisions should include the age or stage of the plant, the soil and climate and the parts of the plant (Zhang et al. 2006). In the case of trees, depending on the size, the location of the sample and the number of samples are likely to influence the outcomes. To date, there are no reports on the spatial diversity within a branch or root of a tree. However, as the abundance of endophytes is low (Kaewkla and Franco 2013a), it is recommended that a large amount of plant sample is collected to be able to increase the number and diversity of the strains cultivated.

Surface sterilisation of plant material is an obligatory step for endophytic actinobacterial isolation in order to kill all the surface microbes. It is usually accomplished by treating the plant tissues with an oxidising agent or general sterilant for a specific period, followed by repeated sterile water rinses. Commonly used surface sterilants include ethanol (70–95 %), sodium hypochlorite (3–10 %) and also hydrogen peroxide (3 %). Some surfactants such as Tween 20, Tween 80 and Triton X-100 can also be added to enhance the effectiveness of surface sterilisation (Sturz 1995; Hallmann et al. 2006). A general protocol involves a three-step procedure similar to that described by Coombs and Franco (2003a). It was recommend that a five-step procedure is optimum, and addition of sodium thiosulfate solution following the sodium hypochlorite treatment will improve cultivation efficiency because thiosulfate can neutralise the detrimental effects of residual NaOCl on the growth of microorganisms emerging from within the tissue (Qin et al. 2009). After this treatment, plant tissues can be soaked in 10 % $NaHCO_3$ solution to inhibit any endophytic fungi, which can outgrow the actinobacteria on isolation medium plates (Nimnoi et al. 2010a). The effectiveness of surface sterilisation should be checked to confirm the isolates are true endophytes. In general, the sterilisation procedure should be standardised for each plant type and tissue, especially the sterilisation time, as the sensitivity varies with plant species, age and tissue type. The concentration of the hypochlorite and the length of exposure should be adjusted to the type of plant tissue. For example, many leaves are more ‘porous’ than their root or stem surfaces and are prone to infiltration by the sterilant.

Samples containing extraneous material such as soil can be sonicated before sterilisation to remove any attached soil or microorganisms. Surface-sterilised plant samples are routinely air-dried or heated at 80 or 100 °C for 15–30 min to kill bacteria, resulting in a lowering of vegetative bacterial number if present. Commonly, plant materials are septically sectioned into small fragments of about 0.2×1.0 cm size (Coombs and Franco 2003a; Cao et al. 2004; Verma et al. 2009; Fialho de Oliveira et al. 2010) and then placed/distributed into various actinobacterial isolation media. In another method, surface-sterilised plant tissues can be aseptically crumbled into smaller fragments by commercial blender (Qin et al. 2008a, b; Li et al. 2009), to expose organisms from within the plant material and increase their recovery. These two preferred methods could recover a higher number of less commonly detected genera among the endophytic actinobacteria. One of the main objectives is to release the endophytes from the inner parts of plant tissue material and expose them to the growth medium. Some sterile samples were mixed in a mortar with 0.5 g of sterile powdered calcium carbonate and then placed in a Petri dish, and two millilitres of sterilised tap water was added to the sample to create a moist environment. After 2 weeks at 28 °C, the samples were air-dried at room temperature and placed in media plates, or samples were also placed in a glass dish and flooded with 50 ml of 10 mmol phosphate buffer containing 10 % plant or soil extract at 28 °C to liberate actinobacterial spores (Qin et al. 2009). Endophytes can also be separated from plant tissue using the method of Jiao

et al. (2006) by grinding the plant material and subjecting it to enzymes that break down plant cell walls. The bacterial pellet is separated out by differential centrifugation, diluted and plated onto isolation media.

All the methods examined gave different populations, and none of them was recommended as being superior to any other.

3.1.2 Composition and Combination of Culture Media and Incubation Conditions

Successful culturing of microorganisms on laboratory media is dependent on the nutritional composition of the media and the incubation conditions. The use of a medium composition that mimics the micro-environments of inner part of the plants is a good strategy for isolation of endophytic actinobacteria. Some of the established media for isolation of actinobacteria from soil samples include humic acid vitamin B (HV) (Hayakawa 1990), International *Streptomyces* Project media 2 and 5 (Shirling and Gottlieb 1966), raffinose-histidine agar (Vickers et al. 1984) and starch casein agar (Küster and Williams 1964). Low-nutrient medium TWYE was found effective for isolation of endophytic actinobacteria from many plant species (Coombs and Franco 2003a; Qin et al. 2009; Li et al. 2009), due to the fact that high nutrient concentration allowed fast-growing bacteria to overgrow slower growing actinobacteria. Inside the plant, amino acids are the major source of nitrogen, and cellulose and xylan are the primary sources of carbon. Media containing amino acids (proline, arginine and asparagine) as nitrogen sources and cellulose, xylan, sodium propionate and sodium succinate as carbon sources improved isolation effectiveness and yielded uncommon and rare endophytic actinobacterial genera (Qin et al. 2009). Similarly, addition of plant or soil extracts into the isolation medium could help meet specific requirements of actinobacteria from plant tissues and soil environments (Okazaki 2003). Janso and Carter (2010) used arginine vitamin agar supplemented with 3 % soil extract to isolate several phylogenetically unique endophytic actinobacteria such as *Sphaerisporangium* and *Planotetraspora* from tropical plants of Papua New Guinea and Mborokua Island, Solomon Islands. In another example, the use of media with low concentrations of plant polymers (gellan gum, xylan and pectin), their constituent sugars (glucose, galactose, xylose, arabinose, glucuronate, galacturonate, ascorbate, gluconate and carboxymethylcellulose), and 17 amino acids improved the isolation of 16 rare actinobacterial genera including a new genus *Flindersiella* in the family *Nocardioides*, while other 11 strains were accepted as new species of endophytic actinobacteria (Kaewkla and Franco 2013a).

Kaewkla and Franco (2013a) recommend incubation of isolation plates under moist conditions for up to 16 weeks with removal of colonies every week, as they found that the majority of non-streptomycetes emerged after 6 weeks of incubation.

A list of isolation protocols and media used to study the diversity of endophytic actinobacteria is shown in Table 2.2.

4 Diversity of Endophytic Actinobacteria in Plants of Terrestrial Ecosystems

4.1 Agricultural/Field Crops

Early studies on endophytic actinobacterial associations in agricultural crop plants were reported from Italy by Sardi et al. (1992) who isolated 499 strains from surface-sterilised root samples of 28 plant species including different field crops such as barley, rye, oats and soybean, with the majority of the isolates belonging to the genus *Streptomyces*. Okazaki et al. (1995) isolated endophytic actinobacteria from other part of crop plants, e.g. leaves and leaf litter, with the majority belonging to the genera *Streptomyces* and *Microbispora*. *Microbispora* spp. was the most common actinobacteria isolated from the surface-sterilised roots and leaves of field-grown maize plants

Table 2.2 Methodology used in culture-based studies for the isolation of endophytic actinobacteria from different plant species

Plant type	Methods	Media used	List of reported/cultured genera	References
Australian endemic trees (*Callitris preissii*, *Eucalyptus camaldulensis*, *Eucalyptus microcarpa*, *Pittosporum phylliraeoides*)	Surface sterilisation and prolonged incubation at 27 °C up to 16 weeks	Mannitol mung bean yeast extract mineral salt agar (MMYA), yeast extract casamino acid glucose agar (YECG), humic acid vitamin B agar (HVA), HVA with gellan gum (HVG), VL 70 gellan gum with different combinations of sugar, amino acid mixtures	*Actinomadura*, *Actinomycetospora*, *Actinopolymorpha*, *Amycolatopsis*, *Flindersiella*, *Gordonia*, *Kribbella*, *Micromonospora*, *Nocardia*, *Nocardioides*, *Nocardiopsis*, *Nonomuraea*, *Polymorphospora*, *Promicromonospora*, *Pseudonocardia* and *Williamsia*	Kaewkla and Franco (2013a)
Cabbage (*Brassica campestris*, China)	Surface sterilisation and incubation at 30 °C up to 3 weeks	Humic acid vitamin B agar (HV) and corn meal agar (CMA)	*Microbispora*, *Streptomyces*, *Micromonospora*, *Nocardia*, *Verrucosispora*, *Nonomuraea*, *Actinomadura* and *Thermonospora*	Lee et al. (2008b)
Ethanobotanical trees (*Cinnamomum zeylanicum*, *Zingiber spectabile*, *Elettariopsis curtisii* and *Labisia pumila*) Thailand	Four different surface sterilisation procedures and incubation at 28 °C up to 3 weeks	Starch yeast casein agar (SYCA), actinomycetes isolation agar (AIA), HV agar, tap water yeast extract agar (TWYA) and coal vitamin agar	*Streptomyces* and one unknown genus	Zin et al. (2010)
Lentil, chickpea, pea, faba bean and rye (Parksville, South Australia)	Surface sterilisation and incubation at 27 and 37 °C up to 4 weeks	HV agar, starch casein medium and TWYA	*Streptomyces* and *Microbispora*	Misk and Franco (2011)
Medicinal plants (Hainan, China)	Surface sterilisation and incubation at 28 °C up to 3 weeks	ATCC 172 agar, Gauze's No. 2 agar, glucose-asparagine agar, HV agar and starch-casein-mineral salts agar	*Amycolatopsis*, *Micromonospora*, *Nocardia*, *Nonomuraea* and *Streptomyces*	Huang et al. (2012)
Medicinal plants (Xishuangbanna, China)	Surface sterilisation followed by four different selective isolation procedures and incubation at 28 °C for 2–8 weeks	TWYE, modified TWYE with plant extract, glycerol-asparagine agar (ISP 5), HV agar, M5 inorganic salts-starch agar (ISP 4), YIM 38 medium, raffinose-histidine agar, sodium propionate agar, cellulose-proline agar, trehalose-proline medium, xylan-arginine agar	*Actinocorallia*, *Blastococcus*, *Dactylosporangium*, *Dietzia*, *Jiangella*, *Oerskovia*, *Promicromonospora* and *Saccharopolyspora*	Qin et al. (2009)

