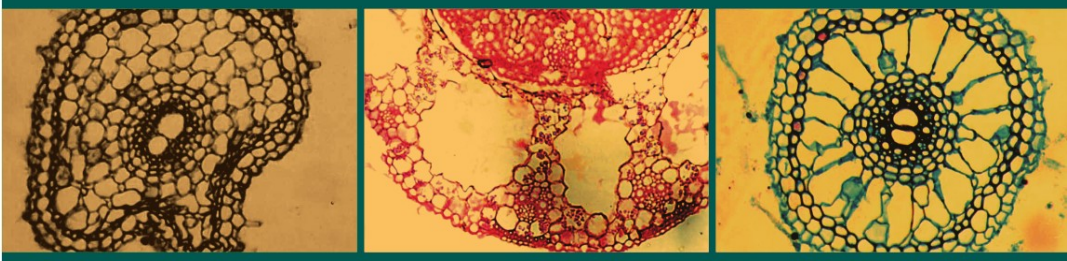
Dinesh K. Maheshwari *Editor*

Bacteria in Agrobiology:



Plant Growth Responses

Springer

Bacteria in Agrobiology: Plant Growth Responses

Dinesh K. Maheshwari
Editor

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Cover illustration: Optical micrograph showing cross sections of intercellular colonization rice calli and regenerated plantlets by A. caulinodans: CS view of root uninoculated control; magnified cross section view of leaf colonized by A. caulinodans in regenerated rice plant; possible sites of infection and colonization of rice root (from left to right); see also Fig. 3.1 in "Endophytic Bacteria – Perspectives and Applications in Agricultural Crop Production", Senthilkumar M, R. Anandham, M. Madhaiyan, V. Venkateswaran, Tong Min Sa, in "Bacteria in Agrobiology: Crop Ecosystems, Dinesh K. Maheshwari (Ed.)"

Background: Positive immunofluorescence micrograph showing reaction between cells of the rhizobial biofertilizer strain E11 and specific anti-E11 antiserum prepared for autecological biogeography studies; see also Fig. 10.6 in "Beneficial Endophytic Rhizobia as Biofertilizer Inoculants for Rice and the Spatial Ecology of this Bacteria-Plant Association", Youssef Garas Yanni, Frank B. Dazzo, Mohamed I. Zidan. in "Bacteria in Agrobiology: Crop Ecosystems, Dinesh K. Maheshwari (Ed.)"

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Preface

The microbial world is the largest unexplored reservoir of biodiversity and acts as a major source for agriculture, industrial, and medicinal applications. Bacteria are the most dominant group of this diversity, which exist in different ecological niches. The growing interest in microorganisms promoting plant growth provides a fascinating biological system for the enhancement of their growth and development. Efforts have been made in this scenario for the selection of organisms and their selective genera suitable for sustainable plant growth.

With limited arable land coupled with rising demand of a steadily increasing human population, food supply is a global challenge and, therefore, production of high-quality food void of unacceptable chemicals is a pressing need. Knowledge of different mechanisms involved in support of plant growth promotion and disease suppression is essential from both societal and scientific point of view.

This volume comprises 15 chapters that cover application of symbiotic, free-living, rhizospheric, endophytic, methylotrophic, diazotrophic, and filamentous bacteria in plant growth promotion and protection and other related genera associated with orchids and coniferous tree. The effects of ecological consequences of the plasmid plasticity influence the adaptation of azospirilla to the complex plant–soil ecosystem, which have proved to be a great success in improving plant growth. A due account is provided with respect to *Lysobacter* spp., which uniquely is displayed in traits distinguishing them from other similar microbes in the practical field of agriculture. Complex knowledge relating to biology and biotechnology of methylotrophy has remained under infancy. A brief picture on the diversity of methylotrophs and their interactions that imply at both plant and ecosystem level is also provided, and the selection of suitable microbial partner to host orchids proved beneficial for the establishment of a stable community.

Substantial information is provided on the knowledge of different groups of bacteria and other related microbes such as *Actinobacteria*, *Actinomycetes*, and insect gut bacteria, which also have an added advantage due to their unexplored niche.

Spectacular progress has been made in phytobacteriology particularly in the area of agrobiology. The subject has evidently grown enormously, but the role of different group of bacteria and related organisms on plant growth responses also needs to be studied.

This book will benefit the students, teachers, and researchers and those interested to strengthen their learning of the subject of Agriculture, Plant physiology, Plant protection, Agronomy, Microbiology, Biotechnology, and Environmental science.

I am grateful to all the leading experts who have contributed their chapters in the preparation of the aforesaid volume. This pragmatic approach for investment in research from seed to soil and then raising plants will boost sustainability.

Appreciations are due to my research students, Abhinav, Rajat, Pankaj, Narendra, and Dr. Sandeep for their sincere efforts. I am also thankful to C.S.I. R., U.G.C., and UCOST for their financial support in the execution of the research projects on the subject that emerged as a prelude in compilation of this volume. I owe my special thanks to Dr. Jutta Lindenborn, Springer, for the excellent support in bringing out the volume in its current shape.

Haridwar, Uttarakhand, India Dinesh K. Maheshwari

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Chapter 1
Genetic and Phenotypic Diversity of Plant Growth Promoting Bacilli

Anelise Beneduzi and Luciane M.P. Passaglia

1.1 Introduction

Aerobic endospore-forming bacteria (AEFB) are essentially ubiquitous in agricultural systems. Common physiological traits important to their survival include the production of a multilayered cell wall structure, formation of stress-resistant endospores, and secretion of peptide antibiotics, peptide signal molecules, and extracellular enzymes. However, significant variation exists in other key traits, including nutrient utilization, motility, and physiochemical growth optima. Their microscopic size and omnipresence in soils facilitates their colonization of plants and animals, but the degree of niche localization of most species has not been thoroughly studied. Multiple *Bacillus* and *Paenibacillus* spp. can be readily cultured from both bulk and rhizosphere soils. Indeed, the ecological significance of the genotypic and phenotypic diversity of named species of *Bacillus* and related genera remains largely a mystery (Gardener 2004).

Analysis of the DNA extracted directly from soil samples, especially those that use the sequencing of the 16S ribosomal RNA genes (16S rRNA), has confirmed the occurrence of easily cultivable bacterium species, as well as a wide variety of noncultivable strains of species that belong to the genera *Bacillus* and *Paenibacillus* (Borneman et al. 1996; Felske et al. 1999; Garbeva et al. 2003; Smalla et al. 2001). Nevertheless, evidence of the relative number of cultivable and noncultivable representatives of these genera in different soils is surrounded by controversy. Some studies have suggested that most 16S rRNA sequences of bacilli isolated directly from soil samples are very similar to the sequences of cultivable and named species (Garbeva et al. 2003; Smalla et al. 2001). Other authors report that the

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predominant sequences found in different soils are not the same as those presented by bacilli isolated and easily cultivable (Borneman et al. 1996; Felske et al. 1999).

In turn, niche specificity and important ecological activities in *Bacillus* and *Paenibacillus* spp. appear to span phylogenetic boundaries. Most species can survive as saprophytes in soils, which are considered the primary reservoirs of these bacteria; however, most viable cells probably occur as inactive spores at any given time (Nicholson 2002). Furthermore, multiple species can be recovered as epiphytes and endophytes of plants and animals, as well as foodstuffs and composts derived from them (Priest 1993; Stahly et al. 1992; Slepecky and Hemphill 1992). The rich variety of organic substrates and microniches present in those environments supports a complex milieu of microbial species, so it is perhaps not surprising that multiple species of *Bacillus* and *Paenibacillus* inhabit them (Gardener 2004).

Numerous *Bacillus* and *Paenibacillus* species may contribute to the health status of plants in several ways. A considerable number of isolates belonging to these genera have been used as agents of biological control against phytopathogens (Lacey et al. 2001; Siddiqui and Mahmood 1999). Yet, the successful application of these agents requires comprehensive knowledge of their ecology. Additionally, the safety and efficacy of inoculants are largely determined by the ecological success of strains in the environments they are to be introduced. Better knowledge of the diversity, distribution, and action of bacteria of the *Bacillus* and *Paenibacillus* genera is extremely important to the identification of new strains, to the formulation of inoculants, and to the determination of the kinds of plantation they may be employed in Gardener (2004).

Members of the genus *Paenibacillus* are facultatively anaerobic organisms that produce spores in definitely swollen sporangia and have G + C contents ranging from 45 to 54 mol%. Some of these organisms excrete diverse assortments of extracellular polysaccharide-hydrolyzing enzymes to hydrolyze complex carbohydrates, including alginate, chondroitin, chitin, curdlan, and other polysaccharides (Shida et al. 1997). Also, a number of species under these genera are known to produce polysaccharides (Yoon et al. 2002), antifungal, and antimicrobial agents, such as polymyxin, octopityn, and baciphelacin (Chung et al. 2000).

Paenibacillus species have been isolated from a wide range of sources, including soil, water, plant rhizosphere, tree roots, plant material, foods, forage, feces, and insect larvae (Daane et al. 2002). Bacterial isolates from this genus have also been detected in Lake Vostok, Antarctica (Christner et al. 2001), in the ice caps of mountains, China (Christner et al. 2000), in paintings that have undergone biological deterioration (Heyrman and Swings 2001), in seawaters (Siefert et al. 2000), in mercury-reducing biofilms (Wagner-Döbler et al. 2000), and in estuarial sediments contaminated with crude oil (Daane et al. 2002). The genus was also observed to be abundant in companies that produce paper used in the food industry (Raaska et al. 2002).

Several species described as nitrogen-fixing bacilli belong to the *Paenibacillus* genus, as for example *P. polymyxa* (Grau and Wilson 1962), *P. macerans*, *P. durus* (*P. azotofixans*), *P. peoriae* (Montefusco et al. 1993), *P. borealis* (Elo et al. 2001),

P. graminis and *P. odorifer* (Berge et al. 2002), *P. brasilensis* (von der Weid et al. 2002), *P. massiliensis* (Roux and Raoult 2004), *P. wynnii* (Rodríguez-Diaz et al. 2005), *P. sabinae* (Ma et al. 2007a), *P. zanthoxyli* (Ma et al. 2007b), *P. donghaensis* (Choi et al. 2008), *P. forsythiae* (Ma and Chen 2008), *P. sonchi* (Hong et al. 2009), and *P. riograndensis* (Beneduzi et al. 2010).

Concerning the bacteria belonging to the *Bacillus* genus, in 1998, Xie et al. detected the activity of the nitrogenase enzyme in *B. megaterium*, *B. cereus*, *B. pumilus*, *B. circulans*, *B. licheniformis*, *B. subtilis*, *B. brevis*, and *B. firmus*. These same authors reported, in 2003, the isolation of 14 *Bacillus* strains that were able to reduce acetylene in paddy fields of eight locations on the banks of river Yangtze, China. Li et al. (1992) had also identified a *Bacillus* species that fixed nitrogen in association with ectomycorrhizas, while Ahmad et al. (2008) have detected nitrogen-fixing *Bacillus* isolates in different rhizosphere soils in Aligarh, India. *Bacillus fusiformis* (strains PM-5 and PM-24) has also been identified as a nitrogen-fixing bacterium using the acetylene reduction test. This species was shown to exhibit an intense nitrogenase activity in different plant cultures in the province of Chungbuk, South Korea. Diazotrophic *Bacilllus* were even found in the rhizosphere of pine and oak trees (Rózycki et al. 1999). Ding et al. (2005) detected biological nitrogen activity in *B. marisflavi* and *Paenibacillus massiliensis* and also identified fragments of the *nifH* gene in *B. megaterium* and *B. cereus*. *B. alkalidiazotrophicus* (Sorokin et al. 2008a), a low salt-tolerant alkaliphile isolated from Mongolian soda soil, was also described as a nitrogen-fixing bacterium. Therefore, it was demonstrated that both genera, *Bacillus* and *Paenibacillus*, presented species that are able to fix nitrogen. It is interesting to note that *Natronobacillus*, a new bacilli genus, was created specifically for *N. azotifigens*, its only species, which is an anaerobic diazotrophic haloalkaliphile bacterium that was isolated from soda-rich habitats (Sorokin et al. 2008b).

In recent years, the interest in soil microorganisms has increased as they play an important role in the maintenance of soil fertility. A major challenge for the development of sustainable agriculture lies in the use of nitrogen-fixing bacteria, which are able to assimilate gaseous N_2 from the atmosphere (Seldin et al. 1998).

1.2 Plant Growth Promoting Rhizobacteria

Rhizosphere can be defined as any volume of soil specifically influenced by plant roots and/or in association with roots and hairs, and plant-produced material. This space includes the soil surrounding plant roots, often extending a few millimeters from the root surface and can include as well the plant root epidermal layer. Plant exudates in the rhizosphere, such as amino acids and sugars, provide a rich source of energy and nutrients for bacteria, resulting in higher bacterial populations in this area (Gray and Smith 2005). In fact, the concentration of bacteria (per gram of soil) found around plant roots (i.e., in the rhizosphere) is generally much bigger than the bacterial density, or concentration, detected in the rest of the soil (Lynch 1990).

Despite the large number of bacteria in the rhizosphere, only 7–15% of the total root surface is generally occupied by microbial cells (Gray and Smith 2005).

The interaction between bacteria and plant roots may be beneficial, harmful, or neutral for the plant, and sometimes the effect of a particular bacterium may vary as a consequence of soil conditions (Lynch 1990). Thus, for example, a particular organism that facilitates plant growth by fixing nitrogen, which is usually present in the soil in limited amounts, is unlikely to provide benefit to a plant in a place where exogenous fixed nitrogen is added to the soil (Glick 1995).

Bacteria that provide some benefit to plants are of two general types: those that establish a symbiotic relationship with the plant and those that are free-living in the soil, but are often found near on or even within plant roots (Kloepper et al. 1988). Symbiotic bacteria, especially rhizobia, have been deeply studied, and although the world market for these microorganisms is nowadays relatively small, they have been developed as a biological means of increasing crop yields in certain circumstances. Beneficial free-living soil bacteria are usually referred to as plant growth promoting rhizobacteria (PGPRs, Kloepper et al. 1989), or, according to Piao et al. (1992), as yield increasing bacteria (YIBs). However, for Gray and Smith (2005), PGPRs also include rhizobia, due to the well-characterized legume–rhizobium symbiosis. Independently of the mechanisms of vegetal growth promotion, PGPRs have to colonize the rhizosphere around the roots, the rhizoplane (root surface), or the root itself (within radicular tissues). Among rhizobacteria, there is a gradient of root proximity and intimacy as follows: (1) bacteria living in the soil near to the roots, which utilize metabolites released by the roots as C and N sources, (2) bacteria colonizing the rhizoplane (radicular surface), (3) bacteria residing inside the root tissues, inhabiting spaces between cortical cells, and lastly (4) bacteria living inside cells in specialized root structures, or nodules, which generally fall into two groups: the legume-associated rhizobia and the woody plant-associated *Frankia* species. The cyanobacterial nitrogen-fixing symbionts of the cycads could also be included as PGPR (Gray and Smith 2005). A number of different bacteria may be considered to be PGPR, including species of the genera *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Acetobacter*, *Burkholderia*, and bacilli (Bashan and Levanony 1990; Brown 1974; Elmerich 1984; Kloepper et al. 1988, 1989; Okon and Labandera-González 1994; Tang et al. 1994).

To aid in this conceptualization, two simple terms have been adopted: intracellular PGPRs (iPGPRs), which are bacteria that live inside plant cells, being localized in the nodules, and extracellular PGPRs (ePGPRs), which are those bacteria that live outside plant cells, being able to enhance plant growth through the production of signal compounds that directly stimulate plant growth, to the improvement of plant disease resistance, or to the mobilization of soil nutrients to the plant. ePGPR can be subdivided into three types, based on the degree of association with plant roots: those living near but not in contact with the roots; those colonizing the root surface; and those living in the spaces between cells of the root cortex (Gray and Smith 2005).

PGPRs can affect plant growth in two different ways: indirectly or directly. The direct promotion of plant growth by PGPRs entails either providing the plant with

a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment (Glick 1995). PGPRs can fix atmospheric nitrogen and supply it to plants; they synthesize siderophores that can solubilize and sequester iron from the soil and provide it to plant cells; they synthesize several different phytohormones that can act to enhance various stages of plant growth; they may have mechanisms for the solubilization of minerals such as phosphorus that then become more readily available for plant growth; and they may synthesize some less well-characterized low-molecular mass compounds or enzymes that can modulate plant growth and development (Brown 1974; Kloepper et al. 1988, 1989). A particular PGPR may affect plant growth and development by using any one, or more, of these mechanisms. The indirect promotion of plant growth occurs when PGPRs lessen or prevent the deleterious effects of one or more phytopathogenic organisms.

Researches on ePGPRs were initially focused on *Bacillus* and *Anthrobacter* spp. (Brown 1974). Applications of these associations have been investigated in maize, wheat, oat, barley, peas, canola, soy, potatoes, tomatoes, lentils, radicchio, and cucumber (Gray and Smith 2005). Among the most widely studied ePGPR bacilli are *B. cereus* (Handelsman et al. 1990; Ryder et al. 1999), *B. circulans* (Berge et al. 1990), *B. firmus*, *B. lichenformis* (Chen et al. 1996), *B. subtilis* (Turner and Blackman 1991; Zhang and Smith 1996), and *B. thuringiensis* (Bai et al. 2002a, b).

1.3 Direct Benefits of ePGPRs for Plants

The extensive biochemical and molecular biological studies of symbiotic diazotrophs, such as rhizobia, have served as a conceptual starting point for the understanding of the mechanisms of growth promotion by PGPRs. Since one of the major benefits that rhizobia provide to the plants is fixed nitrogen, it was initially thought that diazotrophic PGPRs might also function in this way. However, not all PGPRs are diazotrophic, and many of those that are diazotrophic fix only limited amounts of nitrogen. Frequently, this amount is only enough for their own needs and is not sufficient to the host plant's nitrogen requirements (Hong et al. 1991). Some diazotrophic PGPRs still provide their plant hosts with a portion of the fixed nitrogen that they require; however, even for these PGPRs, nitrogen fixation is only a minor component of the benefit they offer to the plant (Chanway and Holl 1991).

ePGPRs influence vegetal growth in different ways, depending on the species and strain. To promote plant growth free-living rhizobacteria resort to more than one mechanism (Glick et al. 1999). Apart from nitrogen fixation, several ePGPRs are also capable of providing sufficient iron (Fe) levels to the plant in soils that exhibit low concentrations of this element (Wang et al. 1993) or even of other important minerals, such as phosphates (Singh and Kapoor 1998). Some ePGPRs can produce and secrete low-molecular-weight (400–1,000 Da) iron-binding molecules (siderophores) with a high affinity for Fe^{3+} (Gray and Smith 2005). Although iron is one of the most abundant minerals on Earth, in the soil it is

relatively unavailable for direct assimilation by several organisms. This is because in aerobic soils iron is found predominantly in the form of Fe^{3+}, mainly as a constituent of oxyhydroxide polymers with extremely low solubility, about 10^{-18} M at neutral pH. Minimal concentrations of iron required for normal growth of plants range from 10^{-9} to 10^{-4} M, depending on other nutritional factors (Neilands et al. 1987). Similarly, minimal iron concentrations for the optimal growth of many microbes are approximately 10^{-5} to 10^{-7} M (Lankford 1973). To overcome this problem, soil microorganisms secrete low-molecular-weight iron-binding molecules (siderophores) that bind Fe^{3+}, transport it back to the microbial cell, and then make it available for the microbial growth (Leong 1986).

The iron–siderophore binding also prevents the proliferation of pathogens due to the sequestration of iron from the environment. Some ePGPRs that present this property include *Pseudomonas putida* and *P. aeruginosa*. *P. putida* inhibits the growth of *Fusarium oxysporum*, a pathogen that affects tomatoes (Vandendergh and Gonzalez 1984), while *P. aeruginosa* inhibits the growth of *Pythium*, another pathogen that attacks tomatoes (Buysens et al. 1994).