(continued)

Table 2.2 (continued)

Plant type	Methods	Media used	List of reported/cultured genera	References
Medicinal tree (*Maytenus austroyunnanensis*) (Xishuangbanna, China)	Surface sterilisation followed by enzymatic homogenisation, diluted supernatant used for isolation and incubation at 28 °C for 2–8 weeks	Same as above	*Amycolatopsis*, *Cellulosimicrobium*, *Glycomyces*, *Jiangella*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardiopsis*, *Polymorphospora*, *Pseudonocardia*, *Saccharopolyspora* and *Streptosporangium*	Qin et al. (2012a, b, 2013a)
Native herbaceous plants (South Korea)	Surface sterilisation followed by isolation from homogenised solution of plant materials and incubation at 30 °C for 2 weeks	Starch casein agar	*Arthrobacter*, *Dietzia*, *Herbiconiux*, *Kitasatospora*, *Microbacterium*, *Microbispora*, *Micrococcus*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Rathayibacter*, *Rhodococcus*, *Streptacidiphilus*, *Streptomyces* and *Tsukamurella*	Kim et al. (2012)
Neem tree (*Azadirachta indica*) (India)	Surface sterilisation and incubation at 28 °C for 3–4 weeks	S-agar and water agar	*Microbispora*, *Nocardia*, *Streptomyces*, *Streptosporangium*, *Streptoverticillium* and *Saccharomonospora*	Verma et al. (2009)
Rice (*Oryza sativa*) (China)	Surface sterilisation and incubation at 26 °C for 1 week	S (*Streptomyces*) medium	*Streptomyces* and *Nocardioides*	Tian et al. (2007)
Tomato (*Lycopersicon esculentum*) (Murray Bridge, South Australia)	Surface sterilisation and incubation at 27 °C up to 4 weeks	TWYE agar, HV agar and yeast extract, casamino acid medium	*Microbispora*, *Nonomurae* and *Streptomyces*	Inderiati and Franco (2008)
Tropical native plants (Papua New Guinea, Mborokua and Solomon Islands)	Surface sterilisation and incubation at 23–25 °C up to 8 weeks	Arginine vitamin agar supplemented with soil extract from organic humus	*Actinoplanes*, *Amycolatopsis*, *Dactylosporangium*, *Kibdelosporangium*, *Kitasatospora*, *Lechevalieria*, *Lentzea*, *Microbispora*, *Nonomuraea*, *Planotetraspora*, *Pseudonocardia*, *Sphaerisporangium*, *Streptomyces* and *Streptosporangium*	Janso and Carter (2010)
Wattle tree (*Acacia auriculiformis*) (Thailand)	Surface sterilisation followed by isolation from solution of crushed plant materials and incubation at 28 °C up to 4 weeks	Starch Casein agar containing 100 g/ml ampicillin, 2.5 U/ml penicillin G, 50 g/ml amphotericin B and 50 g/ml cyclohexamide	*Actinoallomurus*, *Amycolatopsis*, *Kribbella*, *Microbispora* and *Streptomyces*	Bunyoo et al. (2009)
Wheat (*Triticum aestivum*) (South Australia)	Sonication followed by surface sterilisation and incubation at 27 °C up to 4 weeks	TWYE agar, HV agar, flour-yeast extract-sucrose-casein hydrolysate agar, flour-calcium carbonate agar	*Microbispora*, *Micromonospora*, *Nocardioides* and *Streptomyces*	Coombs and Franco (2003a)

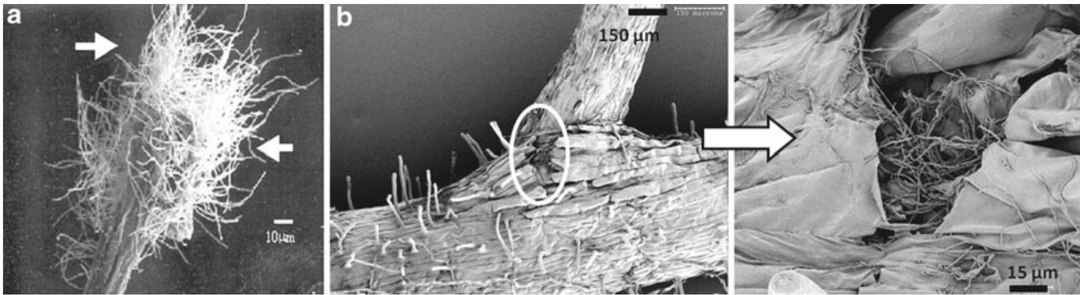

Fig. 2.1 Identification of endophytic actinobacterial colonisation in surface-sterilised wheat plants. (**a**) SEM image of *Streptomyces* aerial hyphal growth on a surface-sterilised root fragment from an isolation agar plate (Coombs and Franco 2003a). (**b**) SEM image showing the endophytic colonisation in a lateral root junction of a wheat plant by *Streptomyces* sp. EN27 (Courtesy V Conn and C Franco)

(*Zea mays* L.) (de Araujo et al. 2000), although *Streptomyces* and *Streptosporangium* spp. were also represented and some of them showed antimicrobial activity against one or more tested bacteria and yeast.

Coombs and Franco (2003a) reported the isolation of filamentous actinobacteria from surface-sterilised root tissues of healthy wheat plants (*Triticum aestivum* L.) (Fig. 2.1). Of the 49 endophytic isolates that belonged to *Streptomyces*, *Microbispora*, *Micromonospora* and *Nocardioides* were strains found to be similar to *S. caviscabies* and *S. setonii* that had been isolated originally from potato scabs. Therefore, detection of pathogenicity was required as the endophytic isolates were potential biocontrol agents. The isolates were found to be nonpathogenic, as they neither had *nec1*, a pathogenicity-associated gene, nor produced the toxin thaxtomin. In other studies, they visually demonstrated the colonisation of germinating wheat seed embryo, endosperm and emerging radicle with one of these endophytic actinobacteria, *Streptomyces* sp. strain EN27, tagged with the *egfp* gene. These observations show that the endophytic actinobacterium was able to associate with its host at a very early stage in the development of the plant (Coombs and Franco 2003b). Similarly, in pea plants, Tokala et al. (2002) showed a remarkable degree of preferential colonisation of pea nodules relative to roots by *Streptomyces lydicus* strain WYEC108 that was isolated from a rhizosphere soil. This observation and other studies indicated that actinobacteria isolated from soil could be capable of endophytic colonisation.

Tian et al. (2007) identified actinobacterial strains from the surface-sterilised stems and roots of rice and described differences in endophytic populations from these plant parts. Strains similar to *Streptomyces cyaneus*, *S. aurantiacus* and *S. paresii* were also isolated from roots and stems, whereas *Nocardioides thermolilacinus*, *S. exfoliates*, *S. glauciniger* and *S. kathirae* were only isolated from roots and *S. caviscabies* and *S. scabies* were isolated from stems only, indicating that more diverse actinobacteria were isolated from roots than stems. Their results also suggest the presence of more diverse communities of uncultured actinobacteria within stems and roots of rice. Velazquez et al. (2008) selected the apoplastic sap of the medullary parenchyma of the stem of healthy sugarcane plants to identify endophytic isolates belonging to the genera *Microbacterium, Micrococcus* and *Kokuria*. Root nodules of the grain legume *Lupinus angustifolius* yielded 136 different orange-pigmented actinobacterial colonies from surface-sterilised nodules which belonged to the genus *Micromonospora,* and a detailed taxonomic study on six of these isolates identified two novel species, *Micromonospora lupini* and *M. saelicesensis* (Trujillo et al. 2007). Misk and Franco (2011) found a physiologically diverse group of endophytic actinobacteria from grain legume plants such as lentil, chickpea, pea and faba bean. Some of the biotic activities observed included siderophore and cyanogen

production, antifungal activity and phosphate solubilisation. These studies exemplify the value of using different approaches to characterise the culturable diversity of endophytic isolates obtained from a few crop plants. A large number of studies have since been reported from most crop plants confirming their ubiquitous presence. This group of microbes can colonise the internal tissue of crop plants and are capable of producing plant growth-promoting chemicals, enhancing nutrient uptake as well as producing secondary metabolites that can inhibit microbial pathogens and induce systemic resistance. Therefore, their functions have been a major factor for their isolation as they promise to offer an advantage in terms of reliability and efficacy as inoculants due their endophytic nature. A summary of these beneficial functions is shown in Table 2.3.

4.2 Horticultural Crops

Cao et al. (2004) compared the endophytic actinobacteria from roots and leaves of healthy and wilting banana plants. Community analysis of the 242 isolates demonstrated increased actinobacterial diversity in wilting leaves compared to that in healthy leaves, although actinobacterial communities in roots were similar. The same laboratory tested a total of 131 strains, identified as *Streptomyces*, *Streptoverticillium* and *Streptosporangium* spp., that were successfully isolated from surface-sterilised banana roots (Cao et al. 2005). About 18.3 % of these isolates inhibited the growth of pathogenic *Fusarium oxysporum* f. sp. *cubense*, the causal organism of Panama wilt disease of banana, on banana tissue extract medium. About 37.5 % of the most frequently isolated *S. griseorubiginosus* strains were antagonistic to this pathogen, but the antagonism was lost when $FeCl_3$ was introduced into the inhibition zone. These findings indicate the potential of developing siderophore-producing *Streptomyces* endophytes for the biological control of *Fusarium* wilt (Panama) disease of banana (Cao et al. 2005).

Actinobacteria were reported for the first time as endophytes of grapevines, with a number of other isolates identified as *Streptomyces* spp. and also the rare actinobacterium *Curtobacterium* spp. (Bulgari et al. 2009; West et al. 2010).

In a survey of endophytic bacteria colonising roots of processing carrot cultivars (Carochoice, Red Core Chantenay) grown at two locations in Nova Scotia, Surette et al. (2003) reported the association of *Arthrobacter*, *Kokuria* and *Microbacterium* as endophytes. In a similar study on potato-associated bacteria, the *Streptomyces* spp. had the highest antagonistic activity among endophytic actinobacteria against most of the fungal as well as bacterial pathogens (Sessitsch et al. 2004). A total of 619 actinobacteria, all *Streptomyces* spp., were isolated from different cultivars of tomato. The *aureus* group of *Streptomyces* was the most frequent isolate group, but the population composition of *Streptomyces* varied according to tomato cultivars, physiological status and soil types (Tan et al. 2006). *Microbispora* spp. (67 %) were the most common isolates of the 81 endophytic actinobacteria from Chinese cabbage roots (Lee et al. 2008b), followed by *Streptomyces* spp. (12 %) and *Micromonospora* spp. (11 %). The three antagonistic isolates were identified as *Microbispora rosea* subsp. *rosea* (A004 and A011) and *Streptomyces olivochromogenes* (A018), which effectively suppressed the disease club root of cabbage caused by *Plasmodiophora brassicae*. Recently, Khan and Doty (2009) reported a diverse array of endophytic bacteria associated with sweet potato plants (*Ipomoea batatas* L.) which included the actinobacterial genus *Arthrobacter*.

Shimizu et al. (2000) explored endophytic actinobacteria from the flowering plant Rhododendron. Nine, six and two isolates, with distinguishing characteristics based on the macroscopic appearance of colonies, were obtained from roots, stems and leaves, respectively, and shown to have antagonism against two major fungal pathogens of rhododendron, *Phytophthora cinnamomi* and *Pestalotiopsis sydowiana*. Similarly, Nishimura et al. (2002) isolated a total of 73 actinobacteria from leaves, stems and roots of the other *Ericaceae* plant called mountain laurel (*Kalmia latifolia* L.), and most of them were *Streptomyces* spp. with a broad and intense antimicrobial spectrum against various yeasts and

Table 2.3 Functional aspects of endophytic actinobacteria isolated from different plant species and habitats

Plant type	Endophytic actinobacterial genera	Functional role established	References
Arabidopsis	*Micromonospora* sp. strain EN43 and *Streptomyces* sp. strain EN27	Induction of defence through SAR and JA/ET pathways	Conn et al. (2008)
Banana	*Streptomyces*	Siderophore production and antibiosis	Cao et al. (2004, 2005)
Cabbage	*Microbispora* and *Streptomyces*	Antibiosis	Lee et al. (2008a, b)
Cucumber	*Actinoplanes campanulatus, Micromonospora chalcea* and *Streptomyces spiralis*	Antibiosis and glucanolytic activity	El-Tarabily et al. (2009)
Eaglewood tree	*Actinomadura*, *Nocardia*, *Nonomuraea*, *Pseudonocardia* and *Streptomyces*	Ammonia, indole acetic acid (IAA) and siderophore production	Nimnoi et al. (2010a)
Epiphytic vine	*Streptomyces*	Antibiosis	Ezra et al. (2004)
Foliose lichens	*Nocardia*, *Nocardiopsis* and *Streptomyces*	Antibiosis	da Silva et al. (2011)
Herbaceous and woody plants	*Microbispora*, *Micromonospora*, *Nocardia* and *Streptomyces*	Antibiosis	Taechowisan et al. (2003)
Lentil, chickpea, pea, faba bean and rye (South Australia)	*Microbispora* and *Streptomyces*	Siderophore and cyanogen production; phosphate solubilisation and antibiosis	Misk and Franco (2011)
Lichens	*Amycolatopsis*, *Actinomadura*, *Micromonospora*, *Streptomyces* and *Streptosporangium*	Antibiotic biosynthetic genes detected and antibiosis	González et al. (2005)
Madagascar periwinkle	*Streptomyces*	Antibiosis	Kafur and Khan (2011)
Mangrove plants in China	*Micromonospora* and *Streptomyces*	Antibiosis and inhibition of anticancer protein synthesis	Hong et al. (2009)
Marine sponges and soft corals	*Streptomyces*	Antibiosis	El-Bondkly et al. (2012)
Medicinal plants	*Amycolatopsis*, *Micromonospora*, *Nocardia*, *Nonomuraea* and *Streptomyces*	Antitumour activity and antibiosis	Huang et al. (2012)
Medicinal plants in Panxi plateau, China	560 isolates belonging to different genera	Antibiotic biosynthetic genes detected and antibiosis	Zhao et al. (2010b)
Medicinal plants in Xishuangbanna, China	2174 isolates belonging to different genera	Antibiosis	Qin et al. (2009, 2012a, b, 2013a)
Native herbaceous plants in Korea	21 straining belong to different genera	Antibiosis, IAA and hydrolytic enzyme production; phosphatase activity	Kim et al. (2012)
Neem tree	*Nocardia*, *Streptomyces* and *Streptosporangium*	Antibiosis	Verma et al. (2009)
Rhododendron	*Streptomyces*	Antibiosis	Shimizu et al. (2000)
Snakevine	*Streptomyces*	Antibiosis	Castillo et al. (2006)
Tomato	*Microbispora*, *Nonomuraea* and *Streptomyces*	Siderophore production and antibiosis	Tan et al. (2006), Inderiati and Franco (2008)
Tropical native plants in Papua New Guinea, Mborokua and Solomon Islands	*Micromonospora*, *Nonomuraea*, *Pseudonocardia*, *Sphaerisporangium*, *Streptomyces*, *Streptosporangium* and *Thermomonospora*	Detection of bioactive extracts and biosynthetic genes for PKS-I, PKS-II and NRPS	Janso and Carter (2010)
Wattle tree	*Amycolatopsis* and *Streptomyces*	Antibiosis	Bunyoo et al. (2009)
Wheat	*Microbispora*, *Nocardioides* and *Streptomyces*	Antibiosis and plant growth promotion	Coombs and Franco (2003a), Coombs et al. (2004)

fungal pathogens of *Ericaceae*. In recent years, members of the genus *Micromonospora* have also been recovered from diverse plant tissues, especially nitrogen-fixing root nodules (Valdes et al. 2005; Trujillo et al. 2010). A new species *Streptosporangium oxazolinicum* sp. nov. in the genus *Streptosporangium* was isolated from the roots of a variety of orchids collected in the subtropical Okinawa prefecture by Inahashi et al. (2011) which was shown to produce a new group of antitrypanosomal antibiotics, spoxazomicins.