Oppositely to microbial pathogens, plants do not suffer negative effects of iron depletion by ePGPRs. Some plants may capture the bacterial iron–siderophore complex, transporting it into its cells, where iron is released from the siderophores and is made available to the plant (Crowley et al. 1988). The production of siderophores has also been reported for *Azospirillum lipoferum* (Saxena et al. 1986; Shah et al. 1992), *A. brasilense* (Bachhawat and Ghosh 1987), and *Azotobacter vinelandii* (Demange et al. 1988; Knosp et al. 1984).

Phosphorus (P) exists in nature in a variety of organic (derived from microorganisms and plants) and inorganic (originating from applied P fertilizers) forms that are insoluble or very poorly soluble (Paul and Clark 1989). In fact, phosphorus is one of the least soluble elements in the natural environment, with less than 5% of the total soil phosphate content being available to the plants (Dobbelaere et al. 2003). Therefore, the addition of phosphate fertilizers has become a common practice in modern agriculture. However, a large portion of the soluble inorganic phosphate applied to soil as fertilizer is rapidly immobilized by the iron and aluminum in acid soils and by calcium in calcareous soils soon after application, thus becoming unavailable to plants (Holford 1997).

Soil microorganisms are able to solubilize insoluble mineral phosphate by producing various organic acids. This results in acidification of the surrounding soil, releasing soluble orthophosphate ions that can be readily taken up by plants. Furthermore, they are able to solubilize organic P compounds through the action of phosphatase enzymes (Garcia et al. 1992). In fact, the major source of phosphatase activity in soil is considered to be of microbial origin. In particular, phosphatase activity is substantially increased in the rhizosphere (Rodríguez and Fraga 1999). Phosphate solubilization has often been cited as a likely mechanism of promotion of vegetable growth by ePGPRs.

A large number of P-solubilizing bacteria (PSB) have been isolated from the rhizosphere of several crops. It was estimated that P-solubilizing microorganisms may constitute 20–40% of the cultivable population of soil microorganisms and that

a significant proportion of them can be isolated from rhizosphere soil (Chabot et al. 1993). There have been a number of reports on plant growth promotion by bacteria that have the ability to solubilize inorganic and/or organic P from soil after their inoculation in soil or plant seeds. The production (by these strains) of other metabolites beneficial to the plant, such as phytohormones, antibiotics, or siderophores, among others, has created confusion about the specific role of phosphate solubilization in plant growth and yield stimulation (Kloepper et al. 1989).

Experiments performed with P-solubilizing diazotrophs are scarce, and the results obtained are quite diverse, varying according to plant or bacterial species. *B. megaterium* and *P. polymyxa* are able to enhance growth and yield but not the P uptake of canola, indicating that P-solubilization is not the main mechanism responsible for positive growth response (de Freitas et al. 1997). Nevertheless, studies with *B. firmus* (Datta et al. 1982) and *P. polymyxa* (Gaur and Ostwal 1972) have confirmed the increase in phosphorus uptake in rice and wheat yields, respectively, after inoculation with these bacteria.

Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate. Among the bacterial genera with this capacity are *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, and *Erwinia*. There are considerable populations of phosphate-solubilizing bacteria in soil and in plant rhizospheres. These include both aerobic and anaerobic strains, with a prevalence of aerobic strains in submerged soils. A considerably higher concentration of phosphate-solubilizing bacteria is commonly found in the rhizosphere in comparison with nonrhizosphere soil (Rodríguez and Fraga 1999).

Phytohormones are natural plant growth regulators that influence physiological processes, when at low concentrations. Plant growth regulators are classified as auxins (cell differentiation, root and fruit growth, and abscission control), cytokinins (growth regulation, cell differentiation, and plant senescence), gibberellins (cell division and elongation, interruption of dormancy, and increase in fruit growth), abscisic acid (transpiration regulation, interruption of dormancy, and initial seed development), and ethylene (ripening of fruits, promotion of leaf, fruit and leaf abscission, and influence in female sexual expression) (Arshad and Frankenberger 1998; Raven et al. 1996). The production of these growth regulators has been reported in several bacteria including *Gluconacetobacter*, *Azospirillum*, *Herbaspirillum*, *Methylobacterium*, *Erwinia*, *Pantoea*, and *Pseudomonas* (Bastián et al. 1998; Cassán et al. 2001; Fuentes-Ramirez et al. 1993; Koenig et al. 2002; Lucangeli and Bottini 1997; Patten and Glick 1996; Verma et al. 2001) and has been consistently observed in bacteria that live in association with plants.

Phytohormone production is a bacterium–plant interaction mechanism influenced by numerous factors, as well as the plant and microorganism genotypes. Jain and Patriquin (1985) showed that pure *Azospirillum* endophytic strains, or culture filtrates, induced the ramification of wheat roots. The authors also observed that this phenomenon was influenced by the bacterial genome, at strain level, and by the plant's genome, at cultivar level. The same work reported the isolation and

characterization of indole acetic acid (IAA), an auxin produced by the bacterium, which was shown to play a role in root growth.

Although some *Paenibacillus* species are highly efficient in fixing nitrogen, the reason for the stimulation of plant growth is not directly related to this ability, but to its capacity to produce and secrete phytohormones, such as cytokinins (Timmusk et al. 1999) and auxins, especially IAA (Lebuhn et al. 1997).

Auxins (from the Greek *auxein*, to increase) are plant hormones originating from the amino acid tryptophan. The natural auxin is called IAA. Tryptophan is the precursor of IAA. Although there are four different pathways for IAA biosynthesis, all of them originated from tryptophan (Raven et al. 1996). The ability to synthesize phytohormones is widely observed in bacteria associated with plants – 80% of the bacteria isolated from rhizospheres are capable to produce IAA (Zakharova et al. 1999). The first studies were conducted in the 1970s. Tien et al. (1979) demonstrated that *Azospirillum brasiliense*, when exposed to tryptophan, produces IAA and lactic acid, and that IAA production increases by the time. *Bacillus* spp. isolated from the *Phaseolus vulgaris* rhizosphere produces significant amounts of IAA (Srinivasan et al. 1996). The isolates obtained from the rhizosphere of the genera *Enterobacter*, *Xanthomonas*, *Pseudomonas*, *Alcaligenes*, *Azotobacter*, *Acetobacter*, and *Agrobacterium* produce higher amounts of IAA than the soil isolates not associated with the roots (Asghar et al. 2002). In phytopathogenic bacteria, such as *Agrobacterium tumefaciens* and *Pseudomonas syringae* pathovars, IAA is produced from tryptophan via the intermediary indole acetamide and has been related to plant tumors. The beneficial bacteria synthesize IAA mainly through a tryptophan-dependent alternative pathway, from indole-pyruvic acid.

Promotion of root growth is one of the major markers by which the beneficial effect of PGPB is measured. Rapid establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, is advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment, thus enhancing their chances for survival. Most root-promoting bacteria synthesize IAA, and their effect on plants mimics that of exogenous IAA (Patten and Glick 2002).

Production of IAA, a plant hormone that does not apparently function as a hormone in bacterial cells, may have evolved in bacteria due to its importance in the bacterium–plant relationship. IAA secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial ACC deaminase activity (1-aminocyclopropane-1-carboxylate, a precursor of acetylene). However, the role of bacterial IAA in plant growth promotion remains undetermined (Patten and Glick 2002).

The response of plants to external (microbially released) IAA can vary from beneficial to deleterious effects, depending on the concentration of IAA in the plant roots. Low IAA concentrations may stimulate growth, while high IAA concentrations may inhibit root development. IAA levels released by rhizosphere bacteria depend on bacterial growth, metabolic activity, and the expression of genes encoding enzymes for IAA biosynthesis (Lambrecht et al. 2000). To assess these effects, different methodologies have been employed, such as the inoculation of

roots with mutant bacteria for the production of IAA or the application of bacterial inocula at different concentrations. Patten and Glick (2002) utilized a *P. putida* strain that secreted high IAA contents and reported an increase of 35–50% in canola's primary root growth. These authors provided direct evidence that bacterial IAA plays an important role in root elongation, when the producing bacterium is associated with the plant. Apart from this, these researchers provided stronger support to the hypothesis that beneficial bacteria produce IAA from pyruvic acid (IPyA), while pathogenic bacteria produce IAA from indole acetamide (IAM) (Manulis et al. 1998; Patten and Glick 1996; Prinsen et al. 1993). Extensive research has been carried out with mutant bacteria lacking the ability to synthesize IAA. These studies reported the existence of more than one tryptophan-dependent biosynthesis pathway for IAA production in the same microorganism. These biosynthesis pathways are classified in terms of their intermediary compounds, such as IAM or IPyA (Dobbelaere et al. 1999; Patten and Glick 1996).

The detection of auxins may be carried out according to a very precise, quantitative method, such as high performance liquid chromatography (HPLC) or even by a less accurate method, a specific and sensitive colorimetric reaction that uses the Salkowski reagent (Crozier et al. 1988). Different methodologies utilize the Salkowski reagent; some use microplates while others resort to nitrocellulose (Bric et al. 1991; Sarwar and Kremer 1992).

In a study with *P. polymyxa* strains isolated from wheat roots, Lebuhn et al. (1997) reported the production of IAA and indolic metabolites related to this compound in media supplemented or not with tryptophan. It was also observed that tryptophan greatly increased auxin production by these strains. These findings point out the importance of tryptophan as an IAA precursor. The isolates obtained from the rhizosphere produced more IAA as compared to those obtained directly from soil not associated with rhizosphere, suggesting that specific bacterial genotypes are selected by the host plants.

1.4 Promotion of Plant Growth by Bacilli

There is a long list of studies that demonstrate the beneficial effects of PGPRs, reporting that they promote significant improvement in plant growth and in the yield of several types of crops.

Beneduzi et al. (2008a) investigated several strains of bacilli, mainly species of the genera *Bacillus* and *Paenibacillus*, displaying important (PGP) characteristics isolated from seven distinct rice production zones of the Rio Grande do Sul State, south Brazil. Of those 296 isolates, 94 and 148 produced between 0.1 and 30 mg of (IAA) ml^{-1} in vitro after 72 and 144 h of incubation, respectively. Twenty-two isolates were able to solubilize phosphate and 32 isolates produced siderophores. *Paenibacillus* and *Bacillus* genera were the most prominent groups in the rhizosphere and soil populations analyzed. *Paenibacillus borealis* was the most frequent species in both locations. The isolate SVPR30, identified by 16S rRNA gene

Fig. 1.1 The effect of inoculation of a native PGPR isolate on rice growth promotion, 30 days after sprouting. (1) Plant was inoculated with SVPR30 strain; (2) positive control (plant was irrigated with mineral fertilizer solution); (3) negative control (plant was irrigated with distilled water); and (4) plant was inoculated with *Paenibacillus polymyxa* ATCC 10343 strain

sequence analysis as a strain of *Bacillus* sp., was chosen for in vivo greenhouse experiments and proved to be very efficient in promoting a significant increase in the roots and shoot parts of rice plants (Fig. 1.1).

In wheat crops, the genus *Paenibacillus* was the most prominent group in both the rhizosphere (77.8%) and soil (79%). *P. borealis* was the most frequently identified species, followed by *P. graminis*. The remainder of the isolated bacteria belonged to the genus *Bacillus* sp. Indolic compound production [indole-3-acetic acid (IAA), indole-pyruvic acid (IPyA), and IAM] was detected in 33.6 and 26% of the isolates from the rhizosphere and soil, respectively. Among the 311 isolates, 9 were able to solubilize phosphate and 48 were able to produce siderophores. The isolates SBR5, CSR16, and EsR7, identified by the 16S rRNA gene sequence as strains of *Paenibacillus* sp., were chosen for in vivo experiments in a greenhouse and proved to be very efficient in promoting a significant increase in the shoot and dry matter of wheat plants (Beneduzi et al. 2008b; Fig. 1.1).

Strains of *Bacillus* and *Paenibacillus*, which are Gram-positive ePGPRs, may be inoculated in separate or in consortium with *Rhizobium* or *Bradyrhizobium* strains, which are Gram-negative iPGPRs, to promote plant growth (Gray and Smith 2005). The inoculation of *P. polymyxa* strain H5, a phosphate-solubilizing bacterium, with *Rhizobium* led to an increase in chickpea yields due to the increase of phosphorus and nitrogen uptake (Alagawadi and Gaur 1988). Colonization and nodulation of soybean with *B. japonicum* strains (Li and Alexander 1988) may increase in media containing *Bacillus* spp., resulting in higher plant dry weight and seed yields.

Bacillus spp. isolated from the rhizosphere of *Phaseolus vulgaris* produced significant amounts of IAA (Srinivasan et al. 1996). IAA promoted root growth and/or nodulation when *R. etli* was added to *P. vulgaris* cultures, causing an increase in nodule weight and number, in nitrogenase activity, and in leghemoglobin content. Applied alone, *Bacillus* spp. also plays a promoting role in the growth of several plants, which may vary depending on the type of soil (Ramos et al. 2003). *Bacillus* strains have also been proved to increase the growth of tomatoes, when used in the plant's culture medium (Yan et al. 2003). *B. subtilis* promoted the growth of peanut seedlings, leading to an increase between 3.5 and 37% in crop yield and to improved germination, seedling emergence, plant nutrition, and root growth (Turner and Blackman 1991). *B. lichenformis* CECT 5106 and *B. pumilus* CECT 5105 inoculation enhanced growth rates of *Pinus* seedlings.

B. cereus UW85 increased soybean nodulation, both in laboratory tests and in the field, as well as in growth chambers (Halverson and Handelsman 1991). Interestingly, field experiments revealed differences in nodulation between 25 and 35 days after seeding; however, as the experiment evolved, differences in nodulation ceased to be detected as of 45 days of seedling. These results evince a promoter effect regarding initial growth, which nevertheless is not sustained in the long run. Yet, initial growth effects may be beneficial in the growth of young plants, assisting them to better cope with environmental stress they may have to face in their future development (Gray and Smith 2005).

Apart from fixing nitrogen, *P. polymyxa* solubilizes phosphorus in soils, produces antibiotics, chitinase, and other hydrolytic enzymes, increases soil porosity, and produces compounds that promote the growth of similar plants in media containing active IAA (Timmusk et al. 1999). It has also been demonstrated that this species increases nutrient uptake by the plant and acts as a biocontrol agent for pathogenic microorganisms (Chanway 1995; Mavingui and Heulin 1994), which is the reason for it been used as a biological control agent against *Fusarium* and *Pythium* (Guemouri-Athmani et al. 2000).

Paenibacillus durus is found in soils and roots of sugarcane, wheat, and other gramineous species. The interesting feature of this species lies in its capacity to biologically fix nitrogen, even in the presence of high nitrate levels, and to produce antimicrobial compounds (Neves and Rumjanek 1998). The capacity to fix nitrogen in vitro exhibited by *P. durus* is higher as compared to other *Paenibacillus* species, and because the bacterium is not affected by the presence of nitrate in the medium, fixing may occur in the presence of fertilizers (Rosado et al. 1996).

Li et al. (2008) conducted a study with soy nodules and isolated 98 nonsymbiotic endophytic bacterial strains that were characterized as being *Pantoea*, *Serratia*, *Acinetobacter*, *Bacillus*, and *Burkholderia*. The inoculation of endophytic bacteria did not lead to any significant effect in soy growth and nodulation, though several strains produced IAA, solubilized phosphate, and fixed nitrogen, a great promise in future studies on the plant growth promotion.

In the search for efficient PGPRs, Ahmad et al. (2008) have isolated and identified 72 bacteria as belonging to *Azotobacter*, *Pseudomonas*, *Mesorhizobium*, and *Bacillus* genera from the rhizospheres of wheat, sugarcane, onion, as well as

chickpea nodules in Aligarh, India. These isolates were tested for antifungal activity. The *Bacillus* and *Pseudomonas* strains isolated were the most efficient and are candidates for further studies on plant growth promotion.

Park et al. (2005) isolated free-living fixing bacteria from the rhizosphere of seven different plants: sesame, maize, wheat, soy, lentil, pepper, and rice in Chungbuk, South Korea. The strains exhibiting nitrogenase activity were identified as *Stenotrophomonas maltophila*, *B. fusiformis*, and *P. fluorescens*. *B. fusiformis* exhibited the highest nitrogenase activity and IAA production rates, demonstrating its potential as rhizobacterium promoter of plant growth.

Other PGPRs isolated from the rhizospheres of melon, alfafa, tomato, cotton, and wheat were analyzed for the production of auxins, nitrogenase and antifungal activity, and also to the improvement in nutrient uptake in cotton and pea in a semiarid region in Uzbekistan (Egamberdiyeva and Höflich 2004). The strains were proved to produce auxins, to fix nitrogen, and to exert antagonistic action against *Verticillium loteritum*. The inoculation of the tested plants with *Pseudomonas alcaligenes* PsA15, *P. denitrificans* PsD6, *P. polymyxa* BcP26, and *Mycobacterium phlei* MbP18 strains led to a significant increase in the growth of root and aerial parts of plants, as well as a higher content of N, P, and K in these plants.

Several strains of *B. subtilis* and *B. cereus* have been isolated and selected in China, due to their ability to promote plant growth and control fungal diseases that affect wheat roots (Ryder et al. 1999). Among these strains is *B. cereus* A47, which has been used in several cultures in that country, stimulating wheat production by around 11%. In turn, *B. subtilis* B908 was used to control rice sheath blight, caused by *Rhizoctonia solani*. Both strains were also tested in two different soils in Australia, in experiments conducted in a greenhouse. These bacteria considerably reduced the severity of diseases caused by *R. solani* and *Gaeumannomyces graminis* var. *tritici*, stimulating also the growth of wheat seedlings (Ryder et al. 1999).

Mena-Violante and Olalde-Portugal (2007) investigated the effect of the inoculation of tomato roots with *B. subtilis* BS13 on the fruit's quality and showed that production, weight, and length of tomatoes were higher in the inoculated group as compared to the negative control. The texture of fruits was also improved, demonstrating that BS13 strain plays a positive role in improving tomato quality, especially concerning size and texture.

Based on the presence of multiple PGP traits, Islam et al. (2009) isolated ten strains from paddy fields, screened for multiple PGP traits, and evaluated for their inoculation effects on canola and rice plants. *Paenibacillus* sp. RFNB4 significantly increased plant height and dry biomass production by 42.3 and 29.5%, respectively. Canola plants and rhizosphere soils inoculated with *Bacillus* sp. RFNB6 exhibited significantly higher nitrogenase activity.

B. megaterium B388, isolated from rhizosphere soil of pine belonging to a temperate Himalayan location, has been characterized by Trivedi and Pandey (2008). The carrier-based formulations of the bacterium resulted in increased plant growth in bioassays. The rhizosphere colonization and the viability of the

cells entrapped in alginate beads were greater in comparison to coal- or broth-based formulations.

A greenhouse experiment was performed by Figueiredo et al. (2008) to evaluate the effects of PGPR on nodulation, biological nitrogen fixation (BNF), and growth of the common bean (*Phaseolus vulgaris* L. cv. *tenderlake*). Beans coinoculated with *Rhizobium tropici* (CIAT899) and *P. polymyxa* (DSM 36) had higher leghemoglobin concentrations, nitrogenase activity, and N_2 fixation efficiency and thereby formed associations of greater symbiotic efficiency. Inoculation with *Rhizobium* and *P. polymyxa* strain Loutit (L) stimulated nodulation as well as nitrogen fixation. PGPR also stimulated specific-nodulation (number of nodules per gram of root dry weight) increases that translated into higher levels of accumulated nitrogen. The activities of phytohormones depended on their content and interactions with *R. tropici* and *Paenibacillus* and/or *Bacillus* (PGPR) strains, which affect the cytokinin content in the common bean.