4.3 Medicinal Plants

It is believed that the greatest diversity of bacterial endophytes is likely to occur in the plant species of tropical and temperate regions (Strobel and Daisy 2003). From 36 medicinal plant species in Thailand, Taechowisan et al. (2003) isolated 330 strains belonging to four genera of endophytic actinobacteria, namely, *Streptomyces*, *Microbispora*, *Nocardia* and *Micromonospora*. Medicinal plants in Xishuangbanna tropical rainforest of China were subjected to diverse pretreatment methods and selective media, resulting in an unexpected variety of 10 different suborders and 32 genera, including at least 19 new taxa (Qin et al. 2009, 2010b). Huang et al. (2012) carried out the isolation of endophytic actinobacteria from the surface-sterilised tissues of 12 medicinal plants in Hainan, China, using different media. Of the 280 isolates recovered, 154 were from roots, 73 from stems and 53 from leaves, and they were identified as *Streptomyces, Micromonospora, Nocardia, Nonomuraea* and *Amycolatopsis* spp.

A total of 38 endophytic actinobacteria were isolated from surface-sterilised leaves of *Catharanthus roseus* (L.) (Kafur and Khan 2011). Similarly, from the medicinal plant *Artemisia annua*, a total of 228 isolates representing at least 19 different genera of actinobacteria were obtained and several of them were novel taxa (Li et al. 2012). From the plant *Maytenus austroyunnanensis* alone, a total of 312 endophytic actinobacteria were obtained and they were affiliated with the order *Actinomycetales* (distributed into 21 genera). Notably, a new genus *Polymorphospora* and seven new species were also isolated (Qin et al. 2012a).

Similarly, Kim et al. (2012) reported on the diversity of endophytic actinobacteria and their physiological properties in various Korean native plant species. Using a culture-based approach, the members of the genus *Rhodococcus* and the family *Streptomycetaceae* were found to be the main constituents of the endophytic actinobacterial community. In addition, *Arthrobacter*, *Dietzia, Herbiconiux*, *Mycobacterium*, *Nocardia*, *Rathayibacter*, *Tsukamurella, Streptacidiphilus* and *Kitasatospora* were reported for the first time as endophytes.

Higashide et al. (1977) isolated an actinomycete *Actinosynnema pretiosum* that produces maytasinoid compounds. These compounds are usually found in the Chinese medicinal tree *M. austroyunnanensis,* but no endophytic actinobacteria producing this compound were isolated from this plant (Qin et al. 2012a). Similar is the case with *Artemisia annua*, where many endophytic actinobacteria were reported, but none of them produced the compound artemisinin, an antimalarial drug.

4.4 Perennial Trees

Recent studies suggest that many of the perennial trees are an untapped source of endophytic actinobacteria of the non-*Frankia* type. Eleven strains of endophytic actinobacteria were isolated from the healthy roots of wattle trees *Acacia auriculiformis*, collected from Bangkok and Nakhonpathom, Thailand. Analysis of 16S rRNA sequences of those strains revealed that they belong to the genera *Streptomyces*, *Actinoallomurus*, *Amycolatopsis*, *Kribbella* and *Microbispora* (Bunyoo et al. 2009). Similarly, Verma et al. (2009) reported the isolation of endophytic actinobacteria from a neem tree *Azadirachta indica*. A total of 55 separate isolates were obtained from 20 plants, and 60 % of these showed inhibitory activity against one or more pathogenic fungi and bacteria. Actinobacteria were most commonly recovered from roots (54.5 % of all isolates), followed by stems (23.6 %) and leaves (21.8 %). The dominant genus was *Streptomyces* (49.09 % of all isolates), while *Streptosporangium* (14.5 %),

Microbispora (10.9 %), *Streptoverticillium* (5.5 %), *Saccharomonospora* (5.5 %) and *Nocardia* (3.6 %) were also recovered.

Zin et al. (2010) carried out the isolation of endophytic actinobacteria from the root and stem samples of ethanobotanical trees, namely, *Cinnamomum zeylanicum*, *Zingiber spectabile*, *Elettariopsis curtisii* and *Labisia pumila*, in the northern part of the Malay Peninsula. Sixty six *Streptomyces* spp., and one unidentified isolate were successfully isolated. Of the total isolates obtained, 61.2 % were isolated from root and 38.8 % from the stem. Of these 56.7 % of the endophytic actinobacteria were isolated from the outermost parts of the surface-sterilised plants and 43.3 % were from the internal part of the plants.

Chen et al. (2011) revealed species diversity of endophytic actinobacteria from cinnamon trees *Elaeagnus angustifolia*, mainly distributed in northwest of China and western inner parts of Mongolia. Eight strains of endophytic actinobacteria were successfully isolated from root nodules of *Elaeagnus angustifolia* by the method of nodule slicing, and the result showed that five of these strains belonged to *Micromonospora* and the other three strains were *Nonomuraea*, *Pseudonocardia* and *Planotetraspora*, respectively.

Recently, Kaewkla and Franco (2013a) reported the presence of a wide range of actinobacterial genera as endophytes by incubating plates for up to 16 weeks, but removing emerging colonies as soon as they were 1 mm in diameter. The majority of 576 actinobacterial isolates from leaf, stem and root samples of four Australian endemic trees—*Callitris preissii* (native pine tree), *Eucalyptus camaldulensis* (red gum), *Eucalyptus microcarpa* (Grey Box) and *Pittosporum phylliraeoides* (native apricot tree)—were *Streptomyces* spp., and the others belonged to 16 other actinobacterial genera, namely, *Actinomadura*, *Actinomycetospora*, *Actinopolymorpha*, *Amycolatopsis*, *Gordonia*, *Kribbella*, *Micromonospora*, *Nocardia*, *Nocardioides*, *Nocardiopsis*, *Nonomuraea*, *Polymorphospora*, *Promicromonospora*, *Pseudonocardia*, *Williamsia* and a novel genus *Flindersiella*. One of the strains represented a novel genus in the family Nocardioides and the other 11 strains were accepted as novel species. The literature from the limited number of studies with a limited number of trees has indicated the need for more research and the strong prospect for the culturing of diverse endophytic actinobacteria, including novel and rare genera residing in perennial trees.

The majority of agricultural crops, or other small medicinal, herbaceous weeds, are mostly seasonal, annual or biennial plants. In comparison, trees are perennial and growing for many years and exposed to varying soil conditions (with depth) and changing environmental conditions over many growth cycles. Both belowground and above-ground parts of perennial trees are exposed to continuous changes which occur with respect to climatic and environmental conditions. These spatio-temporal interactions may lead to the enrichment of many rare bacterial groups or more fastidious actinobacteria in their interior as endophytes.

5 Diversity of Endophytic Actinobacteria in Mangrove Ecosystems, Lichens and Mosses

5.1 Mangrove Ecosystems

Mangroves are the coastal wetland forests mainly found in the intertidal zone of estuaries, backwaters, deltas, creeks, lagoons, marshes and also mudflats of the tropical and subtropical latitudes. It is estimated that mangrove forests cover a total area of over one fourth of the world's coastline (Spalding et al. 1997; Alongi 2002). Mangroves are highly productive ecosystems, and little is known about the microbial communities living therein. Mangrove sediments contain populations of *Streptomyces*, *Micromonospora* (Eccleston et al. 2008) and other novel actinobacteria, as illustrated by the isolation of *Asanoa iriomotensis* (Han et al. 2007), *Nonomuraea maheshkhaliensis* (Ara et al. 2007) and *Micromonospora rifamycinica* (Huang et al. 2008). Hong et al. (2009) isolated over 2,000 bioactive actinobacteria from both rhizosphere soil and plant materials (including

endophytes) of 23 plant species collected from 8 mangrove sites in China. The highest number of bioactive strains was observed from the plant tissues of *Bruguiera*. Taxonomic diversity of these bioactive actinobacteria assigned most of them to the genera *Micromonospora* and *Streptomyces* and less to the other genera *Actinomadura, Nocardia, Nonomuraea, Rhodococcus* and *Verrucosispora*.