Orhan et al. (2006) studied the growth promoting effects of two *Bacillus* strains (OSU-142, a nitrogen-fixing bacterium, and M3, which also fixes nitrogen and solubilizes phosphorus) used in consortium or individually in organic raspberry. The plants were evaluated for production, growth, nutrient content in leaves, and variation in nutrient composition. The results showed that *Bacillus* M3 stimulated plant growth and significantly increased yields. The inoculation of raspberry roots and rhizosphere with M3 and OSU-142 (in consortium or individually) caused an expressive improvement in yield (33.9 and 74.9%, respectively) as compared to the control. The levels of N, P, and Ca in leaves were also significantly increased. Similarly, inoculations affected the total levels of N, P, K, Ca, Mg, Fe, Mn, and Zn in soil. These results demonstrated that *Bacillus* M3, whether alone or in consortium with OSU-142, exhibited a high potential to increase yield, growth, and nutrition parameters in organic raspberry.

Karlidag et al. (2007) also tested the effects of *Bacillus* M3 and OSU-142 strains and that of *Microbacterium* FS01 isolated or in consortium on the growth of apple trees. The inoculation of these strains alone or in consortium increased plant growth (aerial part: 16.4–29.6%; diameter of aerial part: 15.9–18.4%) as well as yield (26–28%) and fruit size (13.9–25.5%). Of the nutrients investigated (N, P, K, Mg, Ca, Fe, Mn, and Zn), all were significantly affected (except Mg). The results of that study indicated that *Bacillus* M3, OSU-142, and *Microbacterium* FS0 strains are potential candidates to increase yield, growth, and nutrition of apple trees.

Bacillus OSU-142 was also investigated individually or in consortium with *Pseudomonas* BA-8 strain in yield, growth, and nutrient composition of cherry trees (Esitken et al. 2006). Both bacteria used individually as well as in consortium stimulated plant growth and led to a significant increase in yield. The use of BA-8, OSU-142, or a combination thereof on foliage and flowers led to a marked elevation in yield numbers (16.3, 10.9, and 21.76%), in fruit weight (4.14, 5.37, and 1.24%), and in the plants' aerial parts (11.3, 11.8, and 29.6%), respectively, when compared to controls. Additionally, expressive increases were observed in N, P, and K contents in cherry leaves, while Fe and Zn levels increased with applications of BA-8 and OSU-142 strains in consortium. Mn contents were higher when these two

bacterial strains were used separately. These results suggest that the inoculation of *Pseudomonas* BA-8 and *Bacillus* OSU-142 alone or in consortium may increase yield, growth, and nutrition levels of cherry trees.

1.5 Conclusions

The growing interest in PGPRs emerges from the wish to shed more light on a complex and fascinating biological system and from the potential these organisms have to increase productivity of crop cultures. The strains that exhibit a specific desired characteristic are selected, tested, and then used as an element in agricultural practices, though so far these strategies have not been extensively adopted – in spite of the several well-succeeded examples mentioned in this chapter. In a scenario where a greater number and more efficient bacterial strains are used, agricultural applications of PGPRs shall increase. Efforts made in the last decade have provided better insight into PGPRs and the mechanisms these bacteria utilize to promote plant growth, increasing the commercial interest in these strains (Glick 1995). While PGRPs have been characterized within several different bacterial taxa, many PGPRs developed for commercial applications are *Bacillus* species. These products utilize bacilli in their endospore form, which lends population stability during formulation and shelf life. Among bacilli, the strains of *B. subtilis* are the most widely used PGPRs, due to their capacity to produce antibiotics and consequent reduction in disease occurrence, when they are used as components of seed treatment strategies (Kokalis-Burelle et al. 2006).

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Chapter 2
Bacillus mojavensis: Its Endophytic Nature, the Surfactins, and Their Role in the Plant Response to Infection by *Fusarium verticillioides*

Charles W. Bacon and Dorothy M. Hinton

2.1 Introduction

Preharvest fungicides are used to control or reduce pathogenic fungi, their inoculum, and infection in hosts that also should reduce the formation of undesirable contaminants such as mycotoxins. However, the emphasis in greater sustainability and an increase in public concern for hazards associated with synthetic chemical pesticides and transgenic plants have produced a resurgence of interest in the use of introduced microorganisms for biological control of plant pathogens. Biological control is another one of the several measures used as pre- and postharvest control of several pathogens including the species of *Fusarium*. Most of these microorganisms are inconsistent in their performance in biological control resulting in reduced commercial development and widespread use. The major reason for this lack of performance is inadequate colonization of the target site, variation in expression of control at that site, and the need for numerous applications. Most of the biocontrol organisms are either soil or surface dwellers and have very little affinity to plants as specific colonizers as evidenced by ineffective controls of disease following repeated applications (see Thomashow and Weller 1990 and Hallmann et al. 1997 for review).

There is a unique group of bacteria that form endophytic associations with plants, and several of these have been reported to be successful in preventing disease development. The uses of bacterial endophytes in agriculture for general and specific biological control applications are current, widespread (Chanway 1996; Hallmann et al. 1997; Kobayashi and Palumbo 2000; Reinhold-Hurek and Hurek 1998; Sturz et al. 2000), and might afford protection to host plants similar to those provided by fungal endophytes.

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Bacterial endophytes can be distinguished from nonbacterial endophytes by their unique behavior with plant hosts. Large numbers of bacterial species are endophytic and are regarded as symbiotic with plants as biotrophic and several of these form mutualistic endophytic associations (Hallmann et al. 1997; Chanway 1996, 1998; Bacon and Hinton 2006; Kobayashi and Palumbo 2000; Bacon and Hinton 2006). These bacterial endophytes actively colonize above- (foliage) and below-ground (root) host tissues and establish long-term associations, actually lifelong natural associations, without doing substantive harm to the host. These associations are to be distinguished from transient bacteria, usually dormant or latent infections, which form associations as happenstances that will not survive long. Endophytic bacteria are further distinguished by controlling the growth of several fungi, bacteria, and nematodes (Bacon and Hinton 2006; Hallmann et al. 1997). Endophytic bacteria include both obligate and facultative species. These bacteria offer several advantages for control of pathogenic fungi, and they are expected to control both endophytic fungi, such as *Fusarium verticillioides*, and epibiotic to endophytic species of other *Fusarium*.

Bacillus mojavensis Roberts, Nakamura, and Cohan was distinguished from the *Bacillus subtilis* complex on the basis of whole-cell fatty acid composition, divergence in DNA sequence, and resistance to genetic transformation (Roberts et al. 1994). The type of this bacterium was an isolate from the Mojave Desert, California, which served as the specific epithet for this and the other desert isolates as *B. mojavensis* Roberts, Nakamura, and Cohan. This group of species in addition to the desert origins has additional physiological traits that serve as characteristics of the species. All strains of this species are antagonistic to fungi and are all endophytic. These traits suggest that this species has outstanding traits as an endophytic biocontrol agent.

The genus *Bacillus* has several traits in common and one that is emerging is the ability to produce specific lipopeptide biosurfactants such as surfactins A, B, and C, pumilacidin, esperin, lichenysin, fengycin, iturin, the bacillomycin, mycosubtilin, surfactants 86, arthrofactin, and the fungicines. We recently discovered that this species also produced a mixture of closely related cyclic lipopeptide isoforms of the biosurfactant, surfactin A (Snook et al. 2009; Fig. 2.1). Among the many anticipated uses of surfactin, this biosurfactant is inhibitory to fungi, some bacteria, mycoplasmas, and viruses (Bonmatin et al. 2003; Desai and Banat 1997; Georgiou et al. 1992; Peypoux et al. 1999; Seydlova and Svobodova 2008).

In addition to *B. mojavensis*, the surfactins are produced by *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens*. Only two other genera are reported as producer of lipopeptide biosurfactants and these include *Serratia marcescens* that produces serrawettin W2 and species of *Pseudomonas* that produces the putisolvins and arthrofactin (Desai and Banat 1997; Matsuyama et al. 1992).

A major thrust is on bacterial endophytes that offer biocontrol potential for fungal endophytes such as *Fusarium* species. This genus consists of several species that are endophytic (Kuldau and Yates 2000), and one species in particular is *F. verticillioides* (synonym *Fusarium moniliforme*), Holomorph: *Gibberella*

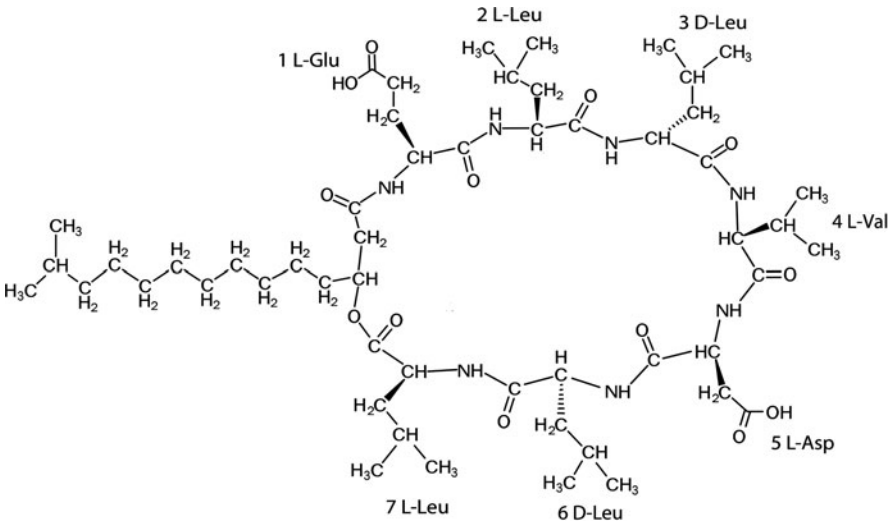

Fig. 2.1 Structure of surfactin A, Leu[7]-surfactin with the amino acid sequence of Glu, Leu, Leu, Val, Asp, Leu, Leu. Surfactin B has an amino acid sequence Glu, Leu, Leu, Val, Asp, Leu, and note for this isomer Val at position 7. Surfactin C has an amino acid sequence of Glu, Leu, Leu, Val, Asp, and note for this isomer Ile at position 7

moniliformis Wineland, a pathogen of maize and other crops. In maize, it produces the fumonisin mycotoxins that are toxic to horses, swine, poultry, and are correlated with human toxicity (Bullerman 1996; Marasas et al. 1988, 2004; Riley et al. 1993; Ross et al. 1992; Voss et al. 2001).

Since the fumonisins are common occurrence on maize, most probably due to the endophytic nature of *F. verticillioides*, it is imperative to find a control for both the endophytic infection and the saprophytic state. The endophytic state serves both as a source of mycotoxin production and as an infection source. The endophytic state also makes it difficult to control by fungicides registered for use on food crops. *B. mojavensis* has the potential to control infections by *F. verticillioides* and for reducing the content of fumonisin in maize (Bacon et al. 2001). However, the practical uses of bacterial endophytes under field conditions are problematic. The basis for this we feel is due to the unknown mechanism or mechanisms responsible for biocontrol by endophytic bacteria. Such mechanisms obviously are complicated by genetics of biocontrol and target organisms, interacting biotic and abiotic factors, and most importantly competing organisms. Further, there might be several mechanisms operating within a given environment, each operating for specific species of biocontrol organism. Intuitively, competitive exclusion is used to describe competition between two organisms for the same niche. What is described as competitive exclusion is envisioned as replacing one organism with another is too simplistic. The method of obtaining dominance or excluding another from a niche is operationally a complex scenario of which very little is known. In this regard, nutrition is often used as the explanation, although most theories encompass

an antagonistic response such as the production of an antibiotic. In the instance of *B. mojavensis* versus *F. verticillioides*, antibiotic production by the biocontrol organisms is suggested since the production of antagonistical substances is observed on Petri plates.

Surfactins are produced by the majority of strains of *B. mojavensis* tested (Snook et al. 2009). Further, since all *B. mojavensis* strains examined are endophytic, we believe this species can offer valuable biocontrol information from which we can draw conclusions and extend to other bacterial endophytes. Moreover, *B. mojavensis* along with other *Bacillus* species produce surfactins, which implies that there is a natural role in the ecology of a species for surfactin and others in this class of biosurfactants (Ron and Rosenberg 2001). We review the chemistry of surfactin, surfactin production in this natural endophytic species, as well as the biocontrol potential for surfactins. We also review the biocontrol activities and host–bacterial relations along with in planta growth properties. The target of our control is primarily *F. verticillioides* and other endophytic species of fungi, although other pathogens might equally fit the model and be controlled as well.

2.2 Surfactin Chemistry and Structural Features

The surfactins consist of a group of several surface-active agents produced by microorganisms and conveniently grouped as biosurfactants because they are surfactants of microbial origin, and they are readily biodegradable. Thus, they are environmentally friendly. Surfactin is one of the small molecular weight lipopeptide biosurfactants that range from 1,003 to 1,127 Da (Cooper et al. 1989). Surfactin was first isolated from *B. subtilis* by Arima et al. (1968), and the name assigned to it is due to its surfactant activity (Arima et al. 1968). The complete structure was determined as a macrolide lipopeptide, which was confirmed by Kakinuma et al. (1969). Since this initial discovery, surfactin serve as the major class of this cyclic lipopeptides.

The surfactins consist of a polar end of seven α-amino acids and an acyl chain of hydrophobic β-hydroxy fatty acids. The amino acids are arranged in the chiral sequence LLDLLDL with hydrophobic amino acids located at carbons 2, 3, 4, 6, and 7, while hydrophilic amino acids are located at carbons 1 and 5 as glutamyl and aspartyl, respectively (Fig. 2.1). Surfactin is rated one of the most biologically active biosurfactant that has been discovered (Kakinuma et al. 1969).

Surfactin is soluble in polar and nonpolar solvents due to the hydrophilic and hydrophobic moieties, which explains most of its many attributes. Either moiety has the chemical ability to partition interfaces between different surface polarities. This class is a highly active compound with high ratings for detergency, foaming, dispersing, and emulsifying qualities. While surfactins belong to the major group of biosurfactants, they are microbial in origin and have several advantages over chemical derivatives or surfactants and these include lower toxicity, higher biodegradability, and ability to remain biologically active over a wide range of pH values

and temperatures (Cameotra and Makkar 1998). Again this is due to the amino acid composition, and specifically aspartic acid and glycine are the major amino acids that make surfactin amendable to microbial degradation and chemical decompositions.

According to Seydlova and Svobodova 2008, surfactin is the most powerful biosurfactants. It lowers the surface tension of water from 72 to 27 mN m^{-1} at a concentration as low as 10 μM. Further, surfactin is amphoteric, possessing both a positive and negative charge. This low molecular weight biosurfactant is characterized due to this weight as having the basic property of lowering surface, interfacial tensions efficiently (Cooper 1980) and other physiochemical properties (Bonmatin et al. 2003). Additional structural features of surfactin and other small molecular weight biosurfactants such as binding to heavy metals, detachment and attachment to and from surfaces, increasing surface, biofilm, and emulsifier production have been reviewed (Ron and Rosenberg 2001).

2.2.1 In Vitro Production and Heterogeneity of Surfactins

Surfactin was originally discovery from a nutrient broth culture of *B. subtilis* (Arima et al. 1968). Current procedures for most producing organisms are based on strains of *B. subtilis* cultured on a variety of synthetic and natural media (Das and Mukherjee 2007) and mixed fermentation procedures and with a variety of incubation temperatures (Makkar and Cameotra 1998). The initial media used to screen strain were the synthetic minimal mineral salt medium (Cooper et al. 1981) or on a semisynthetic medium (Landy et al. 1948). The yield of surfactin by *B. mojavensis* on either of these is very low, and in most instances, there is no surfactin produced at all by some strains, including submerged or stationary cultures. In our experience, the synthetic media reported above do not induce the synthesis of surfactin in *B. mojavensis*. However, the nutrient broth and nutrient broth amended with agar are excellent media and more surfactin is produced on the nutrient agar than broth (Snook et al. 2009). Nutrient agar is by far the best in terms of yield, although another medium that works well is a modified nutrient solution made from cottonseed formulation, Pharmamedia (Traders Protein, Southern Cotton Oil Company), amended with nutrient broth (Snook et al. 2009). On these media, most *B. mojavensis* strains tested produced a variety of isomers (Table 2.1). Some strains produce high levels of the desirable C-14 and C-15 alkyl chain lengths (unpublished data).

The biosynthesis of surfactins is nonribosomal (Desai and Banat 1997; von Dohren 1995) as is the synthesis of most secondary metabolites of bacteria. Regulation of this process is very complex because genes encoding nonribosomal peptide synthetases are organized into operons (Marahiel et al. 1997). The synthesis and regulation involve modular nonribosomal peptide synthetases that are transcriptionally induced under nutritionally limiting or stressful conditions (Desai and Banat 1997; Davis et al. 1999). Thus, the poorer the medium usually the better the product, which is the case of *B. mojavensis* as attempts at increasing the yield of

Table 2.1 Percentage of isolates producing specific surfactin isoforms by *B. mojavensis* in culture

Acyl chain length	% Producing[a] surfactins	% Producing none
C11	28	72
C12	28	72
C13	58	42
C14	51	49
C15	58	42
C16	12	88
C17	15	85

[a]A total of 33 strains from all the major deserts were cultured on the nutrient broth amended with Pharmamedia, a cotton seed product, and tested for specific isoforms of surfactin after 30 h of incubation

surfactins by increasing or modifying carbohydrate and nitrogen levels result in luxuriant growth but no surfactin. Under each domain of a module, the seven amino acids of surfactin are bound to carriers, activated and chemically united to produce the characteristic head. Additional domains such as epimerizations within the module modify or catalyze specific amino acids to produce either D- or L-amino acids.

It was also reported that stressful conditions also induce sporulation by *Bacillus* species and indeed whose sporulation specific locus have been identified and shown to transcribe the synthesis of surfactin due to promoting transcription of the surfactin synthetase operon but only when glucose is in excess (Marahiel et al. 1993). In general, surfactin is induced by nitrogen starvation (Davis et al. 1999), although Marahiel et al. (1993) indicate that it is repressed by glutamate. There are variations in specifics of surfactin fermentation requirements, which are strain specific. For example, in one strain of *B. mojavensis* (synonym *B. licheniformis* JF-2), maximum biosurfactant synthesis occurs during the log phase, but when the cells enter stationary phase, which is controlled by a promoter gene, it stops the synthesis of surfactin and that which is produced is absorbed by the producing cells (Lin 1996). This indicates that the series of genes are controlled by extracellular environmental factor during dense cell growth and its associated biochemical pathways suggesting a "quorum sensing mechanism."

Resorption of surfactin might be an explanation for data published earlier (Bacon and Hinton 2002), indicating that on nutrient agar all strains were antagonistic to *F. verticillioides*, but 42% were negative for surfactin C15, the most biologically active form (Table 2.1). Perhaps these stains reabsorbed the surfactins, but remained toxic when the fungal and bacterial cells interact during the in vitro assay for toxicity on nutrient agar surfactin. This form of inhibition was referred to as surface contact inhibition in that early work (Bacon and Hinton 2002). Yet to be explained is the ability of strains of any species to tolerate massive production of surfactin without self-destruction.

In summary, surfactin biosynthesis is regulated by genes relating to nutrient limiting conditions, which apparently are under transcriptional control. Surfactin biosynthesis and sporulation are coregulated under nutrient poor condition. Thus,

measures to increase its production under laboratory conditions should include a limit of nitrogen sources and concentration as well as amounts of carbon available to cell, usually under log phase. Strategy to improve strain for product formation might be achieved by altering specific cultivation conditions and by genetic manipulation (Abu-Ruwaida et al. 1991; Das and Mukherjee 2007; Davis et al. 1999).

2.2.2 *Surfactin Biocontrol Features*

The acyl chain length varies from C13 to C16 fatty acids that are linear, *iso*-, or *anteiso*-branched. The terminal amino acid is linked to the carboxyl group of the fatty acid and the carboxyl terminus is linked to the hydroxy or amino group of the acid resulting in a lactone cyclic peptide configuration with a hydrophobic tail. There are three structural analogues of surfactin, A (Fig. 2.1), B, and C, in which each differs in the sequence of amino acid, particularly in position 7. The essential difference is that Leu located at position 7 of surfactin A is replaced by valine and isoleucine in surfactin B and C, respectively (Baumgart et al. 1991).