A study of 19 different mangrove plant species in Bhitarkanika, Orissa, India, revealed that three species of *Streptomyces*, namely, *S. halstedii*, *S. longisproflavus* and *S. albidoflavus*, were found to be associated with *Kandelia candel*. Similarly, *S. atroolivaceous* was found in phyllosphere of *Sonneratia apetala* and *S. caseolaris* of Dangmal and Khola region respectively. Two species *S. exfoliates* and *S. aurantiacus* were found to be associated with almost all mangrove plants studied (Gupta et al. 2009). An endophytic actinobacterial strain *Nocardiopsis* sp. A00203 isolated from the leaves of mangrove plant *Aegiceras corniculatum* collected from Jimei, Fujian Province, China, was shown to produce three biologically active 2-pyranone compounds (Lin et al. 2010). In another study, Mangamuri et al. (2012) isolated a rare actinobacterium closely related to *Pseudonocardia endophytica* from a mangrove ecosystem of Nizampatnam, India, which produced bioactive metabolites with broad-spectrum inhibitory effects on Gram-positive, Gram-negative bacteria and fungi. Baskaran et al. (2012) reported a higher proportion of actinobacterial endophytes in the mangrove plant *B. gymnorrhiza* of the Andaman Islands. However, the ecto- and endorhizosphere of plants in the mangrove ecosystems are still largely an unexplored source for screening and isolation of novel endophytic actinobacteria with rich potential to produce active secondary metabolites.

5.2 Lichens and Mosses

As pioneers of the colonisation of terrestrial habitats, lichens are found from the Arctic to tropical regions and are present on stones, in arid soils or as epiphytes on plants (Ahmadjian 1993). About 10 % of lichen-forming fungi are associated with nitrogen-fixing cyanobacteria (e.g. *Peltigerales* and *Lichinomycetes*); however, the remaining 90 % of lichen-forming fungi are not known for their intimate association with many other bacteria (Richardson and Cameron 2004; Liba et al. 2006). Studies have described the isolation of different species of the actinobacteria of the genera *Micromonospora* and *Streptomyces* from this environment (Hirsch et al. 2004). González et al. (2005) reported on the diversity in actinobacterial population from three regions: Within tropical lichens studied, *Micromonospora* strains were isolated with similar frequencies from different types of lichens, whereas arboricolous lichens from Hawaii were richer in *Streptomyces* than saxicolous samples. In addition, members tentatively assigned to the order Pseudonocardiaceae and the genera *Actinoplanes* and *Actinomadura* were isolated. Other genera isolated from lichens collected in Alaska belonged to *Rhodococcus* spp., from Hawaii belonged to *Saccharopolyspora* spp. and *Geodermatophilus* spp. and from Reunion Island belonged to *Planobispora* spp. and *Streptosporangium* sp. Two lichen-derived actinobacteria identified as new species of *Streptomyces* produced novel angucycline and butenolide compounds having cytotoxic activities against cancer cells and antibacterial activity. Two novel actinobacterial strains *Actinomycetospora iriomotensis* and *Actinomycetospora rishiriensis* were isolated from a lichen sample from Iriomote Island and Rishiri Island of Japan, respectively (Yamamura et al. 2011a, b). Recently, da Silva et al. (2011) isolated 71 isolates of actinobacteria associated with the foliose lichens from an Amazonian ecosystem in Brazil. The morphological characteristics and characterisation of cell wall amino acid of actinobacteria isolated from foliose lichens indicated that from the total of 71 actinobacteria, 91.5 % were *Streptomyces*, 4 % *Nocardia* and 1.5 % *Nocardiopsis* (1.5 %). Janso and Carter (2010) isolated 123 endophytic actinobacteria from tropical native plants including ferns and club mosses collected from several locations in Papua New Guinea and Mborokua Island,

Solomon Islands. 16S rRNA gene sequence analysis revealed that 17 different genera were represented and rare genera such as *Sphaerisporangium* and *Planotetraspora*, which have never been previously reported to be endophytic, were prevalent.

There are approximately 12,000 species of moss distinguished by their multicellular rhizoids (Theissen et al. 2001). Mosses are abundant on the forest floor in a broad range of boreal forest types (Bach et al. 2009). A high diversity and complexity in phyllosphere bacterial communities was recently described for the sphagnum moss (Opelt et al. 2007). Park et al. (2013) studied the endophytic bacterial diversity of an Antarctic moss *Sanionia uncinata* through pyrosequencing of amplified 16S rRNA genes and showed that *Proteobacteria* was the most dominant phylum with 65.6 %, followed by *Bacteroidetes* (29.1 %) and *Actinobacteria* (11.7 %).

6 Diversity of Endophytic Actinobacteria in Aquatic Ecosystem

Aquatic ecosystems contribute to a large proportion of the planet's biotic productivity, and aquatic plants are largely an unexplored environment for endophytic actinobacterial diversity and their biotic potential.

Freshwater ecosystems cover 0.80 % of the Earth's surface and inhabit 0.009 % of its total water. They generate nearly 3 % of its net primary production (Alexander and Fairbridge 1999). Three basic types of freshwater ecosystems are lentic (include pools, ponds and lakes), lotic (streams and rivers) and wetlands. In the littoral zone of lakes, where rooted plants occur, ponds are typically small lakes of shallow water with abundant marsh and aquatic plants. Food webs are based both on free-floating algae and upon aquatic plants (Sculthorpe 1985; Chapman and Reiss 1998). However, the diversity of the microbial community, in particular endophytes, associated with planktons and aquatic plants in the freshwater ecosystems is poorly understood.

Wetlands are the most productive natural freshwater ecosystems in the world because of the proximity/availability of water and fertile (nutrient rich) soil. Hence, they support large numbers of plant and animal species. Wetlands are dominated by vascular plants that have adapted to saturated soil (Keddy 2010). Among the wetlands, the rice ecosystem microbial communities have been extensively studied due to its importance both for food production and also for its anaerobic methanogenesis causing global climate change (Bernstein et al. 2007).

Marine ecosystems cover approximately 71 % of the Earth's surface and contain approximately 97 % of the planet's water and an exceptional biological diversity, accounting for more than 95 % of the whole biosphere (Qasim 1999). Recent studies have identified a diverse community of actinobacteria associated with marine sponges and soft corals (Lee et al. 1998; Dharmaraj et al. 2010; Webster et al. 2001; EI-Bondkly et al. 2012; Nithyanand et al. 2011). However, as they are not considered to be plants, they are not included in this chapter.

Most of the research on seagrass root-associated microbiology includes communities present on the outside and inside of the root material; hence, the findings are not specific for endophytes only. Similar to results from terrestrial plants, actinobacteria were found to be one of the most abundant groups of bacteria in the roots of seagrass, such as *Zostera marina* (Jensen et al. 2007). Lee et al. (2008b) isolated *Phycicolagilvus* from living seaweed collected along the coast of Jeju, Republic of Korea, which represented a novel species of a new genus within the family *Microbacteriaceae*. From the seaweeds of the Gulf of Mannar, Saravanakumar et al. (2010) isolated 12 strains of actinobacteria, of which 9 represented the genus *Streptomyces* and 3 belonged to the genus *Micromonospora*, which showed strong antagonism against bacterial fish pathogens *Vibrio harveyi, V. fisheri, Aeromonas hydrophila* and *A. sobria*. Recently, Wu et al. (2012) reported that most of the 110 actinobacterial isolates from the seagrass, *Thalassia hemprichii*, harboured polyketide synthetase (PKS) and nonribosomal peptide synthetase (NRPS) gene sequences indicating their bioactive potential. Most of them

belonged to ten genera of actinobacteria including *Streptomyces*, *Micromonospora*, *Saccharomonospora*, *Mycobacterium*, *Actinomycetospora*, *Nonomuraea*, *Verrucosispora*, *Nocardiopsis*, *Microbacterium and Glycomyces*.

As indicated before, the chemicals (e.g. NaCl and hypochlorite) used and the timing of treatment may vary depending upon the plant and organ type (Kaewkla and Franco 2013a), and a proper standardisation of sterilisation and isolation procedures appropriate for aquatic plants is essential for discovering the true diversity of their endophytes.