Substitution of specific amino acids with other L- or D-amino acids exerts a change on the entire lipopeptide structure that modifies both the chemistry and biological activity of the molecule (Kikuchi and Hasumi 2002). For example, lichenysin is another cyclic heptapeptide with similarity to surfactin, except Glu in surfactin as opposed to the presence of Gln in lichenysin which confers a remarkable decrease in biological activity compared to surfactin (Konz et al. 1999). However, in lichenysin the makeup and length of the fatty acid tail also affect antimicrobial activity (Seydlova and Svobodova 2008; Yakimov et al. 1996), which was not measured in the Konz et al. (1999) work, but speculated here as being important for activity of surfactin to which it is structurally similar. Further, it was noted that surfactant activity also varied with the branching type in an increasing order from *anteiso* to *iso* to *normal*, which imparts the highest biocontrol activity relative to fungal toxicity (Maget-Dena and Ptak 1995; Yakimov et al. 1996). Thus, *normal* alkyl chains are more active than *iso* and *anteiso* being last active, but as observed in Table 2.1, several isomers coexist in the same extract that might be more active due to synergism. Although, of several strains tested, 42% did not produce any surfactins, they were all antagonistic to *Fusarium verticillium* (Bacon and Hinton 2002), suggesting the role of additional inhibitors, resorption of surfactin as described above, or failure of an inhibitory substance to diffuse into the test medium (and see below). The pattern of β-hydroxy fatty acids synthesized depends on the branched amino acids in the medium such as valine and isoleucine (Besson et al. 1992).

The physical characteristics of surfactins increase the benefits as biocontrol agents. Most lipopeptides are physically active over a variety of conditions and it is expected that surfactins will follow this generalization. Surfactin is active at below room temperature, and up to 80 or 100°C, and over a range of pH values from 3 to 11 (Cameotra and Makkar 1998; Joshi et al. 2008; Makkar and Cameotra

1998). *B. mojavensis* is an osmophile that tolerates seawater and salt concentrations up to 50 g l^{-1} and capable of anaerobic growth at 45°C (Javaheri et al. 1985; Janneman et al. 1983). However, we do not know if surfactin and other biocontrol modulators are produced or active at this concentration of salt. Further, *B. mojavensis* JF-2 is capable of anaerobic growth, provided DNA or deoxyribonucleoside are supplied to the medium (Folmsbee et al. 2004). The ability to tolerate high salt concentrations, extreme pH, and other factors describes the versatility of the bacterium to most environments.

The major uses of biosurfactants such as surfactin are for bioremediation of oil recovery and spills as well as emulsifiers, detergents, and cleaners, with some small uses in the cosmetic industry (Rahman et al. 2006). Worldwide well over 17 million tons of biosurfactants are produced and used for these industrial purposes (Whalley 1995). Surfactins specifically have a large range of applications for use in medicine (Rodrigues et al. 2006).

Very little of this was used for agriculture and almost none for biocontrol uses as advanced here. The anticipated uses in agriculture are expected to include biocontrol of phytopathogenic fungi, mycoplasmas, viral, and as an antitumor agent. Surfactin is also inhibitory to some extracellular enzymes, and evidence indicates that it has biocontrol activity over mosquito larva (Das and Mukherjee 2006; Geetha et al. 2010). To our knowledge, the only reported use of a biosurfactant as a biocontrol is against three zoosporic plant pathogens: *Pythium aphanidermatum*, *Phytophthora capsici*, and *Plasmopara lactucea-radicis* (Stanghellini and Miller 1997). The uses of endophytic bacteria as biocontrol agents while widespread are not specifically designed for use in the in situ endophytic production of a biosurfactant as a biocontrol for specific disease of plants. Thus, the field is wide open for uses of specific biosurfactants for the control of specific diseases.

2.3 Plant–Bacterial Endophytic Relationships

Bacterial endophytes as intended here are those that are strictly intercellular (Bacon and Hinton 2002; Chanway 1998; McCully 2001), although the bacterium may colonize either below, above, or both plant axes. Our definition continues to exclude those species that are only transient dwellers of the intercellular habit which can and do within their life cycle become intracellular, oftentimes invading xylem tissues which usually become problematic and cause diseases in plants. Further, xylem endophytes, while not causing a disease, are viewed as a problem since endophytes that live within it are considered not ideal endophytes for biocontrol uses since such infected vessels are weakened and become nonfunctional and therefore detrimental to the plant (McCully 2001). *B. mojavensis* is associated with plants as biotrophic symbionts, but it is not known if this association is obligate or facultative, since all strains are readily isolated from soils (as spores) and their natural colonization from desert habitats such as plants have not been

documented. It is logical to assume that this species naturally inhabits desert plants as endophytes. However, we do know that under experimental conditions, the preferred niches colonized are roots and seedling stems immediately upon germination and the association remains throughout the life of the plant.

The method by which infection occurs is extremely important not only from a biotechnological viewpoint but also from understanding the basic biology of this bacterium. The infection process of *B. mojavensis* occurs from the topical application to seed, where we assume the infection takes place directly from cuts and abrasion of roots during the germination process. Upon germination the roots and mesocotyl are already infected and this infection is perpetuated throughout the plant axis during the entire season (Bacon and Hinton 2002). There are no direct studies on how plants are initially infected and most studies suggest that infection is accomplished through breaks, tares, or wounds of the roots of seedling during either germination or shortly thereafter (Patriquin and Dobereiner 1978). Foliage and stem infection can take place through the pores and stomatas. As we are dealing with nonpathogenic bacteria, there is very little evidence that they possess the arsenal of hydrolytic enzymes that are used to gain entry into roots and leaves characteristic of plant pathogens. We see no evidence for the activity of hydrolytic enzymes characteristic of pathogenic bacteria during or after infection of plants by *B. mojavensis*.

B. mojavensis and other bacterial endophytes benefit from inhabiting the plant's interior because it is a protected niche in which there is a constant source of nutrition. However, the concentrations of such nutrients have been reported as being sparse, suggesting that intercellular bacteria might live under oligotrophic conditions. This, however, is not correct as intercellular spaces are actually high in organic and inorganic nutrients (see review of Bacon and Hinton (2006)) necessary to support the growth of a complex intraspecific mixture of endophytes (Bacon and Hinton 2006; Canny 1995; Dong et al. 1994; Kursanov and Brovchenko 1970). This is due to the recent consideration that the apoplasm, the intercellular spaces, and symplast are structurally the same. Nutrients within the apoplasm are similar to and in similar concentrations as those in the symplasm. Nutrient concentrations in the apoplasm and symplasm are interactive with the phloem, dispelling the earlier notion that the apoplasm is relatively free of nutrients. Indeed, current research indicates that nutrient transport within plant tissues is considered to occur through an apoplastic route via the cell wall continuum and via the symplastic route via the plasmodesmata (Canny 1995).

Bacterial endophytes in general and *B. mojavensis* in particular are not oligotrophs, but rather copiotrophs. Thus, the intercellular space in maize is rich in substances necessary to support the oligotrophic growth of *B. mojavensis* supporting the observation of large numbers of bacterial cells observed in the several intercellular spaces (Fig. 2.2). Additional information on bacterial-induced morphological and chemical changes within cellular types of the endophytic niche has been described in an earlier review (Bacon and Hinton 2006). These morphological changes are presumably harmless as diseases are not observed, not even under stresses such as drought.

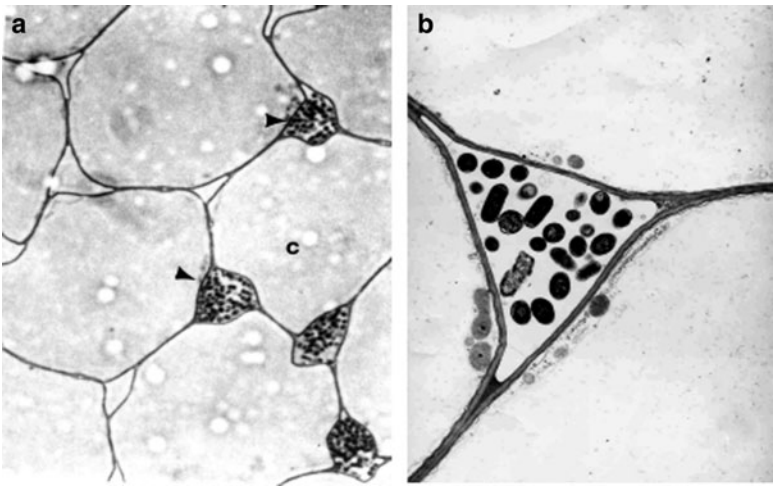

Fig. 2.2 *Bacillus mojavensis* in the intercellular spaces of maize: (**a**) Light microscopy showing bacterial cells located within the intercellular spaces of the cortex, c, of maize root (*arrow*), 40×. (**b**) Transmission electron micrograph of maize root showing bacteria between the intercellular spaces formed by three adjacent cells, 7,250× (Hinton and Bacon 1995)

2.4 Plant Growth Effects

Plant growth responses to bacteria are well established for several crop plants and we presented evidence of the effect of *B. mojavensis* on maize and bean growth indicating growth enhancements on both a monocot and a dicot (Figs. 2.3 and 2.4). The factors responsible for growth enhancements are unknown, but might reflect the exogenous production of phytohormones such as ethylene, auxins, and cytokinins. We have not examined the in vitro production of plant growth hormones by *B. mojavensis*, and it has been demonstrated that a plant-associated bacteria (Kuklinsky-Sobral et al. 2004) but not necessarily endophyte as defined in this review can produce hormones. However, it is not uncommon for bacteria to produce phytohormones, which have been demonstrated for several species (Arshad and Frankenberger 1991). Growth enhancements are also observed on *B. mojavensis*-infected maize plants grown in the presence of pathogenic isolates of *F. verticillioides* (Fig. 2.3).

In addition to the production of phytohormones plant growth, other factors of abiotic nature should also be considered. Other plant growth effects attributed to endophytic bacteria are enhanced mineral uptake such as the solubilizing of bound soil iron and phosphorus and by providing the plant with siderophores and nitrogen (Sturz et al. 2000; Surette et al. 2003). The siderophore producing ability of *B. mojavensis* has been demonstrated, but its interaction at the maize root level has not been determined (unpublished). Additional effects are those that interact with soil bacterial population, prior crop history and the buildup of soil bacteria and

Fig. 2.3 Effects of *Bacillus mojavensis* endophytic infection on plant growth: *Left*, growth of maize shoot inoculated with bacterium; *Middle*, inoculated with *Fusarium verticillioides*; *Right*, inoculated with the fungus and bacterium (Bacon and Hinton 2002)

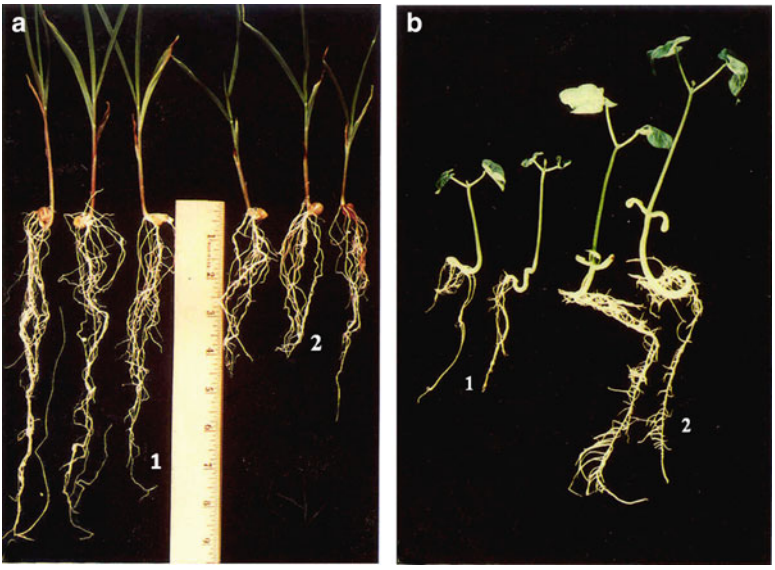

Fig. 2.4 (**a**) Effects of bacterium on maize root growth, (1) and without the bacterium (2). (**b**) Effects of bacterium on 2-week-old growth of beans inoculated without (1), and with bacterium (2); (Bacon and Hinton 2002)

the resulting benefits derived from allelopathy as indicated in the above section on transformation. In addition to soil nitrogen, i.e., a root effect, recent evidence has indicated the endophytic fixation of nitrogen in rice by a leaf endophyte isolated from wild rice by a species of *Herbaspirillum* (Elbeltagy et al. 2001), and similar

analyses by other foliage endophytes such as in sorghum (James et al. 1997). In rice it has been estimated that as much as 200 kg N ha^{-1} $year^{-1}$ can be produced by the use of endophytes in plants (Ladha and Reddy 1995). Thus, nitrogen fixation can be transferred to endophytic bacteria, which can be accomplished through the use of *B. mojavensis* and if necessary by genetic alteration to accomplish the task.

The discussion above describes direct effects on plant growth by the bacterium. There are other effects that relate to plant health, which bacterial endophytes such as *B. mojavensis* might relieve. It is anticipated that with the control of pathogenic organisms, plant growth can be maximized with basic cultural condition. The resulting growth is due to lack of parasitisms and diseases, as well as decreased susceptibility to abiotic stresses such as drought and frost tolerance or resistance.

2.5 Product Transformations

Maize and other cereals produce a group of phytoanticipins the benzoxazinones and their decomposition products (benzoxazolinones and their methyl derivatives) as part of a constitutive defense system capable of deterring insects and pathogenic fungi (Niemeyer 1988). In addition to serving as defensive metabolites, they possess allelopathic qualities (Singh et al. 2003). Due to this very wide spectrum of biological activity, they are commonly referred to as a group of allelochemicals and offer a great deal of promise for natural controls of pests. The main benzoxazinones produced by maize are 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one and its dimethoxy derivative 2,4-dihydroxy-6,7-dimethoxy-1,4-benzoxazin-3 (4*H*)-one with the former occurring in greater concentrations (Cambier et al. 2000; Whitney and Mortimore 1959). Since these benzoxazinones are highly toxic and reactive, they are also toxic to the plant but are stored as the nontoxic conjugate, a glycoside. The mechanism is put into operation from injury from insects or pathogenic response and are degraded to their unstable and highly toxic agluconic forms, which spontaneously decompose to the corresponding nonplant toxic benzoxazolinones, such as MBOA (6-methoxy-2-benzoxazolinone) and BOA (benzoxazolin-2-one) (Niemeyer 1988). These toxic decomposition products are very stable and remain in debris and maize stubble. Over time apparently maize pathogens have developed resistance to these benzoxazolinones, which is offered as the major reason why maize fields are characterized by fungi that are predominantly maize pathogens with resistance to MBOA and BOA (Glenn 2001; Glenn and Bacon 1998) as observed in *F. verticillioides* (Glenn et al. 2001). Thus, MBOA and BOA are found in plant debris and soil as stable degradation products of DIMBOA and DIBOA, which are considered useful allelochemicals for other uses (Fomsgaard et al. 2004) but are of no consequence for biocontrol of maize pathogens.

It was recently discovered that *B. mojavensis* has the ability to tolerate the benzoxazolinones and in fact transforms BOA and MBOA into 2-amino-3H-phenoxazin-3-one (APO), which is stable and very toxic transformation product to

F. verticillioides and other maize pathogens (Bacon et al. 2007). This substance is now a much better fungicide than the original BOA since *F. verticillioides* is not resistance to it. The results indicate that the bacterium metabolizes a plant metabolite, preventing the usual series of transformations of this same metabolite by *F. verticillioides* and other maize fungal pathogens to nontoxic products, resulting in the accumulation of APO. Thus, an enhanced biocontrol is suggested by this in vitro study. Additional evidence of biotransformation by *B. mojavensis* and other novel enzymatic activities have yet to be reported presumably due to the relative recent attempts at exploiting this species.

2.6 Fungal–Bacterium Interaction

The successful use of a biocontrol bacterium depends on its value under field condition to control the target species, usually a pathogenic fungus that may be equally competitive within specific environments. *F. verticillioides* produces a number of secondary metabolites some of which are considered as antibiotics providing this fungus with a competitive edge. Fusaric acid (5-butylpicolinic acid) was first implicated in the pathogenesis of tomato wilt cause by *F. oxysporum* f.sp. *lycopersici*. Fusaric acid is now known to be a common metabolite of the genus *Fusarium* (Bacon et al. 1996) and other biological activities are assigned to it. It is a mild mycotoxin (Porter et al. 1995; Voss et al. 1999) and is pharmacologically active (Porter et al. 1995; Ballio 1981; Bungo et al. 1999).

In addition to mammalian toxicity, fusaric acid is toxic to vegetative growth of plants in general (Corden and Diamond 1959; D'Alton and Etherton 1984; Drysdale 1984; Marrè et al. 1993) as well as to a variety of physiological processes of plants (Luz et al. 1990; Marrè et al. 1993; Mace and Solit 1966; MacHardy and Beckman 1981; Sanwal and Waygood 1961). Thus, plants infected with *Fusarium* species, endophytic and otherwise, should also show degrees of growth restrictions, provided fusaric acid is produced in planta.

Fusaric acid also has antibiotic effects on both Gram-negative and Gram-positive bacteria including *Bacillus*, *Paenibacillus*, and *Pseudomonas* species (Arias 1985; Landa et al. 2002; Luz et al. 1990; Notz et al. 2002; Schnider-Keel et al. 2000). We have determined that high levels of fusaric acid exhibited toxicity to some strains of *B. mojavensis* (Table 2.2) (Bacon et al. 2006). This indicates that the endophytic states of *Fusarium* species might have a competitive edge over *B. mojavensis* and any other biocontrol bacterium sensitive to fusaric acid. Fusaric acid sensitivity is not established since the data use what might be considered high concentrations of fusaric acid. These levels are, however, low enough and do not show a response to maize and are within the concentrations used by others to demonstrate. We have developed mutants of *B. mojavensis* that are tolerant to fusaric acid at these concentrations and higher. These mutants might prove superior under field conditions provided all the other qualities such as endophytism and surfactin production are maintained. Fusaric acid is a common contaminate in

Table 2.2 Inhibitory activity of fusaric acid (100 μg ml^{-1}) on the specific rate of growth of *Bacillus mojavensis* strains and their antagonism to *F. verticillioides* (Bacon et al. 2006)

Strains	Origin	% Fusaric acid inhibition[a] (100 g ml^{-1})
RRC 101	Corn, Italy	59a
RRC 112	Mutant of 101	81b
NRRL B-14698^T	Mojave	92b
NRRL B-14699	Mojave	21c
NRRL B-14700	Mojave	76a
NRRL B-14703	Gobi	91a
NRRL B-14708	Gobi	0d
NRRL B-14706	Gobi	26c
NRRL B-14710	Gobi	87b
NRRL B-14714	Sahara	64a
NRRL B-14716	Sahara	77a
NRRL B-14817	Sahara	62a
NRRL B-14824	Sahara	69a

[a]Percentage of fusaric acid inhibition was determined within a 48-h incubation period at 30°C. Values represent the means of six replicate cultures. Means within a column followed by a different letter were significantly different at a *P* value of 0.05 according to Fisher's protected least significant difference test

feedstuffs made from maize (Smith and Sousadias 1993), indicating its natural occurrence. Nevertheless, fusaric acid and possibly other *Fusarium* specific metabolites must be considered a major problem with the use of this and other bacteria. The manner by which fusaric acid tolerance is expressed, i.e., production of an extracellular degrading enzyme or it is impermeable to membrane-regulated transport, is not known but must be determined before such mutant will have a valid place to control the growth of *Fusarium* species under field conditions.