7 Methods for Diversity Analysis of Culturable Endophytic Actinobacteria

In the last 4 years alone, more than 50 new taxa have been identified from various terrestrial plants (Table 2.4). The identification of a pure actinobacterial culture is achieved with a polyphasic approach using techniques described in Fig. 2.2. However, not all of these techniques offer the discrimination required for the rapid characterisation of a large number of freshly isolated strains. In order to achieve this in an economical way, a combination of morphological, chemo-taxonomical and molecular fingerprinting methods are available for the characterisation and diversity analyses of actinobacteria (Embley and Stackebrandt 1994; Rademaker et al. 2000; Cook and Meyers 2003; Brusetti et al. 2008; Yuan et al. 2008).

Some of these methods can be employed to reduce the number of strains sent for sequencing and still be able to identify all the isolates. Culture morphology can be used to distinguish a number of genera such as *Micromonospora*, *Microbispora*, *Rhodococcus, Streptosporangium* and *Strepto-myces* spp., as well as a basis to form groupings of strains with similar morphological features. Representatives of each groups are subjected to molecular fingerprinting techniques such as RAPD (Mehling et al. 1995), AFLP, BOX or REP-PCR (Savelkoul et al. 1999; Rademaker et al. 2000) or the analysis of restriction patterns of PCR products of rRNA genes or ARDRA (Vaneechoutte et al. 1993) to identify strains that are similar to each other. Tian et al. (2007) used RFLP technique to characterise actinobacterial-specific 16S rRNA gene clone libraries constructed from the roots and stems of rice. RFLP analysis based on single digestion with restriction enzymes *Sma1* and *Pst1* grouped clones with similar patterns together. Clones from each RFLP group were chosen for further identification by 16S rRNA gene sequencing. Amplified rDNA (Ribosomal DNA) Restriction Analysis (ARDRA) was originally developed by Vanee-choutte et al. (1993) to characterise *Mycobac-terium* species.

ARDRA has been used successfully in identifying several species of endophytic actinobacteria belonging to the genera *Actinomadura*, *Gordonia*, *Nocardia*, *Rhodococcus*, *Saccharomonospora*, *Sa ccharopolyspora*, *Streptomyces* and *Tsukamurella* (Steingrube et al. 1997; Wilson et al. 1998; Laurent et al. 1999; Harvey et al. 2001). Cook and Meyers (2003) identified four restriction endonucleases, *Sau*3AI, *Asn*I, *Kpn*I and *Sph*I, that significantly differentiated the genus *Streptomyces* from all other actinobacteria genera by using ARDRA. ARDRA can be useful in reducing ambiguity in isolate similarities based on morphological characterisations. Kaewkla and Franco (2013a) used ARDRA of partial 16S rRNA genes to distinguish both non-streptomycete- and streptomycete-like isolates obtained from Australian native trees. In this study, initial ARDRA with *Hha1*digestion yielded 13 ARDRA patterns for the total 579 isolates. However, second ARDRA patterns based on a second digestion with the enzymes *Rsa1* and *Pst1* more effectively differentiated the genera within the ARDRA patterns based on single enzyme digestion, indicating the necessity to use more than one restriction enzyme and judicious selection of isolates for identification by 16S rRNA gene sequencing.

Nimnoi et al. (2010a) employed random amplification of polymorphic DNA (RAPD) to determine the genetic relatedness up to the genus level for the endophytic actinobacterial isolates obtained from healthy shoots and roots of *Aquilaria crassna.* Though RAPD is a simple,

Table 2.4 New genera and species isolated as endophytic actinobacteria (from 2010 to till date)

Endophytic actinobacterial species	Name of the host plant	Plant types	Plant part	References
Actinoallomurus acacia	*Acacia auriculiformis*	Wattle tree	Leaves	Thamchaipenet et al. (2010)
Actinoallomurus oryzae	*Oryza sativa*	Rice	Roots	Indananda et al. (2011)
Actinomycetospora iriomotensis	–	Lichens	–	Yamamura et al. (2011a)
Actinomycetospora rishiriensis	–	Lichens	–	Yamamura et al. (2011b)
Actinophytocola oryzae	*Oryza sativa*	Rice	Roots	Indananda et al. (2010)
Actinoplanes rishiriensis	–	Lichens	–	Yamamura et al. (2012)
Actinopolymorpha pittospori	*Pittosporum phylliraedoies*	Australian apricot tree	Leaves	Kaewkla and Franco (2011b)
Allonocardiopsis opalescens	*Lonicera maackii*	Medicinal plant	Fruit	Du et al. (2013a)
Amycolatopsis endophytica	*Jatropha curcas*	Oil-seed	Seeds	Miao et al. (2011)
Amycolatopsis jiangsuensis	*Dendranthema indicum*	Coastal salt marsh plant	–	Xing et al. (2013)
Amycolatopsis samaneae	*Samanea saman*	Medicinal plant	Roots	Duangmal et al. (2011)
Brachybacterium saurashtrense	*Salicornia brachiata*	Extreme halophyte	Roots	Gontia et al. (2011)
Dietzia maris	*Viola mandshurica*	Manchurian violet	Roots	Kim et al. (2012)
Flindersiella endophytica	*Eucalyptus microcarpa*	Grey Box eucalyptus tree	Roots	Kaewkla and Franco (2011a)
Herbiconiux ginsengi	*Artemisia princeps var. orientalis*	Mugwort	Roots	Kim et al. (2012)
Jatrophihabitans endophyticus	*Jatropha curcas*	Oil-seed	Stem	Madhaiyan et al. (2013)
Jishengella endophytica	*Acanthus illicifolius*	Holy mangrove	Roots	Xie et al. (2010)
Kibdelosporangium phytohabitans	*Jatropha curcas*	Oil-seed	Roots	Xing et al. (2012a)
Kineococcus endophytica	*Limonium sinense*	Coastal halophyte	Roots	Bian et al. (2012b)
Kitasatospora viridis	*Lamium purpureum*	Purple henbit	Roots	Kim et al. (2012)
Kribbella endophytica	*Pittosporum phylliraedoies*	Australian apricot tree	Leaves	Kaewkla and Franco (2013b)
Micromonospora pisi	*Pisum sativum*	Pea	Root nodules	Garcia et al. (2010)
Micromonospora tulbaghiae	*Tulbaghia violacea*	Wild garlic	Leaves	Kirby and Meyers (2010)
Modestobacter roseus	*Salicornia europea*	Coastal halophyte	Roots	Qin et al. (2013a)
Nocardia callitridis	*Callitris preissii*	Pine tree	Roots	Kaewkla and Franco (2010c)
Nocardia endophytica	*Jatropha curcas*	Oil-seed	Roots	Xing et al. (2011)
Nocardioides caricicola	*Carex scabrifolia*	Halophyte	Roots	Song et al. (2011)
Nocardioides panzhihuaensis	*Jatropha curcas*	Oil-seed	Stem	Qin et al. (2012a)
Nocardioides perillae	*Perilla frutescens*	Medicinal plant	Roots	Du et al. (2013b)
Nonomuraea endophytica	*Artemisia annua*	Medicinal plant	Roots	Li et al. (2011b)
Phytohabitans flavus	–	Orchids	Roots	Inahashi et al. (2012)
Phytohabitan shouttuyneae	*Houttuynia cordata*	Orchids	Roots	Inahashi et al. (2012)
Phytohabitans rumicis	*Rumex acetosa*	Orchids	Roots	Inahashi et al. (2012)
Phytohabitans suffuscus	–	Orchids	Roots	Inahashi et al. (2010)
Phytomonospora endophytica	*Artemisia annua*	Medicinal plant	Roots	Li et al. (2011a)

(continued)

Table 2.4 (continued)