2.7 Conclusion

Endophytic bacteria as defined in this review describe *B. mojavensis* as a bacterium that dwells within the intercellular spaces of plants, is highly compatible with plants, it does not impose negative reactions to the plant, and its interactive metabolism with the host influences growth, maturations, and tolerance to diseases. *B. mojavensis* offers additional qualities of being competitive with some fungi, particularly those that are also endophytic by the production of isomers of surfactins, a biosurfactant that is described as being the most potent known of the lipopeptide group. The surfactins are very active, and are readily degradable, thus increasing its desirability for use in biological controls. The target group of fungi such as *F. verticillioides* and the other *Fusarium* species are also endophytic and competent in production of similar antibiotics some of which are toxic to bacteria. However, there are strains naturally resistant and laboratory generated mutants that should obviate this problem. These should compete well under field conditions with

F. verticillioides resulting in mycotoxin reduction because under controlled conditions the fumonisin mycotoxins were considerably reduced. Several plasmids have been identified useful for transformation of *B. mojavensis* with several fluorescent proteins to serve as ecological marker for field studies (Olubajo and Bacon 2008). This species of bacteria also produces a battery of extracellular specific enzymes capable of the transformation of maize phytoanticipins into more stable and fungitoxic compounds. Its role in related environmental processes such as phytoremediation is noteworthy, which while not a direct plant growth response is an important process that should facilitate plant growth. Current technology can be used to modify *B. mojavensis* for application in increasing the nutritional value of agronomic plants that are lacking in specific animal and human growth requirements. The process, surrogately transformation of plants, offers an alternative to genomic transformation of plants, which is controversial and objectionable to some.

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Chapter 3
Use of Plant-Associated *Bacillus* Strains as Biofertilizers and Biocontrol Agents in Agriculture

Rainer Borriss

3.1 Introduction

In spite of limited arable land coupled with rising demand of a steadily increasing human population, food supply is a global challenge making production of high-quality food, free from unacceptable levels of chemicals, a pressing need (Table 3.1).

Paradoxically, this increased demand has led to the development of agricultural practices that are undesired and increase disease pressure on plants. An extreme example, impressively illustrating our situation, was recently reported (Guo et al. 2010). Overuse of N-fertilizer contributes substantially to regional soil acidification in China. Since 1980, crop production has increased with rapidly increasing N-fertilizer consumption. Decreasing N use efficiency, mainly due to increasing soil acidification, results from this practice. More and more N-fertilizer is being lost to the environment, causing further negative environmental impacts.

It has been estimated that approximately one third of the food crop is destroyed every year due to attack by insects, pathogenic fungi, bacteria, and nematodes. Current worldwide potato crop losses, due to late blight caused by pathogenic fungus *Phytophthora infestans*, are at $6.7 billion, for instance (Haverkort et al. 2008). At present, the major strategies against damages caused by plant pathogens are chemical pesticides or resistant plant cultivars. However, there are major limitations in using both strategies. Firstly, agrochemicals do not prevent all diseases, and toxic residues can accumulate in the soil and food chain. Therefore, the use of many agrochemicals was banned or restricted, because of environmental and health risks.

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Table 3.1 Development of world population and arable land for food production

Year	World population (billion)	Arable land and permanent crops (billion hectares)	Farmland per person (hectares)
1950	2.5	1.3	0.52
1975	4.1	1.4	0.34
2000	6.1	1.5	0.25
2025	8.0	1.5	0.19
2025	9.2	1.5	0.16

Source: United Nations, Riggs J (2009) 8th International PGPR workshop, Portland

Secondly, resistance of genetically resistant cultivars is often broken by the pathogen within a few years and frequently accompanied by a reduction in yield (Fry 2008). Typically, there is a lack of acceptance among the public for genetically modified (GM) crops.

Environment-friendly biotechnological approaches, such as the use of microbial biopesticides (Box 3.1), offer alternatives to chemical control of plant diseases and pests. Among these alternatives, the development of bioformulations, which are manufactured from plant-growth-promoting rhizobacteria (PGPRs) with biocontrol activity (Lugtenberg and Kamilova 2009), is exemplary and their use is steadily increasing.

Although development of biological products based on beneficial microorganisms seems to be a very recent and "modern" approach, the first product named "bacteriological fertilizer for the inoculation of cereals" was marketed, as early as 1897, under the proprietary name "Alinit" by "Farbenfabriken vorm. Friedrich Bayer & Co." of Elberfeld, Germany, today's Bayer AG. The product was based on a *Bacillus* species now known by its taxonomic name *Bacillus subtilis*. According to the contemporary literature, the use of Alinit raised yields up to 40% (Kilian et al. 2000). Although biological control is subject of academic research for more than 50 years, the next successful attempt to apply endospore-forming bacilli in large scale was performed nearly 100 years after Alinit was commercialized. In the 1990s, several PGPR-based products became commercially available in the USA. Earlier attempts to commercialize products containing fluorescent pseudomonads failed due to lack of long-term viability (Kloepper et al. 2004). Intensive screening and field testing led to commercial development of diverse *Bacillus* strains as biological control agents (McSpadden Gardener and Fravel 2002) (Table 3.2).

Plant rhizosphere is a highly competitive environment in which bacteria are abundantly present due to availability of nutrients secreted by the plant root. Some of these bacteria which are living within or in the vicinity of plant roots and supporting plant growth are generally referred as being "PGPRs" (Kloepper et al. 1980). In many cases, their plant-growth-promoting activity is linked with their ability to suppress soil-borne plant pathogens (bacteria and microfungi), occurring in the competing microflora ("biocontrol"). Different mechanisms are discussed in this context. Besides production of antimicrobial ("antibiotics") and nematocidal compounds, also stimulation of plant-induced systemic resistance (ISR) and subtle

Box 3.1 Chemopesticides and Biopesticides

The total market for global and synthetic pesticides is valued at $26.7 billion in 2005 and is expected to decline to $25.3 billion in next years. The challenge of new and more stringent chemical pesticide regulations, combined with increasing demand for agriculture products with positive environmental and safety profiles, is boosting interest in biopesticides.

Biopesticides are defined by the US Environmental Protection Agency (EPA) as pesticides derived from natural materials, such as animals, plants, bacteria, and certain minerals. The industry is still considered a niche sector, accounting for around 2% of the overall global pesticide market. It is expected that its share of the market will increase, at an annual average growth rate 9.9%, to over 4.2% corresponding $1 billion by now (2010).

The EPA defines three kinds of biopesticides: microbial, consisting of microorganisms; biochemical, which are naturally occurring substances such as extracts and pheromones; and plant-incorporated protectants, which are substances that plants produce from genetic material added to the plant. Some players include macrobials, such as beneficial insects and nematodes, as another biopesticide type. Genetically modified crops, however, are generally excluded.

While biopesticides are typically seen as an alternative to synthetic chemicals, some as the medium-sized biopesticide company AgraQuest see biopesticides as complementary to conventional pesticides already on the market. It is possible that the emerging low-chemical pesticide sector, where biopesticides can be added in a spray program to reduce the amount of synthetics to their lowest label rate, becomes an interesting alternative to selling biopesticides exclusively into the niche organic market. According to Meadows-Smith (AgraQuest), this sector will grow to be worth $5–10 billion by 2017 [http://www.icis.com, June 9, 2009 (Source: ICB)].

pathogen–biocontrol interactions contribute to the suppressive effect (Haas and Defago 2005). In other PGPRs, termed "biofertilizer," plant growth promotion (PGP) dominates. The mechanisms that are involved in this process can include nitrogen fixation, phosphate and mineral solubilization, and the production of macromolecule degrading enzymes (amylases, proteases, and hemicellulases), phytohormones (auxin, cytokinin, and gibberellins), and volatile growth stimulants (such as ethylene and 2,3-butanediol). In recent years, PGPRs are increasingly commercially applied in agriculture to enhance yield of crops and vegetables and to reduce use of harmful agrochemicals (e.g., chemical fertilizers and pesticides; see Box 3.2). Representatives of such plant-beneficial bacteria are widely spread among Gram-negative and Gram-positive bacteria, but in recent years representatives of *Pseudomonas* and *Bacillus* have been attracting main attention. Main subject of present and past research concerning molecular principles, which are underlying beneficial interactions between bacteria and the plant, is *Pseudomonas fluorescens*

Table 3.2 Examples for commercial use of *Bacillus*-based bioformulations in agriculture

Commercial name	*Bacillus* strain	Known properties	Company
Kodiak™	*Bacillus subtilis* GB03	EPA-registered (1992) biological seed treatment fungicide (e.g., *Rhizoctonia* and *Fusarium*) with demonstrable PGR activity. Efficient in cotton, beans and vegetables	Bayer Crop Science, North Carolina, NC (former Gustafson, LLC)
Yield Shield®	*Bacillus pumilus* GB34 (=INR7)	EPA-registered biofungicide, elicits ISR	
BioYield™	*Bacillus amyloliquefaciens* GB99 + *Bacillus subtilis* GB122	Combination of strong ISR activity (IN937a = GB99) with PGR activity (GB03 = GB122)	
Subtilex®	*Bacillus subtilis* MBI600, isolated from phylloplane of Vicia faba	EPA-registered biofungicide (*Fusarium*, *Rhizoctonia*, *Alternaria*, *Aspergillus*)	Becker Underwood Saskatoon, Saskatchewan, Canada
VAULT®	*Bacillus subtilis* MBI600 + rhizobia strains	Produced by "BioStacked® Technology" (2004), enhancing growth soy beans and pea nuts	
SERENADE®	*Bacillus subtilis* QST713	Biofungicide for prevention, suppression, and control of soil born plant pathogens. It protects vegetables, fruit, nut and vine crops	AgraQuest Inc., Davis, California, CA
SONATA®	*Bacillus pumilus* QST2808	EPA-registered biofungicide, powdery mildew control	AgraQuest Inc., Davis, California, CA
RhizoVital® 42 liquid	*Bacillus amyloliquefaciens* FZB42	Biofertilizer, plant-growth-promoting activity, provides protection against various soil-borne diseases	ABiTEP GmbH, Berlin, Germany
RhizoPlus®	*Bacillus subtilis* FZB24[a]	Plant-growth-promoting rhizobacterium and biocontrol agent. It can be used for potatoes, corn, vegetables, fruits and also turf. Registered by BBA	ABiTEP GmbH, Berlin, Germany
Taegro®	*Bacillus subtilis* FZB24[a]	EPA-registered biofungicide for use in North America	Earth Biosciences Inc., USA/now acquired by *Novozymes Biologicals, Inc*., Salem, Virginia, VA
POMEX	*Bacillus subtilis* CMB26	Microbial fungicide, control and inhibition germination effect on powdery mildew, *Cladosporium fulvum* and *Botrytis cinerea*	NIN Co. Ltd., South Korea

[a]FZB24 has to be renamed "*B. amyloliquefaciens*" due to its similarity to the group of plant-associated *B. amyloliquefaciens* strains (Idriss et al. 2002). In fact, the whole genomic sequence of FZB24 is nearly 100% identical with that of FZB42 (Michael Rey, Novozymes, Inc., personal communication)

Box 3.2 Agriculture Chemical Firms' Interest in Biopesticides Rises
In recent years, use of biologicals in plant protection is steadily increasing and begins to replace in part chemical pesticides. This development is also more and more recognized by large chemical companies, hitherto exclusively focused on the production of chemicals. Combining traditional chemical seed treatment with *Bacillus*-based treatments becomes an interesting alternative. Bayer Crop Science sells two EPA-registered biofungicides that are composed of PGPR: Kodiak® concentrate and Yield Shield®. Moreover, the same company acquired BioNem, the biological assets, and technology of AgroGreen, Ashdod, Israel, in March 2009. AgroGreen is a leading company in the bionematocides business, including the development of BioNem (active ingredient: *Bacillus firmus*). Novozymes has acquired Earth Biosciences Inc., a small company devoted to the production of biopesticides, in October 2006, and started to sell *B. subtilis* FZB24, for instance. Other large crop protection industry players – Arysta Life Science (Japan), Makhteshim Agan (Israel), and FMC (USA), just to name a few – have collaborated with smaller biopesticide companies to develop their own biocontrols. DuPont signed an agreement in June 2007 to provide Marrone Bio Innovations (MBI) with exclusive access to natural product discoveries for development as biopesticide products. In April 2009, BASF signed a licensing, supply, and distribution deal for California, US-based AgraQuest's Serenade biofungicide, which has received Annex I registration in the EU. Through the agreement, BASF gains right to Serenade for foliar and drench applications in countries throughout Europe, Africa, Middle East, Asia, and Latin America.
http://www.farmchemicalsinternational.com September 2009.

(Haas and Defago 2005). The whole genome of strain *P. fluorescens* Pf5 was sequenced in the year 2005 (Paulsen et al. 2005), as the first representative of PGP rhizobacteria. Unfortunately, despite the great progress already obtained in *Pseudomonas* research, the commercial use of this bacterium in agriculture is limited due to difficulties in preparing stable and long-living bioformulations, competitive to chemical pesticides (see above). Focus is made on describing our present knowledge about endospore-forming PGPRs (*Bacillus* spp. and *Paenibacillus polymyxa*).

3.2 Bacilli as Biofungicides and Biofertilizers: Advantages and Disadvantages

At present, bacilli are by far the most widely used bacteria on biopesticide market in North America. Advantages and disadvantages of their application are summarized in Table 3.2. Vast majority of these products (market share 79%) are

Table 3.3 Microbial products on Annex 1 Dir. 91/414/EEC or in the registration process (*–xx*) and time frame for processing of dossiers in the EU and the USA (EPA)

Product	Organism	EU period (month.year)	EU time frame (months)	EPA time frame (months)
Preferal®	*Paecilomyces fumosoroseus*	5.94–6.01	85	60
Contans®	*Coniothyrium minitans*	11.98–8.03	57	15
Cedomon®	*Pseudomonas chlororaphis*	1.96–4.04	99	–
AQ10®	*Ampelomyces quisqualis*	2.96–10.04	104	?
Spodex®	*Baculovirus*	7.97–*xx*	>90	12
Prestop®	*Gliocladium catenulatum*	3.99–10.04	67	13
Serenade®	*Bacillus subtilis*	5.00–*xx*	>56	14
Sporodex®	*Pseudomyza flocculosa*	3.01–*xx*	>46	39
Bioact®	*Paecilomyces lilacinus*	10.02–*xx*	>27	11
Average time period			>70	23.4

According to Ehlers (2006)

Table 3.4 Importance of biopesticides in North America

Estimated North American biopesticide market by product type				
	Sales (US$)			
	Canada	Mexico	USA	Total
Bt aizawai/kurstaki	4,100,000	12,000,000	35,150,000	51,250,000
Bt H14	500,000	1,000,000	20,000,000	21,500,000
Bacillus sphaericus	30,000	–	4,000,000	4,030,000
Bacillus subtilis	20,000	–	10,000,000	10,020,000
Other bacteria	50,000	50,000	1,050,000	1,150,000
Virus-based products	10,000	20,000	1,000,000	1,030,000
Fungus-based products	10,000	2,000,000	9,750,000	11,760,000
Protozoa-based products	–	–	50,000	50,000
Nematodes	200,000	50,000	8,000,000	8,250,000
Total	4,920,000	15,120,000	89,000,000	109,040,000

Report prepared by CPL Business Consultants, July 2006. http://www.bio-pesticides.org

still bioinsecticides mainly prepared from *Bacillus thuringiensis*, *B. kurstaki*, and *B. sphaericus*. However, *B. subtilis* and its closest relatives are increasingly used as biofertilizers and biofungicides with a market share exceeding 9% in the year 2006 (Table 3.4). The successful use of such spore formulations is sometimes hampered by varying success after application due to our insufficient knowledge about the molecular principles underlying their beneficial effects (Emmert and Handelsman 1999). Another problem hindering use of biopesticides, especially in

Europe, is the costly and time-consuming registration procedure, which largely follows rules developed for synthetic pesticides; thus, many possibly irrelevant investigations, e.g., on the ecotoxicology are requested. Costly risk assessment studies and long-term evaluation of dossiers keep these products off the market. EU Annex I inclusion for the microorganism *Pseudomonas chlororaphis* (the active ingredient of the product Cedomon®) took more than 8 years and an investment of over 2.5 million €. This situation cannot attract monetary inputs into the biocontrol sector. Compared to the biocontrol market in the USA (59 BCAs registered), the market in Europe is exposed to major restrictions, resulting in only five BCAs included in Annex I. More strikingly, the time frame for the EU evaluation of dossiers is >70 months compared with ca. 23 months for the same products in the USA (Table 3.3).

3.3 *Bacillus amyloliquefaciens* and *Paenibacillus polymyxa*: Plant-Growth-Promoting Endospore-Forming Bacteria with Biocontrol Activity

These two Gram-positive, aerobic, endospore-forming bacteria with plant-growth-promoting activity are investigated more deeply for their potential to suppress plant-pathogenic microflora and to stimulate plant growth. Both bacteria belong to the order Bacillales, but are distantly related to each other. The genera *Bacillus amyloliquefaciens* FZB42 (family 1 *Bacillaceae*) are closely related to *B. subtilis* type strain 168, but are distinguished by its ability to form biofilms and to support plant growth and to suppress plant pathogens living in plant rhizosphere. It was isolated from soil infested with plant pathogens (Krebs et al. 1998) and is successfully commercialized as biofertilizer by ABiTEP GmbH (http://www.abitep.de/). The beneficial action of FZB42 and its closely related "cousin" FZB24 with respect to that of PGP and biocontrol is well documented (Bochow 1992; Dolej and Bochow 1996; Kilian et al. 2000; Schmiedeknecht et al. 1998, 2001; Grosch et al. 1999; Bochow et al. 2001; Yao et al. 2006; Burkett-Cadena et al. 2008). The genome of FZB42 was the first Gram-positive PGPR that has been completely sequenced (Chen et al. 2007). *Paenibacillus polymyxa* E681 (family 5 *Paenibacillaceae*) belongs to a group of facultative anaerobic Bacillales, in that their ellipsoid spores swell the mother cell (Fritze 2004). The full genome of E681, about 5 Mbp in size, was also sequenced at the same time as FZB42 by the Genome Research Center at Korea Research Institute of Bioscience and Biotechnology (S-H Park personal communication), but unfortunately, the whole sequence is not published till now. *P. polymyxa* E681 was isolated from the rhizosphere of winter barley grown in Korea and is known to suppress plant diseases and produce antimicrobial compounds and phytohormones (Phi et al. 2008a, b). Besides the beneficial effects exerted by *P. polymyxa*, e.g., against oomycete plant pathogens, inoculation of *Arabidopsis thaliana* by *P. polymyxa* (in the absence of biotic or abiotic stress) results in 30%

reduction in plant growth, indicating a "mild" pathogenic effect (Timmusk and Wagner 1999; Timmusk et al. 2009). Our experiments performed with the natural *P. polymyxa* isolate M1 and *A. thaliana* corroborated this surprising finding suggesting that *P. polymyxa* is to be considered as a "deleterious" rhizobacterium (DRB, Timmusk et al. 2005).

Comparison of the several findings obtained with the two bacteria should allow some general conclusions about the molecular basis of plant–bacilli interactions. In our comparative genome analysis, we will include recent results obtained from the genome of nonplant-associated *B. amyloliquefaciens* type strain DSM7.

3.4 *B. amyloliquefaciens* FZB42: Taxonomy and Comparative Genomics

The complete genome sequence of the *B. subtilis* strain 168 (Kunst et al. 1997) is used successfully to advance our understanding of *Bacillus* physiology, but strain 168 has no known activities related to PGP and biocontrol. Therefore, the genome sequences of *Bacillus* strains, successfully used in improving plant growth and health, should provide insights into the genetic basis for biological control in this species. Pioneering work of Joshi and McSpadden Gardener (2006) reveals that sequences of genes involved in antibiotic production are useful in discriminating the plant-beneficial bacilli from common soil bacteria. The genomes of two commercialized strains were compared with *B. subtilis* 168 by suppressive subtractive hybridization. It rules out that the most effective biocontrol strains, GB03, QST713, MB1600, and FZB42, are closely related and share multiple genetic elements responsible for pathogen suppression.