Endophytic actinobacterial species	Name of the host plant	Plant types	Plant part	References
Plantactinospora endophytica	*Camptotheca acuminata*	Happy tree	Leaves	Zhu et al. (2012)
Promicromonospora endophytica	*Eucalyptus microcarpa*	Grey Box eucalyptus tree	Roots	Kaewkla and Franco (2012)
Promicromonospora xylanilytica	*Maytenus austroyunnanensis*	Medicinal plant	Leaves	Qin et al. (2012b)
Pseudonocardia adelaidensis	*Eucalyptus microcarpa*	Grey Box eucalyptus tree	Stem	Kaewkla and Franco (2010a)
Pseudonocardia artemisiae	*Artemisia annua*	Medicinal plant	Roots	Zhao et al. (2011a)
Pseudonocardia bannensis	*Artemisia annua*	Medicinal plant	Roots	Zhao et al. (2011b)
Pseudonocardia eucalypti	*Eucalyptus camaldulensis*	Red gum tree	Roots	Kaewkla and Franco (2010b)
Pseudonocardia kunmingensis	*Artemisia annua*	Medicinal plant	Roots	Zhao et al. (2011d)
Pseudonocardia nantongensis	*Tamarix chinensis*	Coastal halophyte	Leaves	Xing et al. (2012b)
Pseudonocardia serianimatus	*Artemisia annua*	Medicinal plant	leaves	Zhao et al. (2011c)
Pseudonocardia sichuanensis	*Jatropha curcas*	Oil-seed	Roots	Qin et al. (2011)
Pseudonocardia tropica	*Maytenus austroyunnanensis*	Medicinal plant	Stem	Qin et al. (2010b)
Pseudonocardia xishanensis	*Artemisia annua*	Medicinal plant	Roots	Zhao et al. (2012a)
Rathayibacter festucae	*Conyza canadensis*	Horseweed	Roots	Kim et al. (2012)
Rhodococcus artemisiae	*Artemisia annua*	Medicinal plant	Roots	Zhao et al. (2012b)
Saccharopolyspora dendranthemae	*Dendranthema indicum*	Coastal salt marsh plant	–	Zhang et al. (2013)
Saccharopolyspora gloriosae	*Gloriosa superba*	Medicinal plant	Stem	Qin et al. (2010a)
Saccharothrix yanglingensis	*Cucumis sativus*	Cucumber	Roots	Yan et al. (2012)
Streptacidiphilus anmyonensis	*Chelidonium majus* var. *asiaticum*	Greater celandine	Roots	Kim et al. (2012)
Streptomyces artemisiae	*Artemisia annua*	Medicinal plant	Roots	Zhao et al. (2010a)
Streptomyces endophyticus	*Artemisia annua*	Medicinal plant	Roots	Li et al. (2013)
Streptomyces halophytocola	*Tamarix chinensis*	Coastal halophyte	Stem	Qin et al. (2013b)
Streptomyces phytohabitans	*Curcuma phaeocaulis*	Medicinal plant	Roots	Bian et al. (2012a)
Streptosporangium oxazolinicum	–	Orchids	Roots	Inahashi et al. (2011)
Tsukamurella suncheonensis	*Iris rossii* var. *rossii*	Caudate-bracted iris	Roots	Kim et al. (2012)

inexpensive and useful typing method for genetic studies of bacteria, it has low resolving power, limited applicability in species-specific comparisons and variable experimental reproducibility.

BOX-PCR is a version of the rep-PCR techniques that uses the BOX-A1R primer targeting the BOX dispersed-repeat motif, common in a number of actinobacterial groups (Van Belkum et al. 1998). The BOX-PCR genomic fingerprints generated from culturable isolates of endophytic actinobacteria permit identification, classification and differentiation to the species, subspecies and strain level. Yuan et al. (2008) characterised the endophytic actinobacteria isolated from medicinal plants through BOX-PCR fingerprinting and revealed more genetic diversity among the closely related strains belonging to the two genera, *Streptomyces* and *Micromonospora*. Endophytic actinobacterial isolates obtained from *Lupinus angustifolia*

Approach	Sample Required	Taxonomic resolution				
Culturable		Family	Genus	Species	Sub-sp.	Strain
	Genomic DNA			<------------------------RAPD------------------------>		
	" "			<-------------------AFLP/RFLP----------------->		
	" "			<--------------Rep- and BOX-PCR------------>		
	Proteins			<---------------Isozyme analysis-------------->		
	Whole cell Proteins			<----Transcriptome / protein profiling---->		
	Genomic DNA		<---------------DNA-DNA Hybridization--------------->			
	" "		<--------------------ARDRA---------------------->			
	" "		<---16S rRNA/ tRNA PCR- Sequencing---->			
	" "			<---16S-23S rRNA-ITS/ tRNA-ITS PCR--->		
	" "		<-------------*cyt C1, ctp syn, glu-tRNA syn, PKS-I, PKS-II, NPRS* genes sequencing (specific to actinobacteria)- ------------>			
	Whole cell lipids		<------FAME/ other chemical analysis ---->			
	Whole genome	<-Whole genome sequencing and Multi-Locus Sequences Analysis ->				
Unculturable	Microbial community DNA		<------------16S rRNA PCR- DGGE------------>			
	" "		<-16S rRNA/Functional genes PCR- TRFLP->			
	" "		<-16S rRNA clone libraries sequencing-->			
			<-------Direct shot-gun/ Pyro-sequencing (16S rRNA/ Functional genes)-------------->			

Fig. 2.2 Relative applicability of different molecular biological techniques used in the taxonomic identification and diversity analysis of endophytic actinobacteria (Modified from the Rademaker and De Bruijn 1997)

were analysed using BOX-PCR fingerprinting technique, and results revealed on unexpectedly high genetic diversity among the strains belonging to the genus *Micromonospora* (Trujillo et al. 2010). BOX-PCR patterns are not affected by the culture age of the strain to be analysed and have a similar or even better strain differentiation power than other molecular techniques (Kang and Dunne 2003). BOX-PCR is easier to perform and fingerprinting outputs can be easily analysed by computer-assisted methods. Recently, Brusetti et al. (2008) developed a fluorescent BOX-PCR, in which the amplified fluorescent-labelled products can be separated in an automated DNA sequencer which helps overcome limitations from poor band resolution on agarose gel electrophoresis.

Chemotaxonomical methods are more labour intensive, but the identification of the LL- or *meso*-form of the cell wall compound 2,6-diaminopimelic acid (DAP) can be effective in discriminating between *Streptomyces* and non-*Streptomyces* strains. The amino acid and sugar composition of cell walls provide information suitable for the

classification of pure isolates of actinobacteria but are not diagnostic.

The fatty acid composition is another unique chemotaxonomic marker used for the identification and diversity characterisation of major genera of actinobacteria (Vestal and White 1989; Embley and Wait 1994). However, it is labour intensive and better suited to discriminating between species within a genus, although it can also be used to identify specific genera that are present in the Sherlock Microbial ID System (www.midi-inc.com), or when a small number of genera are present (González et al. 2005).

7.1 New Molecular Approaches for Strain Characterisation

In the last two decades, the whole genome sequence of number of bacteria has been decoded, and attempts are underway to test whether the data from whole genome comparison can be used for diversity characterisation and taxonomy of culturable bacteria. For example, pairwise comparison of complete whole genome sequences showed that the 'average nucleotide identity' (ANI) of all conserved genes between any two genomes correlated well with 16S rRNA sequence identity and DNA-DNA similarity values. It has also been shown that 70 % DNA-DNA similarity corresponds to 95 % ANI (Konstantinidis and Tiedje 2005). Moreover, all pairs of genomes showing 95 %, or higher, ANI also showed at least 98.5 % 16S rRNA gene identity (Goris et al. 2007). This approach of comparative genomics information has also been generated from the available whole genome sequences of well-known actinobacterial taxa including some of the endophytic actinobacterial genera like *Frankia, Leifsonia, Streptomyces* and *Nocardia* (Ventura et al. 2007).

Multilocus sequence analysis (MLSA), a phylogenetic characterisation based on sequence comparison of multiple housekeeping genes in bacterial genome, has been proposed as a replacement for DDH technique in the classification of prokaryotes (Gevers et al. 2006). In the recent *Bergey's Manual of Systematic Bacteriology*, the MLSA has been used in redefining phylogeny of actinobacterial genera like *Mycobacterium* and *Bifidobacterium* (Ventura et al. 2007). The concatenation of four gene fragments encompassing the 16S rRNA gene, *hsp65*, *rpoB* and *sod* has been used to create a supertree of the *Mycobacterium* genus, and species such as *Mycobacterium fortuitum* and *M. avium* are well separated by a super tree approach than using a single gene-based tree, i.e. 16S rRNA gene-based tree (Devulder et al. 2005). In the super tree of the genus *Bifidobacterium*, concatenation of seven conserved genes, i.e. *clpC*, *dnaB*, *dna*G, *dnaJ1*, *purF*, *rpoC* and *xfp*, has been used to infer its phylogeny (Ventura et al. 2006). Several recent MLSA studies showed that in addition to 16S rRNA gene, the concatenation of four genes such as *gyrB*, *rpoB*, *recA* and *atpD* genes has found useful in phylogeny of other actinobacterial genera like *Micromonospora* and *Steptomyces* (Rong et al. 2009; Rong and Huang 2010; Carro et al. 2012). More recently, Curtis and Meyers (2012) included the *relA* gene for the first time in MLSA of actinobacteria and generated the concatenated sequence super tree to examine the phylogenetic relationships of 17 type strains within the genus *Kribbella*, one of the known endophytic actinobacterial genus.

8 Culture-Independent Approaches for Diversity Analysis

Studies of diversity and functions of plant-associated microbes, especially prokaryotes, are impeded by difficulties in cultivating most of them, and endophytes inside host tissues are not easily amenable to biochemical or genetic analyses. Recent advances in methods for endophytic bacterial enrichment and direct applications of 16S rRNA gene-based culture-independent molecular techniques are helping to unravel the complex endophytic actinobacterial community (Table 2.5). Some of these methods include polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment

Table 2.5 Endophytic actinobacteria from different plants identified using culture-independent methods

Plant species/ habitats	Method and source of microbial community DNA	Molecular techniques used	List of endophytic actinobacterial genera identified	References
Eaglewood tree (*Aquilaria crassna*)	Extraction of total DNA of root materials	PCR-DGGE	*Actinomadura*, *Nocardia*, *Nonomuraea*, *Pseudonocardia* and *Streptomyces*	Nimnoi et al. (2010b)
Grape vine (*Vitis vinifera*)	Endophyte enrichment from both leaves and roots and DNA extraction	PCR-DGGE	*Curobacterium* and *Streptomyces*	West et al. (2010)
Grape vine (*Vitis vinifera*)	Endophytes enrichment from whole plant and DNA extraction	16S rRNA gene clone libraries	*Curtobacterium*	Bulgari et al. (2009)
Medicinal tree (*Maytenus austroyunnanensis*)	Endophytes enrichment from root, stem and leaves and DNA extraction	16S rRNA gene clone libraries	*Actinokineospora*, *Marmoricola*, *Modestobacter*, *Pseudokineococcus*, *Pseudosporangium*, *Sanguibacter* and *Serinibacter*	Qin et al. (2012a, b, c)
Potato (*Solanum tuberosum*)	Bead beating of tubers and DNA extraction	PCR-DGGE (actinobacterial specific)	Mainly *Streptomyces*	Sessitsch et al. (2002)
Rice (*Oryza sativa*)	Extraction of total DNA of root and stem materials	16S rRNA gene clone libraries	*Actinoplanes*, *Amycolatopsis*, *Corynebacterium*, *Dactylosporangium*, *Frankia*, *Micromonospora*, *Mycobacterium*, *Nocardioides*, *Rhodococcus*, *Streptomyces* and other uncultured actinobacteria	Tian et al. (2007)
Soybean (*Glycine max*)	Enrichment through homogenisation roots, root nodules, stem and leaves, filtration and DNA extraction	16S rRNA gene clone libraries	Wide range of actinobacteria genera belonging to three suborders, namely, Frankineae, Propionibacterineae and Micrococcineae	Ikeda et al. (2009, 2010)
Wheat (*Triticum aestivum*)	Homogenisation of root samples with mini-bead beater and DNA extraction	PCR-TRFLP	*Arthrobacter*, *Kitasatospora*, *Micromonospora*, *Microbispora*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Streptomyces* and *Tsukamurella*	Conn and Franco (2004a)

length polymorphism (T-RFLP) analysis, construction and sequencing of 16S rRNA gene clone libraries and next-generation sequencing/ pyrosequencing. A combination of culturable and culture-independent approaches may be needed for in-depth understanding of the diversity and functional relevance of endophytic actinobacteria (Fig. 2.2).

8.1 Methods for Enrichment of Endophytes and Community DNA Isolation from Plants

Endophytic bacteria reside inside the plant tissues mainly in intercellular spaces, rarely in intracellular spaces and interior of vascular tissues (Thomas and Graham 1952). They are tightly attached to host cells and are difficult to extract and separate from plant tissues and prone to contamination from surface-associated bacteria. Mechanical removal of rhizoplane populations by vigorous shaking with glass beads can help overcome the contamination from surface bacteria (Reinhold et al. 1986). Initial studies on the unculturable endophytic diversity were carried out with the extraction of total DNA using general CTAB procedure with certain modifications (Xie et al. 1999; Sessitsch et al. 2002) and subsequent PCR amplification of 16S rRNA genes using prokaryotic universal primers (Dent et al. 2004; Sun et al. 2008). Since DNA obtained using such methods includes material from the plant nuclei, the plastids, the mitochondria and the plant-associated microbes, it is essential to design highly specific primers for endophytic bacteria alone. The high sequence homology between plant chloroplast 16S rRNA gene, mitochondrial 18S rRNA gene and bacterial 16S rRNA can cause interference with specific analysis of endophytic bacteria (Sun et al. 2008). Therefore, enrichment of endophytic bacteria prior to PCR amplification has been suggested to overcome the above-described problems and improve the sensitivity of analysis.

Jiao et al. (2006) enriched bacterial cells from plant tissues by enzymatic hydrolysis of the plant cell wall, followed by differential centrifugation. Subsequently, a variety of mild and specific enzymatic treatments have been successfully used to remove intact bacterial cells from the medicinal plant *Mallotus nudiflorus* (Wang et al. 2008) and grapevine leaf tissues (Bulgari et al. 2009). This method of endophyte enrichment has also helped in the culturing of rare/novel endophytic actinobacteria (Qin et al. 2009; Ikeda et al. 2009). Another technique suitable for enriching bacterial cells from fresh plant tissues was developed by using a bacterial cell extraction buffer containing Triton X-100 for tissue homogenisation with subsequent Nycodenz density gradient centrifugation. Here, the enrichment is based on the speculation that less green colour of the supernatant and interface is an indication of less contamination of plastids in the bacterial fraction obtained from homogenised plant samples (Ikeda et al. 2009). This enrichment technique has been successfully applied to clarify the diversity of endophytic actinobacterial communities in stems and leaves of soybean and rice (Ikeda et al. 2009, 2010). Recently, Nikolic et al. (2011) cut sterilised potato plant material into small pieces and then the endophytic bacteria were dislodged by overnight shaking at room temperature in 0.9 % NaCl. Bacteria were separated from the plant material by filtration and collected by centrifugation. The enrichment procedure allows the extraction of bacterial cells from large amounts of plant material thereby reducing variation associated with specific plant parts and collects rare members of the endophytic community. As a result next-generation sequencing operations which require large amounts of high-quality DNA can be conducted, e.g. for metagenomic analysis (Sessitsch et al. 2012).

8.2 Next-Generation Sequencing and Pyrosequencing

Recent developments in high-throughput sequencing (or next-generation sequencing) technologies enable rapid sequencing analysis of whole genomes and environmental DNA samples (Mardis 2008; Shendure and Ji 2008; Miller et al. 2009; Lauber et al. 2010; Robinson et al. 2010). Some of these methods include massively parallel signature sequencing or MPSS (Lynx Therapeutics), Polony sequencing (Agencourt Biosciences), 454 pyrosequencing (Life Sciences), Illumina (Solexa) sequencing (Illumina), SOLiD sequencing (Applied Biosystems), ion semiconductor sequencing (Ion Torrent Systems Inc.), DNA nanoball

sequencing and HeliScope single molecule sequencing.

In 2010, pyrosequencing was used for the first time to examine the bacterial endophyte community in the roots of 12 different potato cultivars revealing an unprecedented level of diversity among the bacterial root endophytes. Interestingly, the presence of five of the ten most common eubacterial genera (*Rheinheimera*, *Dyadobacter*, *Devosia*, *Pedobacter* and *Pseudoxanthomonas*) revealed by pyrosequencing has not been previously reported as potato root endophytes (Manter et al. 2010). Analysis of endophytic bacterial diversity of an Antarctic moss, *Sanionia uncinata,* using 16S rRNA pyrosequencing technology, indicated that *Proteobacteria* was the most dominant phylum with 65.6 %, followed by *Bacteroidetes* (29.1 %) and *Actinobacteria* (11.7 %) (Park et al. 2013). Actinobacteria were found to be in higher abundance in the endophytic compartment (EC) of the *A. thaliana* rhizosphere microbiome, followed by *Proteobacteria, Firmicutes* and other minor bacterial taxa (Bulgarelli et al. 2012; Lundberg et al. 2012). Lower-order taxonomic analysis demonstrated that enrichment of a low-diversity actinobacteria community in the EC was driven by a subset of families, predominantly *Streptomy-cetaceae*, and the selective enrichment of actinobacteria in the roots community was suggested to depend on the colonisation cues from metabolically active host cells as well (Bulgarelli et al. 2012; Lundberg et al. 2012). These research advances in molecular biological techniques greatly improve our understanding of the complexity and ecological distributions of plant-associated actinobacteria. In spite of these advances, the true functional diversity and capabilities of actinobacteria in different endophytic habitats of various ecosystems remain to be fully discovered.

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