Taxonomic trees calculated from *gyrA* and *cheA* nucleotide sequences suggested that plant-associated (PGPR) *B. amyloliquefaciens* strains, such as FZB42, and other strains with PGP and biocontrol activity, such as FZB24 (RhizoPlus®), GB03 (Kodiak), and QST713 (Serenade), are closely related to a group of plant-associated or endophytic *Bacillus* strains (Chen et al. 2009a), which are forming an own ecomorph distinct from the *B. amyloliquefaciens* type strain DSM7^T (Reva et al. 2004).

A comparative analysis between the genome sequences of the plant-associated FZB42 (Fig. 3.1) with the genome of *B. amyloliquefaciens* DSM7^T revealed obvious differences in the variable part of the genomes, while the core genomes were found very similar. FZB42 and DSM7^T have in common 3,345 genes (CDS) in their core genomes; 547 coding sequences, CDS (DSM7^T), and 344 CDS (FZB42) were singletons. The core genome shared by both strains exhibited 97.89% identity on amino acid level. *B. subtilis* DSM10^T shared a similar number of CDS with the two *B. amyloliquefaciens* strains, 3,222 CDS with DSM7^T, and 3,182 with FZB42, respectively. The number of genes representing the core genome from strains

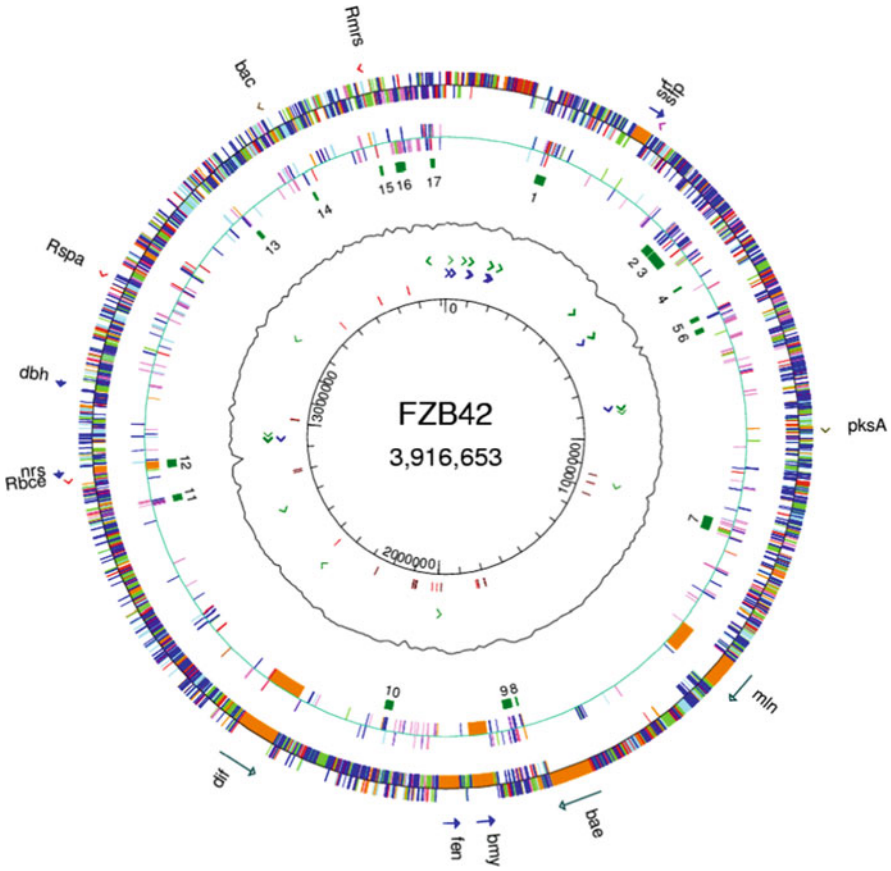

Fig. 3.1 *The genome of B. amyloliquefaciens FZB42*. The likely origin of replication was defined by similarities to the corresponding regions in *B. subtilis* and *B. licheniformis*. The first nucleotide pair of the *B. amyloliquefaciens* genome was defined congruent with *B. subtilis* as the A:T nucleotide pair located 412 bps upstream from *dnaA*. *Outmost circle*: genes and gene clusters involved in synthesis and export (detoxification) of secondary metabolites: *srf* surfactin; *sfp* phosphopantheteinyl-transferase; *pksA* regulator polyketide synthesis; *mln* macrolactin; *bae* bacillaene; *bmy*, bacillomycin D; *fen* fengycin; *dif* difficidin; Rbce, bacitracin export; nrs, hypothetical dipeptide; dbh, bacillibactin; Rspa, subtilin immunity; *bac*, bacilysin; Rmrs, mersacidin immunity. *First circle*: all genes in color code according to their functions: cell envelope and cellular processes, *green*; information pathways, *orange*; intermediary metabolism, *pink*; other functions, *red*; unknown, *black*. *Second circle*: genes not conserved in *B. subtilis* including four giant gene clusters involved in synthesis of secondary metabolites (*orange*); *third circle*: the numbered 17 DNA islands (*green*), *fourth circle*: GC content profile, *fifth circle*: rRNAs (*green*), *sixth circle*: tRNAs (*cyan*), *seventh circle*: prophages (*black*), transposons, and IS elements (*red*), *eighth circle*: scale (bp)

FZB42, DSM7^T, and *B. subtilis* DSM10^T was calculated as being 3,098 and their identity was 92.25% (Borriss unpublished result).

B. amyloliquefaciens strains are distinguished by their potential to synthesize nonribosomally a huge spectrum of different secondary metabolites, many of them with antibacterial and/or antifungal action (Schneider et al. 2007). A survey of the genomes of four other *B. subtilis* strains (http://www.bacillusgenomics.org/bsubtilis/) and that of *B. amyloliquefaciens* DSM7^T corroborated that strains belonging to *B. subtilis sensu stricto* and *B. amyloliquefaciens* type strain did not produce the polyketides difficidin and macrolactin and are often impaired in their ability to produce lipopeptides other than surfactin.

The members of the *B. amyloliquefaciens* DSM7^T-related clade secrete a starch-liquefying alpha-amylase (*amyA*) with high industrial potential, while *B. subtilis* secretes a saccharifying enzyme. Sequences of both genes are not similar. Unlike DSM7^T, the genome of FZB42 did not contain the *amyA* sequence, corroborating an earlier finding of Reva et al. (2004), who were unable to amplify *amyA*-like sequences in plant-associated *B. amyloliquefaciens* strains. Instead, FZB42 and FZB24 genomes possessed an *amyE*-like gene, resembling that of *B. subtilis*.

Environmental and industrial *B. subtilis*-group strains secrete different hydrolases enabling them to use external cellulosic and hemicellulosic substrates present in plant cell walls. Two of these enzymes, endo-1,4-β-glucanase or carboxymethylcellulase (CMCase, cellulase, EC 3.2.1.4) and endo-1,4-β-xylanase (xylanase, 1,4-β-xylan xylanohydrolase, EC 3.2.1.8), are encoded in *B. subtilis* 168 by the genes *eglS* and *xynA*, respectively (Wolf et al. 1995). The same genes were also present in the genome of FZB42, but were absent in *B. amyloliquefaciens* DSM7^T.

Strains *B. amyloliquefaciens* DSM7^T, FZB42, and *B. subtilis* DSM10^T evolved independent restriction modification (RM) systems without sequence similarity. Moreover, we found that other representatives of the plant-associated *B. amyloliquefaciens* ecomorph evolved unique RM systems, different from FZB42. On the contrary, type II restriction modification genes present in DSM7^T shared 98% (methyltransferase) and 100% (restrictase) identity, respectively, with the *Bam*HI system known for *B. amyloliquefaciens* H (Roberts et al. 1977).

The genome of FZB42 harbors nine gene clusters directing nonribosomal synthesis of bioactive peptides and polyketides (Fig. 3.1). In total, FZB42 dedicates about 340 kb, corresponding to 8.5% of the whole genome to nonribosomal synthesis of antimicrobial compounds and siderophores (Chen et al. 2007). For comparison, the genomes of nonplant-associated DSM7^T and *B. subtilis* 168^T devote only 4–5% of their whole-genome capacity for synthesis of such compounds, suggesting that FZB42 became adapted to the highly competitive environment of plant rhizosphere (see below). *P. polymyxa* genome contains two gene clusters, involved in nonribosomal synthesis of fusaridicin and polymyxin, respectively (Choi et al. 2008, 2009, B. Niu, unpublished results).

According to the results of our genome comparison, we assume that *B. amyloliquefaciens* FZB42 and DSM7^T belong to taxonomically related but distinct units, and the high number of unique genes (singletons) supports classifying of both strains into separate taxonomic units.

3.5 Plant–Bacteria Interactions

Land plants and bacteria have shared the same environment for approximately 360–480 million years (Kenrick and Crane 1997). The contact between them has developed into various dependencies on both sides. Competition, predation, parasitism, mutualism, and detritivory can be drawn as the main categories of interactions, although they may be confounded in the course of the relationship (Begon et al. 2006). Plants and certain rhizobacteria (PGPRs) form mutually beneficial associations mediated through an exchange of chemical metabolites.

3.5.1 Colonization of Plant Roots by B. amyloliquefaciens *and* P. polymyxa

Occurrence of *B. subtilis* and its closest relatives is not restricted to soil, but they are common inhabitants of plant root zones, probably because of biofilm formation. Efficient colonization of plant roots and ability to compete with other microorganisms present in rhizosphere is a precondition for beneficial action of PGP bacteria, such as FZB42 and E631. Though plant-associated bacilli are less competitive in the rhizosphere than pseudomonads (see above), several examples for colonization of the roots by representatives of, e.g., *B. subtilis*/*amyloliquefaciens* and *P. polymyxa* were reported. The well-known Kodiak strain GB03 is thought to survive on seeds until planted and then uses seed exudates during seed germination, directionally multiplying to reach young roots, and maintaining a robust population in the presence of field crops via plant–microbial interactions (Kloepper et al. 2004). The minimum bacterial density in the soil for triggering observable plant response is ca. 10^4 colony-forming units (cfu) per root. GB03 can maintain populations of 10^5 cfu/root for over 60 days after planting (Kokalis-Burelle et al. 2006). Biocontrol strain *B. subtilis* 6051 adheres to root surfaces of *Arabidopsis*, where it forms biofilms and produces surfactin (Bais et al. 2004).

Some representatives of several *Bacillus* species are able to invade inner tissues of various species of plants, where these microorganisms play an important role in plant protection and PGP. They belong to species generally recognized as free-living soil organisms including *B. amyloliquefaciens* (Reva et al. 2002), *B. cereus* (Pleban et al. 1997), *B. insolitus* (Bell et al. 1995), *B. licheniformis* (Reva et al. 2002), *B. megaterium* (McInroy and Kloepper 1995), *B. pumilus* (McInroy and Kloepper 1995), *B. subtilis* (Mishagi and Donndelinger 1990), and *Paenibacillus polymyxa* (Shishido et al. 1999). A novel species, named *B. endophyticus*, isolated from the inner tissues of cotton plants, is also described (Reva et al. 2002).

FZB24 strain, closely related to FZB42 (Idriss et al. 2002), was used to coat tomato seeds under in vitro conditions. The seeds were cultivated on Gelrite Murashige-Skoog-medium. FZB24 colonized the root from the treated seeds and closely followed its growth in the rhizosphere region, so that a 0.4–0.8 mm thick

film of bacteria was formed around the root. However, the number of FZB24 cells as a percentage of the total number of microorganisms was found to steadily decrease in the course of time. Potatoes from three practical field trials in 1998 were harvested to determine the number of *Bacillus* sp. found on the potatoes. The number of bacilli from the plots in which the seed tubers had been treated with FZB24® was not higher than that yielded from untreated seed tubers (Kilian et al. 2000). Our experiments performed with maize seedlings treated with green fluorescent protein (GFP), labeled FZB42 strain (B. Fan, unpublished results), revealed that colonization preferentially takes place at the junction area, where lateral roots and root hairs emerge from primary root. The number of FZB42 microcolonies decreased steadily toward root tips. Junction regions in the vicinity of root hairs were preferentially colonized by FZB42. Bacteria grew along root hairs or even forming clusters, circling a root hair. Images obtained by scanning electron microscopy revealed that FZB42 is able to form biofilm-like structures on the root hair surface. In contrast, colonization of *Arabidopsis* roots in a gnotobiotic system took place in the vicinity of root tips (Fig. 3.2), suggesting that colonization strategy is dependent on the host plant. However, no bacteria, invading root epidermis layer cells, were detected indicating that FZB42 grew as a non-endophyte, only able to colonize root surface (B. Fan,

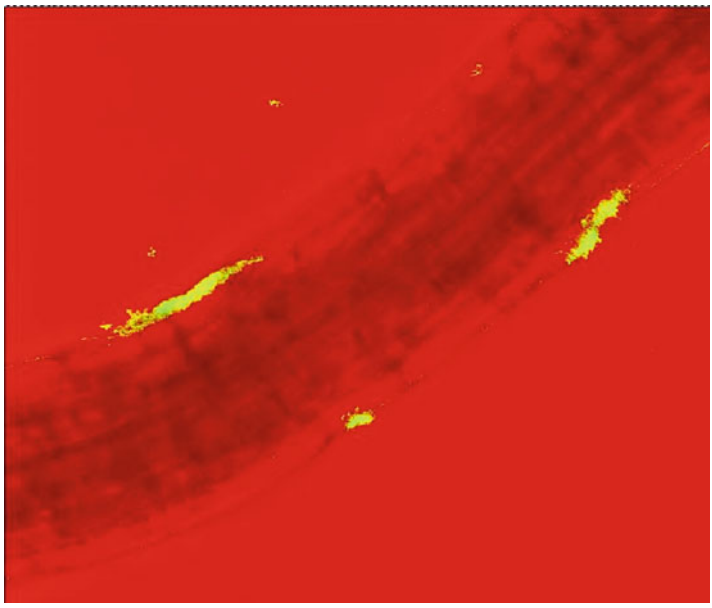

Fig. 3.2 *Colonization of plant roots by GFP-labeled FZB42*. GFP+-labeled strain BF02mut, a derivative of FZB42 (B. Fan, unpublished), forms microcolonies, appearing as *yellow* spots onto the *red* surface of the Arabidopsis primary root, grown in a gnotobiotic system. The confocal scanning laser microscopy (CLSM) image was taken 7 days after inoculation with BF02mut (with courtesy of B. Fan, Bacterial Genetics, Humboldt University Berlin)

unpublished results). Recently, we demonstrated that bacilli used for PGP in culture plants can persist for several months in the natural environment. FZB42 applied to greenhouse cultures of *Antirrhinum majus* was detected 5 months after its application, in soil and at plant remnants, long time after the flowers were harvested (Fig. 3.3).

P. polymyxa was also tagged with the GFP and used for colonizing roots in a gnotobiotic system and in a soil system. In the gnotobiotic system, roots were at first colonized at the tips of the primary roots. A biofilm consisting of bacterial cells and a semitransparent material suggestive of an extracellular matrix was formed within 2 h. *P. polymyxa* cells were able to enter intercellular spaces and to cause a remarkable damage of plant roots. However, they did not spread to the leaves (Timmusk et al. 2005). *P. polymyxa* has a tendency to colonize and to invade plant roots more heavily than FZB42. It is an example for the delicate balance between beneficial and deleterious effects exerted by the same plant-associated bacterium, which can be considered as PGPR and deleterious rhizobacterium, as well.

Rhizosphere competence is linked to the capability of forming sessile, multicellular communities (biofilms) on the surface of root cells ("rhizoplane"). They can be defined as a structured community of cells encased in a self-produced extracellular matrix (Hall-Stoodley et al. 2004). In liquid stand culture, *B. amyloliquefaciens*

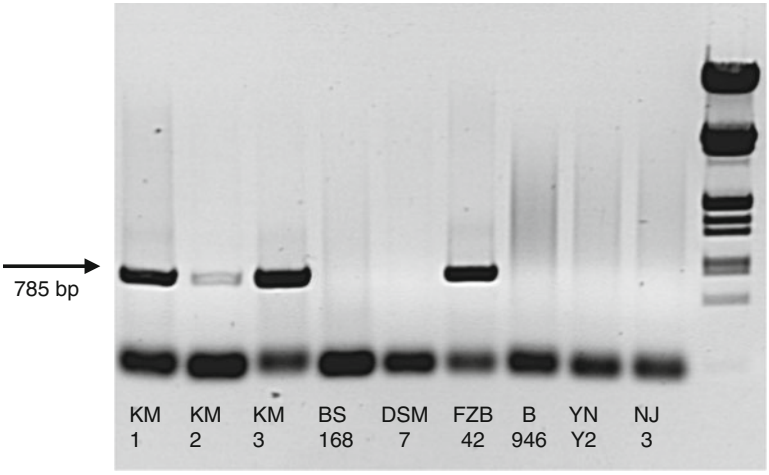

Fig. 3.3 *Detection of FZB42, reisolated from environmental samples*. Five months after its inoculation to Snapdragon plants (Antirhinum major) grown in Chenggong County, Kunming, Yunnan province, China, soil samples were taken and used for reisolation of FZB42. Colonies with the typical morphology of FZB42 were obtained after several steps of enrichment and analyzed for the presence of an unique 785 bp DNA fragment, only present in FZB42, by PCR with the primers: PRBrm5215: tgatggagtaaataataaggctgg, and PRBrm6000: aatacatctaaagttgcatccacc. Agarose gel electrophoresis revealed presence of the 785 bp DNA fragment in the reisolates (KM1-3) and in FZB42. No 785 bp fragment was detected in *B. subtilis* 168, *B. amyloliquefaciens* DSM7T, and other plant-associated *B. amyloliquefaciens* strains isolated from China and belonging to the same ecotype as FZB42 (B946, Chinese Agricultural University, Beijing, Y2, Yunnan Agricultural University, Kunming, and NJ B3, Nanjing Agricultural University, Nanjing)

FZB42 cells formed robust pellicles at the liquid–air interface, whereas domesticated *B. subtilis* strains form thin, fragile pellicles (Chu et al. 2006). It is known that formation of biofilms in *B. subtilis* is a complex process that includes secretion of surfactin lipopeptide (Bais et al. 2004). FZB42 *srf* mutant strains, unable to synthesize surfactin, were impaired in biofilm formation. Double mutants (*sfp yczE*) unable to synthesize 4′-phosphopantetheine-transferase were heavily impaired in forming biofilm and resembled domesticated *B. subtilis* 168. *B. amyloliquefaciens* FZB42 contains the complete set of genes involved in biofilm and fruiting body formation in *B. subtilis*, including the 15-gene exopolysaccharide that holds chains of cells together in bundles (Kearns et al. 2005). An additional gene cluster in the *B. amyloliquefaciens* FZB42 genome, which participates in either exopolysaccharide or lipopolysaccharide biosynthesis, was without counterpart in the nonplant-associated *B. subtilis* 168 and *B. amyloliquefaciens* DSM7^{T}. *Sip* W is a signal peptidase responsible for processing of at least two proteins that play a role in forming the *B. subtilis* biofilm matrix: *Tas* A and *Yqx* M. *Tas* A is a protein component of the biofilm matrix and its correct localization depends on the presence of *Yqx* M (Branda et al. 2006; Chu et al. 2006). The unique genes RBAM00750, RBAM00751, and RBAM00754 encode proteins with a collagen-related GXT structural motif and are probably involved in surface adhesion or biofilm formation (Chen et al. 2007). These and other genes involved in biofilm formation in bacilli are governed by complex, regulatory networks consisting of several transcription factors such as Spo0A, DegU, and AbrB (Hamon and Lazazzera 2001; Hamon et al. 2004).

Motility is another prerequisite for active colonization of plant root cells by bacteria. *B. amyloliquefaciens* displayed a robust swarming phenotype. Both the protein encoded by *swr*A and the lipopeptide surfactin are thought to be essential for swarming motility. These proteins permit colonization of surfaces and nutrient acquisition through their surface wetting and detergent properties (Kearns et al. 2005). Furthermore, a *swr*A gene homologue, sharing 88% identity to *swr*A wild-type alleles present in environmental *B. subtilis* isolates, is in the *B. amyloliquefaciens* genome (Chen et al. 2007).

3.5.2 *Effect of Root Exudates on Root-Colonizing Bacilli*

Interactions between rhizobacteria and the root surface are poorly understood. This is especially true for PGP bacilli, where studies describing plant–bacterial interactions are rare. Signaling events on the root surface, e.g., oxidative burst, lead to crosstalk in plant–pathogen relationship. Oxidative burst is connected with production of large amounts of reactive oxygen species (ROS) and is one of the earliest responses of plants upon microbial attack (Laloi et al. 2004). Beneficial *B. subtilis* FB17 was also recognized by plants by a chemical-dependent cascade, which is independent of the salicylic acid (SA), jasmonic acid (JA), or ethylene pathways. FB17 did not colonize on the roots of NahG, a transgenic Arabidopsis

line for salicylate hydroxylase that produces catechol as the degradation product of SA, suggesting that catechol may play a direct role in inhibiting *B. subtilis* biofilm formation on the NahG roots. The authors concluded that root-derived catechol production elevates ROS, which in turn suppresses *B. subtilis* biofilm formation (Rudrappa et al. 2007).

Root exudates provide energy-rich organic acids that are metabolized within hours by soil microorganisms (Jones et al. 2003), while PGPRs generate an array of biologically active compounds that elicit growth promotion and ISR, in plants.

Constituents of root exudates belong to three classes: (1) low-molecular weight, (2) high-molecular weight, and (3) volatiles. Low-molecular-weight compounds represent the main portion and consist of sugars, amino acids, organic acids, phenolics, vitamins, and various secondary metabolites (Uren 2007). Interestingly, root and seed exudates are different in respect of amino acids (His + Gly, Thr, Ala, Tyr, Val, Phe, Ile, and Leu), citrate, and sugars. Amount of these compounds was found higher in seed than in root exudates (Costa-Carvailhis 2010). High-molecular-weight compounds consist of mucilage and proteins, while carbon dioxide, certain secondary metabolites, alcohols, and aldehydes constitute volatiles (Ortiz-Castro et al. 2009). Root exudates such as malic acid contain signaling molecules attracting *B. subtilis* to the root (Rudrappa et al. 2008).

Our preliminary proteomic analysis suggested that root exudates, prepared from maize seedlings, upregulate enzymes, probably involved in response to oxidative stress generated in plant roots by, for example, thiol peroxidase or enzymes catabolizing compounds secreted by plant roots. Enzymes of this type include bacillopeptidase F, γ-glutamyl transpeptidase, and phosphotransacetylase (which act on organic acids). The hook-associated flagellar proteins HAP1 and HAP2 and the HAG flagellin are differently affected by exudates secreted by plant roots. Although level of the *flgK* gene product HAP1 was reduced in the presence of root exudates, expression of the *fliD* and *hag* gene products (HAP2 and HAG) was upregulated. Flagellin proteins are thought to elicit a host basal defense against potential pathogens. It is likely that variations of the flagellins and other exposed bacterial proteins during colonization surfaces of plant roots might enhance tolerance of *B. amyloliquefaciens* FZB42 against unfavorable plant responses and thereby contribute to its competence in the rhizosphere.

3.5.3 Bacterial Compounds, Beneficial for Plant Growth

Widely accepted mechanisms for PGP include bacterial synthesis of plant hormones (Loper and Schroth 1986), breakdown of plant-produced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate deaminase (Glick et al. 1999), a mechanism mainly investigated in Gram-negative PGPR and here not further discussed, and increased mineral and nitrogen availability in soil (Lin et al. 1983). In addition, a blend of volatiles, especially 2,3-butanediol produced by selected PGP *Bacillus* strains GB03 and IN937a, enhance growth of *Arabidopsis*

seedlings (Ryu et al. 2003). Later experiments demonstrated that besides PGP, the same volatiles also mediate ISR in plants. Volatiles produced by FZB42 exerted the same beneficial effects on plant growth and ISR as described for GB03 and IN937a (Borriss and Ryu, unpublished).

Plants and microorganisms abound with natural chemicals, many of them are volatiles. Ethylene, a molecule from other chemical development, was the first gaseous hormone discovered (Bleeker and Kende 2000). Selected PGP *Bacillus* strains release a blend of volatile components (VOCs). The volatiles 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, released by PGPR *B. subtilis* GB03 and *B. amyloliquefaciens* IN937A, trigger enhanced plant growth (Ryu et al. 2003). To synthesize 2,3-butanediol, *B. subtilis* converts pyruvate into acetolactate by the enzyme-acetolactate synthase, encoded by the *alsS* gene, followed by conversion of α-acetolactate to acetoin by the *alsD*-encoded-acetolactate decarboxylase (Renna et al. 1993). This alternative pathway for pyruvate catabolism is favored under low pH or oxygen-limiting conditions (Ramos et al. 2000). Recently, the metabolic step from acetoin to 2,3-butanediol was shown as being catalyzed by acetoin reductase/2,3-butanediol dehydrogenase (AR/BDH), encoded by the *bdhA* gene (Nicholson 2008). The *B. amyloliquefaciens* FZB2 genome harbors all the three genes involved in 2,3-butanediol synthesis and a plant-growth-promoting effect of volatiles released by FZB42 was demonstrated with divided Petri dishes (I plates) that contain a center partition so that only airborne signals could be transmitted between bacteria and *Arabidopsis* seedlings. FZB42 strains with knockout mutations in *alsS* and *alsD* genes did not trigger plant growth under same conditions (Fig. 3.4).

At present, some progress in elucidating the mechanism of bacterial VOC action on plants is made. Work, performed in the Paré laboratory, showed that emitted bacterial volatiles trigger growth promotion in *Arabidopsis* by regulating auxin homeostasis. Gene expression for auxin synthesis was upregulated in aerial regions of GB03-exposed plants; auxin accumulation decreased in leaves and increased in roots due to activation of basipetal auxin transport. In consequence, cell elongation in leaves was enhanced due to a decrease in indole-3-acetic acid (IAA) concentration, while formation of lateral roots is enhanced at higher auxin concentrations. In addition, cell expansion was also affected positively by activation of cell wall loosening enzymes. In summary, VOCs secreted by selected PGPR may trigger sustained PGP by an optimized coordination between root and leaf development (Zhang et al. 2007).

Besides stress, exerted by different pathogens (biotic stress), plants are often exposed to different kinds of abiotic stress exerted by drought, salt, nutrient deficiency, or excess of heavy metals. Similar as in biotic stress, PGPRs can act as elicitors of tolerance against these kinds of abiotic stress (induced systemic tolerance or IST) as stated by Yang et al. (2009). Inoculation with *Paenibacillus polymyxa* enhances drought tolerance of *Arabidopsis thaliana* (Timmusk and Wagner 1999). Volatiles emitted from PGPR GB03 are involved in IST against salt stress. It is assumed that plant perception of bacterial VOC causes a tissue-specific regulation of Na^+ homeostasis under salt stress (Ryu et al. 2004a). PGP representatives of several *Bacillus* spp., including *B. megaterium*, *B. mucilaginosus*,

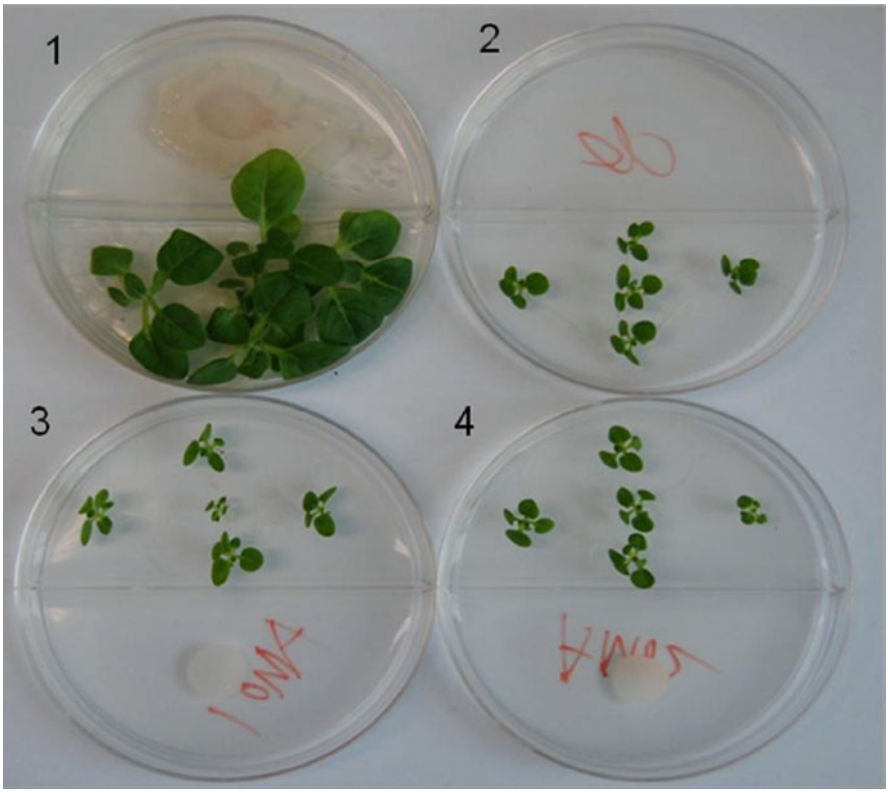

Fig. 3.4 *Influence of volatiles emitted by FZB42 on plant growth*. The picture was taken 4 weeks after inoculation of Arabidopsis with FZB42 (1), and the mutant strains, unable to produce volatiles due to defects in the *alsD* (3) and *alsS* (4) gene, respectively. The water control is indicated by 2. For further details, see text (with courtesy of Ryu C-M, KRIBB, Daejeon, South Korea)

and *B. subtilis*, are successfully used in bioremedation of heavy metals (Zhuang et al. 2007).

The well-documented plant-growth-promoting effect by root-colonizing *Bacillus* and *Paenibacillus* strains (Kloepper et al. 2004; Timmusk and Wagner 1999) is at least partially due to the bacterial production of plant hormones such as IAA, cytokinins, and gibberellins (Bloemberg and Lugtenberg 2001; Bottini et al. 2004). IAA-based PGP effects are detected in 80% of bacteria isolated from the rhizosphere (Loper and Schroth 1986); however, reports demonstrating production of auxins by Gram-positive free-living soil bacteria are scarce. Massive synthesis of IAA by the Gram-positive phytopathogen *Rhodococcus fascians* indicates a delicate balance between beneficial and detrimental effects in dependence on IAA concentration (Vandeputte et al. 2005). GC-MS verifies gibberellin production by *B. pumilus* and *B. licheniformis* (Gutierrez-Mañero et al. 2001). Representatives of the *B. subtilis*/*amyloliquefaciens* group are able to produce substances with auxin

(IAA)-like bioactivity. The presence of IAA-like compounds in the culture filtrates of several members of this group, including FZB42, is detected by enzyme-linked immunosorbent assay tests with IAA-specific antibodies, when those strains are grown at low temperature and low aeration rate (Idris et al. 2004).

Analyses by GC-MS performed with culture filtrates of FZB42 demonstrated the presence of IAA. In the presence of 5 mM tryptophan, a fivefold increase in IAA secretion was monitored. In addition, in the *trp* auxotrophic strains E101 (*trpBA*) and E102 (*trpED*), and in two other strains bearing knockout mutations in genes probably involved in IAA metabolism, the amount of IAA in the culture fluid is diminished. Notably, these mutant strains are less efficient in promoting plant growth, indicating that Trp-dependent synthesis of auxins and PGP is functionally related in *B. amyloliquefaciens* (Idris et al. 2007).

Similar to FZB42, *Paenibacillus polymyxa* synthesizes IAA from the main precursor tryptophan (Lebuhn et al. 1997). Screening of a mini-Tn10 insertional mutant library reveals few genes, possibly involved in the regulation of IAA biosynthesis in this bacterium (Phi et al. 2008a). In contrast to FZB42, the genome of E681 harbors four open reading frames (ORFs) with similarity to indole-3-pyruvate decarboxylase (IPDC), a key enzyme in indole-3-pyruvic acid (IPA) pathway. One of the ORFs, PP2_01257 was expressed in *Escherichia coli* and the purified recombinant protein was characterized as IPDC. Results of the IAA intermediates accumulated in the E681 culture corroborate that the IPA pathway plays a central role in the IAA production by *P. polymyxa* E681 in the presence of exogenous Trp (Phi et al. 2008b). Results obtained with FZB42 and E681 suggest existence of different Trp-dependent pathways of IAA synthesis in both Gram-positive bacteria. While the IPA pathway with IPDC as key enzyme seems to be the major route of IAA synthesis in *P. polymyxa*, the pathway of IAA synthesis in *B. amyloliquefaciens* remains elusive, but results of our gene knockout study favor a pathway in which the gene products of *ysnE* (putative IAA transaminase) and *yhcX* (putative nitrilase) take part. Production of other plant hormones, which can also affect plant growth, e.g., gibberellins, was ruled out in FZB42 (Idris et al. 2007).

3.6 Biofertilizer Function

Bacilli are omnipresent in agricultural soils and many of them are able to colonize plant roots (see Sect. 3.5). Increasing costs for chemical fertilizers and increasing environmental problems are linked with massive use of agrochemicals; PGP bacilli contribute to adequate plant nutrition and reduce negative environmental effect of soil fertilization. There are some encouraging examples of PGPRs that enable crops to maintain productivity with reduced rates of fertilizer applications (Borriss et al. 2006; Kumar et al. 2009; Maheshwhari et al. 2011). Extensive field trials conducted with FZB24 over several years with maize, potatoes, and cotton (Yao et al. 2006) resulted in an increase of yield of about 7.5–10%. Best results were obtained if application of bacilli to potato tubers was combined with use of chemical fungicides.

In such cases, an increase of tuber yield up to 40% was obtained. Reduction of the amount of chemical fertilizer (N–P–K) was also achieved.

3.6.1 Soil Aggregation

The effects of soil aggregation on root growth were extensively studied, because aggregation is involved in plant nutrient uptake. Aggregation of soil in the rhizosphere depends on many factors, such as physical properties, climatic conditions, and biological activities. Close to the surface of roots, soil structure can be modified directly by rhizodeposition or indirectly by stimulation of microbial growth in the rhizosphere. For example, secretion of polysaccharides is characteristic of root cell surfaces and is stimulated by the presence of bacteria in the rhizosphere. In fact, it has been shown that the rhizobacterium *P. polymyxa* utilized sucrose, a major carbon component of wheat root exudates, to synthesize levan, a fructosyl polymer, composed of residues linked to two to six with branches linked two to one. Synthesis of levan is accomplished by a sucrose-inducible β-fructosyl-transferase, called levansucrase, and encoded by the *sacB* gene (Steinmetz 1993). Wheat inoculation experiments with *P. polymyxa* CF43 wild-type and a mutant strain deficient in the levansucrase SacB reveal that both strains display equivalent root colonization efficiency. However, while inoculation with C43 wild type increases root adhering soil mass, addition of the *sacB* mutant strain is without effect, suggesting that levan synthesis by *P. polymyxa* contributes directly to soil adherence at the plant roots (Bezzate et al. 2000). The *sacB* gene is also present in the FZB42 genome.

3.6.2 Nitrogen and Nitrite Extrusion

A major challenge for the development of sustainable agriculture is the use of nitrogen-fixing bacteria, which are able to assimilate gaseous N_2 from the atmosphere. Biofertilization accounts for approximately 65% of the nitrogen supply to crops worldwide (Bloemberg and Lugtenberg 2001). The most efficient nitrogen fixers are root nodulating *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and *Bradyrhizobium*. All these strains form a host-specific symbiosis with leguminous plants and their nitogenase complex is extremely sensitive against oxygen. Free-living nitrogen-fixing rhizobacteria such as *Azospirillum*, *Herbaspirillum*, *Acetobacter*, and *Azoarcus* use a nitrogenase complex that functions under low oxygen conditions. *P. polymyxa* belongs to this group; however, its ability to fix nitrogen does not significantly contribute to the total nitrogen supply and can be neglected in this respect. However, strains belonging to the related species *Paenibacillus azotofixans* were shown to be efficient nitrogen fixers. They are prevalent in the rhizosphere of several crops and forage grasses (Seldin et al. 1998).

Nitrite secreted by FZB42 might enhance root architecture. The strict aerobic *B. subtilis* and *B. amyloliquefaciens* do not possess nitrogenase complex and are unable to support plants in assimilating gaseous N_2 from the atmosphere. However, *B. amyloliquefaciens* FZB42 has a nitrite-extrusion protein, NarK, which is turned on in the presence of plant root exudates (Unpublished). In low amounts, nitrite released by bacteria has been documented to cause phytohormonal effects in graminaceous species, being even more active than IAA in some root assays (Bothe et al. 1992). It is suggested that ascorbate associated with nitrite also plays a role in the enhanced formation of root hairs and lateral roots (Bothe et al. 1992). Under acidic conditions, nitrite forms nitrous acid and nitric oxide (NO) (Lundberg 2008). Nonenzymatic NO-releasing substances enhance root tip expansion in a dose-dependent manner and even act by similar signal transduction pathways than IAA (Gouvea et al. 1997). It is, thus, tempting to speculate that in case of *B. amyloliquefaciens* the extrusion of nitrite is an additional mechanism involved in PGP.

3.6.3 Mineral-Solubilizing Bacteria and Phytase Activity

Phosphorus is often a limiting nutrient in soils, because of processes such as adsorption, precipitation, or conversion to the organic form. Therefore, more than 80% of phosphorus fertilizers can become unavailable for plant uptake (Holford 1997), leading to phosphate accumulation that eventually contaminates surface and groundwater. Phosphate runoff is associated with eutrophication of surface waters, resulting in increased fish mortality (Malakoff 1998). This environmental impact of chemical fertilization can be attributed, in part, to low uptake efficiency by plants. One the other hand, phosphorous is highly reactive with iron, aluminum, and calcium in soils, which can result in precipitation of up to 90% of the soil phosphorous, thus making it largely unavailable to plants. Phosphate-solubilizing microorganisms (PSMs) are ubiquitous in soils and could play an important role in supplying P to plants in a more environment-friendly and sustainable manner (Gyaneshwar et al. 2002).

The two main forms of phosphorus found in soils are inorganic orthophosphate (P_i) and organic phosphate pool, which is mostly constituted by phytic acid (inositol hexaphosphate). In previous in vitro studies, done under soilless, sterile conditions, we showed that biofertilization exerted by extracellular bacterial phytase under conditions of phosphate limitation and in the presence of phytate can contribute to the plant-growth-promoting activity in *B. amyloliquefaciens* FZB45 (Idriss et al. 2002; Makarewicz et al. 2006). Further, Ramirez and Kloepper (2009) applied FZB45 to Chinese cabbage (*Brassica rapa*), grown in soil with known P-related properties. Although there was a significant interaction between inoculum concentration and P-regime, bacterial inoculation only increased fresh shoot weight and plant P_i content at high rate of phytate. Thus, to their results, phytase action is only beneficial under conditions of low soil P content and sufficient phytate availability.

Moreover, plant growth stimulation, found in the presence of FZB45, also depends on IAA produced by the bacteria, suggesting that the two beneficial effects might act together. A transgenic strain, NKTS-3, harboring a chromosomal integrated phytase gene, was found especially efficient in supporting growth of tobacco plants under phosphorous-deficient conditions (Li et al. 2007).

3.7 Biological Control of Soil and Airborne Plant Diseases

Several commercial products such as spores of *B. subtilis* strain GB03 (Kodiak®, Gustafsson, Inc., Plano, TX) are used as biocontrol agent (BCA) for treatment of cotton (*Gossypium* sp.) diseases. The recent success of Kodiak® in the cotton market in the USA has largely been due to the integration of a stable biological control agent (BCA) formulation with standard chemical fungicides as part of integrated pest management (Brannen and Kenney 1997). The principal component of Serenade (Agraquest Inc., Davis, CA) is *B. subtilis* strain QST713. This product is labeled for the management of a variety of plant diseases including early blight, fire blight, downy mildew, and tomato leaf spots. Subtilex (Becker Underwood, Ames, IA) contains *B. subtilis* strain MB1600, which is active against *Fusarium*, *Aspergillus*, and *Rhizoctonia* spp. attack on roots of soybean and peanut and against *Botrytis* spp. infection of vines, strawberry, cucumber, powdery mildew of tomato, and brown rust of cereals. A number of *B. subtilis* strains were integrated successfully into several pest management programs (Jacobsen et al. 2004). For example, Fusarium wilt of chickpea is suppressed more effectively by *B. subtilis* GB03 on the partially resistant cultivar than on a susceptible cultivar (Hervas et al. 1998).

Some progress is made in recent years in understanding molecular basis for biological control of plant pathogens by plant-beneficial bacilli. It is obvious now that two mechanisms are efficient in suppressing plant pathogens, i.e., production of antifungal and antibacterial secondary metabolites, and ISR.

3.7.1 Nonribosomal Lipopeptides

Suppression of the competitive plant-pathogenic microflora within the rhizosphere by secreted antifungal and antibacterial lipopeptides and polyketides is important for promotion of plant growth by FZB42 (Chen et al. 2006). Several hundred wild-type *B. subtilis* strains were collected with the potential to produce numerous antibiotics, including predominantly peptides, e.g., the surfactins, the fengycins, and the iturins (Stein 2005). These lipopeptides are amphiphilic molecules and vary in their peptide and fatty acid moieties (Hofemeister et al. 2004). Surfactins show weak antibiotic activity, but strong hemolytic and surfactant properties (Kowall et al. 1998), whereas fengycins are potent antifungal agents (Vanittanakom et al. 1986) and iturins show a great molecular diversity and differ in their hemolytic,

antibacterial, and antifungal properties (Peypoux et al. 1999) Lipopeptides (Box 3.3) and polyketides (Box 3.4) consist of monomeric building blocks as amino acids or organic acids which are linked together by assembly lines of giant modularly organized peptide synthetases (NRPS) and polyketide synthases (KS), respectively.

The genome of *B. amyloliquefaciens* FZB42 harbors three gene clusters for the nonribosomal synthesis of lipopeptides surfactin, fengycin, and bacillomycin D (Koumoutsi et al. 2004; Chen et al. 2007). The cyclic lipopeptides fengycin and especially bacillomycinD act against soil-borne plant-pathogenic fungi (e.g., *Fusarium* spp. and *Rhizoctonia solani*). Interestingly, bacillomycin D and fengycin can exert a synergistic antagonistic effect when applied together (Koumoutsi et al. 2004).

In the genome of E681, two giant gene clusters directing nonribosomal synthesis for polymyxin (Choi et al. 2009) and fusaricidin (Choi et al. 2008) are present.

Box 3.3 Nonribosomal Synthesis of Lipopeptides

Although diverse in structure, nonribosomally synthesized peptides have a common mode of biosynthesis, the multicarrier thiotemplate mechanism (Stein et al. 1996). They are assembled on very large protein templates called peptide synthetases (NRPS) that exhibit a modular organization, allowing polymerization of monomers in an assembly-line-like mechanism (Doekel and Marahiel 2001). Each elongation cycle in nonribosomal peptide biosynthesis needs the cooperation of three basic domains. (1) The A-domain (adenylation domain) selects its cognate amino acid and generates an enzymatically stabilized aminoacyl adenylated. This mechanism resembles the aminoacylation of tRNA synthetases during ribosomal peptide biosynthesis. (2) The PCP domain (peptidyl-carrier domain) is equipped with a 4′-phosphopantetheine (PPan) prosthetic group to which the adenylated amino acid substrate is transferred and bound as thioester. A 4′-phosphopantetheine-transferase (PPTase) converts the apo-form of the PCP into its holo-form by loading the Ppan-cofactor to an active serine. *B. subtilis* Sfp is a prototype of PPTases with wide substrate tolerance. Sfp-PPTase plays an essential role in priming nonribosomal peptide synthetases, siderophore synthetases (e.g., bacillibactin), and polyketide synthases. It can covalently convert specific serine residues in peptidyl- as well as in acyl- and aryl-carrier proteins/domains (PCP, ACP, and ArCP) to generate their active holo-forms. This occurs by tethering the phosphopantetheinyl moiety of the cosubstrate coenzyme A (CoA) in phosphodiester linkage to the hydroxymethyl side chain of the conserved active serine residue in the CP domain (Walsh et al. 1997). (3) The formation of a new peptide bond is catalyzed by condensation domains (C domains). The linear organization of such core units (1–3) ensures the coordinated elongation of the peptide product. The assembly of the multifunctional proteins of the peptide synthetases is reflected in its genetic organization following the colinearity rule (Duitman et al. 1999).

Box 3.4 Nonribosomal Synthesis of Polyketides
Polyketides belong to a large family of secondary metabolites that include many bioactive compounds with antibacterial, immunosuppressive, antitumor, or other physiologically relevant bioactivities. Their biosynthesis is accomplished by stepwise decarboxylative Claisen condensations between the extender unit and the growing polyketide chain, generating enzyme-bound β-ketoacyl intermediates. Before a subsequent round of chain extension, a variable set of modifying enzymes can locally introduce structural variety. Similar to the nonribosomal synthesis of peptides, the PKS multienzyme system uses ACPs that are posttranslationally modified with the 4′-phosphopantetheine prosthetic group to channel the growing polyketide intermediate during elongation process. Type I PKS are modularly organized giant synthases, each module of which usually contains a β-ketoacyl synthase (KS), an acyltransferase (AT), and the ACP as essential and basic domains that may be complemented by a variable set of additional domains. The order of the modules dictates the sequence of biosynthetic events, and the generally observed colinearity between PKS structure and biosynthetic steps was shown to permit combinatorial manipulation of type I PKS in order to generate novel compounds (Chen et al. 2006).

Polymyxin was also detected in *P. polymyxa* M1 (Dr. Qi Wang, CAU, Beijing) (Ben Niu, unpublished).

Fusaricidin, encoded by the *fus* gene cluster in *P. polymyxa* (Choi et al. 2008), has an excellent antifungal activity against plant-pathogenic fungi, such as *Fusarium oxysporum* and *Leptosphaeria maculans*, causing black root of canola (Beatty and Jensen 2002). Another example for nonribosomal peptide synthesis in bacilli is production of the linear aminopolyol antibiotic zwittermicin A (ZmA) encoded by the *zma*A gene cluster in *Bacillus cereus* UW85. This antibiotic has diverse biological activities, such as suppression of disease in plants caused by protists, inhibition of fungal and bacterial growth, and amplification of the insecticidal activity of the toxin protein from *B. thuringiensis* (Kevany et al. 2009). Use of lipopeptides, produced by bacilli, as sustainable and environment-friendly biofungicides for treatment of fungal diseases in crops and vegetables is a promising approach.

3.7.2 Nonribosomal Synthesized Polyketides

Besides peptides, polyketides are one of the largest families of secondary metabolites having antimicrobial, immunosuppressive, antitumor, or other physiologically relevant bioactivities. Although polyketides are widespread secondary

metabolites from bacteria, only a few of them are isolated from *Bacillus* species. Therefore, their corresponding biosynthetic gene clusters remained hitherto widely unexplored. Recently, we assigned the complete polyketide synthase (PKS) gene clusters to *Bacillus* antibiotics (Chen et al. 2006). This was done with the industrially relevant *B. amyloliquefaciens* FZB42 as a model organism. Three PKS operons were located at sites around 1.4 Mbp (*pks2*), 1.7 Mbp (*pks1*), and 2.3 Mbp (*pks3*) clockwise distant from the replication origin of the *B. amyloliquefaciens* genome (Koumoutsi et al. 2004), which is 3.916 kb in size. All three gene clusters show a modular organization typical for type I PKS systems, implying that FZB 42 has the biosynthetic machinery for the production of at least three different kinds of polyketides. With the help of cassette mutagenesis in combination with advanced mass spectrometric techniques, such as MALDI-TOF-MS and HPLC-ESI-MS, two polyketides, bacillaene and difficidin/oxydifficidin (Fig. 3.5), were identified,

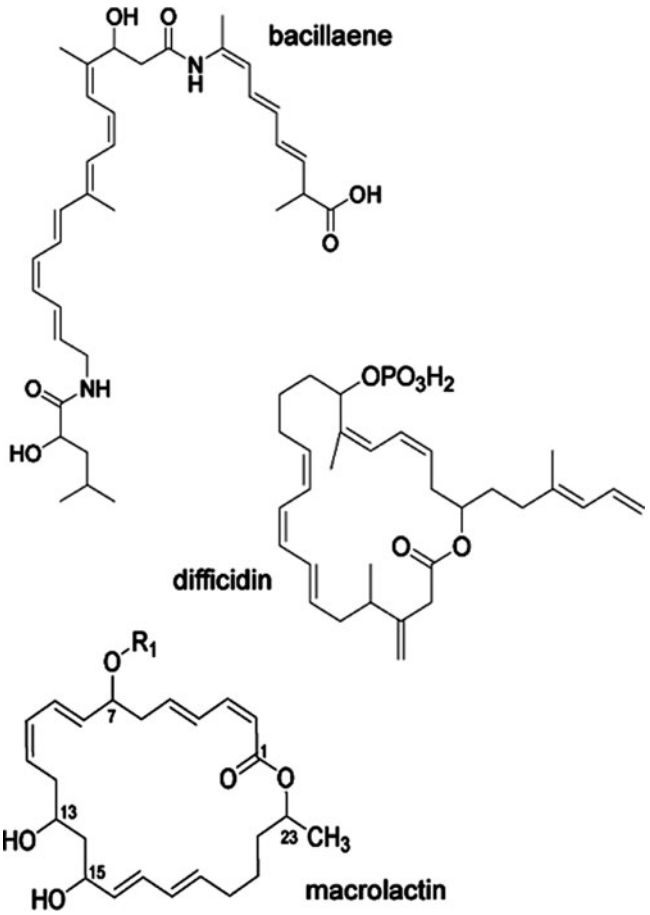

Fig. 3.5 Chemical structure of the polyketides synthesized in FZB42

which are encoded by gene clusters *pks*1 (*bae*) and *pks*3 (*dfn*) (Chen et al. 2006). The *bae* gene cluster is also present in the genome of the model organism *B. subtilis*. Recently, the groups of Clardy and Walsh resolved the structure of bacillaene from *B. subtilis* (Butcher et al. 2007).

Using a similar strategy, the *pks2* cluster is assigned as responsible for synthesis of another polyketide with antibacterial properties, macrolactin (Schneider et al. 2007). The macrolactins consist of 24-membered ring lactones, which also bear modifications, as the attachment of glucose-pyranoside and the appearance of linear analogues. Earlier, macrolactins have gained special interest with the discovery of 7-*O*-malonyl macrolactin A, which is active against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* (Romero-Tabarez et al. 2006).

3.7.3 Ribosomal Synthesized Peptides (Lantibiotics and Bacteriocins)

Peptide antibiotics with interresidual thioether bonds as unique feature are defined as lantibiotics (lanthionine containing antibiotics). Lanthionine formation occurs through posttranslational modification of ribosomally synthesized precursor peptides including dehydration of serine and threonine residues, respectively, and subsequent addition of neighboring cysteine thiol groups. Based on structural properties, two lantibiotic types are distinguishable. Type A lantibiotics (21–38 amino acid residues) exhibit a more linear secondary structure and kill Gram-positive target cells by forming voltage-dependent pores into the cytoplasmic membrane. Self-protection (immunity) against lantibiotics is based on ATP-binding cassette (ABC) transporter homologous proteins that export the lantibiotic from the cytoplasmic membrane into the extracellular space.

B. subtilis harbors several gene clusters involved in ribosomal synthesis of antimicrobial peptides. *SpaS* and *spaBTC* are involved in the biosynthesis of subtilin, a 32-amino-acid pentacyclic lantibiotic, while *spaIFEG* confers self-immunity. The biocontrol *B. subtilis* strain A1/3 produces Ericin (Stein et al. 2002). Ericin S and subtilin only differ in four amino acid residues, and the antimicrobial properties of both the lantibiotics are comparable. The lantibiotic mersacidin belongs to the type B lantibiotics, which exhibit a more globular structure. It inhibits cell wall biosynthesis by complexing lipid II. The mersacidin gene cluster consists of the structural gene *mrsA*, as well as genes involved in posttranslational modification (*mrsM* and *mrsD*), transport (*mrsT*), immunity (*mrsFEG*), and regulation (*mrsR1*, *mrsR2*, and *mrsK2*). Recently, production of mersacidin has been detected in Chinese biocontrol strain Y2 (Vater and Borriss, unpublished), a strain closely related with FZB42. Despite availability of complete genome sequence, for several years no bacteriocins were detected in FZB42. But we have assigned two gene clusters involved in ribosomal synthesis of two

modified peptide compounds in FZB42. One of the two compounds, related to microcinB with a molecular mass 1336 ($M + H^+$), had only weak antibacterial activity, while the other, a representative of group I cyclic bacteriocins with a molecular mass of 6,382, exhibited strong antibacterial activity mainly directed against Gram-positive bacteria (Scholz and Borriss, unpublished).

3.7.4 A Case Study: Combined Use of Bacilysin and Difficidin Against Fire Blight

Fire blight caused by *Erwinia amylovora* is the most serious bacterial disease in apple and pear. During the last four decades, it has spread throughout Europe. In 2007, heavy outbreaks of fire blight led to severe losses for apple growers in Germany, Austria, and Switzerland. Since sanitation methods could not stop the spread of the disease, fire blight management by using appropriate biocontrol agents is a pressing need. Effective control can be achieved through application of streptomycin sulfate, a method widely used in North America. However, the use of streptomycin has been banned by the European authorities, due to risk of development of antibiotic resistance in nontarget bacteria. Search of an environment-friendly biological alternative is a permanent task of present research. Recently, two products based on *B*. "*subtilis*" are registered for blight control in Europe: Serenade® based on strain QST713 and Biopro® based on strain BD170 (Broggini et al. 2005).

We identified two compounds, produced by FZB42, with strong antagonistic effect against *E. amylovora*, the polyketide difficidin (see above), and bacilysin, a dipeptide whose synthesis does not depend on *Sfp*. The bacilysin molecule contains an L-alanine residue at the N terminus and a proteinogenic amino acid, L-anticapsin, at the C terminus. Antibiotic action is achieved by the anticapsin moiety, which becomes released after uptake in susceptible cells and blocks glucosamine synthetase, an essential enzyme of cell wall biosynthesis. Mutant strains with knockout mutation in the Sfp encoding gene (unable to produce any lipopeptides and polyketides) are still able to inhibit *E. amylovora* due to bacilysin, whose production is not affected (Chen et al. 2009b). In contrast to difficidin, bacilysin is rather stable, and search for strains with high bacilysin production is a promising approach in the further development of an efficient and sustainable biocontrol agent, based on PGP bacilli.

3.7.5 Induced Systemic Resistance

Certain bacteria trigger a phenomenon known as ISR. ISR occurs when the plant's defense mechanisms are stimulated and primed to resist infection by pathogens, a

process similar to systemic acquired resistance (SAR). SAR develops when plants successfully activate their defense mechanisms in response to primary infection of their aerial parts by a pathogen, notably when the latter induces a hypersensitive reaction through which it becomes limited in a local necrotic lesion of brown, desiccated tissue. As SAR, ISR is effective against different types of pathogens but differs from SAR in that the inducing PGPR bacterium is colonizing roots and does not cause visible symptoms on the host plant (van Loon et al. 1998). PGPRs that colonize root systems with seed applications and protect plants against foliar diseases include *Pseudomonas fluorescens*, *P. putida*, *B. subtilis*, *B. pumilus*, and *Serratia marcescens* (Ryu et al. 2004a). A major distinction between ISR and SAR is the dependence of the latter on the accumulation of salicylic acid (SA). However, majority of PGPRs that activate ISR appear to do so via a SA-independent pathway involving jasmonate and ethylene signals. Early observations about systemic disease protection in greenhouse experiments were reported for *B. pumilus* and *B. mycoides* (Bargabus et al. 2002, 2004). Field trials showed that *B. pumilus* INR7 reduced the incidence of cucurbit wilt disease caused by *Erwinia tracheiphila* (Zehnder et al. 2001). Ability to act as bioprotectants via ISR has been demonstrated for both Gram-negative and Gram-positive PGPR (Compant et al. 2005). Strains *B. amyloliquefaciens* IN937a, *B. subtilis* IN937b, and *B. pumilus* SE34 and INR7 were found especially efficient in stimulating plant ISR directed against pathogenic fungi, bacteria, viruses, and insects (Kloepper et al. 2004). Several bacterial traits (i.e., flagellation and production of siderophores and lipopolysaccharides) have been proposed to trigger ISR. In endospore-forming Gram-positive bacteria, volatiles play a key role in this process, besides their role in PGP (Ryu et al. 2005). Arabidopsis seedlings exposed in divided Petri dishes to PGPR *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a for 10 days developed significantly less symptomatic leaves 24 h after inoculation with the soft rot-causing pathogen *Erwinia carotovora ssp. carotovora*. Compounds 2,3-butanediol and 3-hydroxy-2-butanone (acetoin) were released consistently as the most abundant volatiles from GB03 and IN937a (Ryu et al. 2004a).

Transgenic tobacco plants with herbicide resistance gene were used to test effect of FZB24 on expression of genes involved in plant defense against pathogens. All three promoters were activated by the treatment with FZB24, though partly with different intensities. The *prp1* promoter responded particularly strongly, independent if bacteria were applied by soil treatment by drenching or leaf treatment by spraying (Kilian et al. 2000). Volatiles released by FZB42 grown in I plates did also elicit ISR in *Arabidopsis thaliana* seedlings, afterward treated with plant pathogen *Xanthomonas campestris*. Moreover, mutant strains bearing *alsS* and *alsD* mutations were found severely attenuated in eliciting ISR in the plants under same experimental conditions, suggesting that, in fact, 2,3-butanediol and acetoin are the main compounds in stimulating ISR response by PGPR *Bacillus* sp. (Ryu, Johti, and Borriss, unpublished).

Ryu et al. (2004a) reported that screening of *Arabidopsis* signaling pathway mutants revealed that only in the ethylene-insensitive line *ein2* when exposed

to VOCs emitted from strain GB03 was the severity of disease symptoms not ameliorated. This suggests that an ethylene-dependent signaling pathway in eliciting ISR by GB03 exists. Interestingly, in case of IN937a ISR signaling pathway was found ethylene-independent (Ryu et al. 2004a). Rhizobacteria-mediated ISR did generally not require SA, but in case of *Serratia marcescens*-mediated protection against Cucumber mosaic virus (CMV), a signaling pathway dependent on jasmonic acid was proposed (Ryu et al. 2004b). Taken together, these results and in spite of the different volatile profiles detected also in closely related species as GB03 and IN937a (Farag et al. 2006), it seems that strain-specific signaling pathways for ISR were induced by specific PGPR strains.

3.7.5.1 Comparison of FZB42 with Related Strains

A survey of genetic markers associated with biological control activity of "*B. subtilis*" revealed that nine markers could be amplified from the commercialized *Bacillus* strains GB03 (Kodiak®), QST713 (Serenade), and MBI600 (Subtilex), except *ituC*, which was not detected in GB03 (Joshi and McSpadden Gardener 2006). Those markers could not be amplified in other *Bacillus* strains including plant-growth-promoting IN937a and IN937b. The authors conclude that the most effective biocontrol strains of *B. subtilis* shared multiple genetic elements responsible for pathogen suppression. Moreover, a close phylogenetic relationship among the three commercialized biocontrol strains was postulated. Another "cousin" of FZB42, plant-associated *B. amyloliquefaciens* strain GA1, resembles FZB42 in nonribosomal synthesis of the same spectrum of lipopeptides and polyketides (Arguelles-Arias et al. 2009).

3.8 Conclusions and Outlook

More than 20 years had passed, since Kloepper et al. (1989) firstly reviewed prospects of PGPR for crop productivity, and many of the concepts, discussed in that early review, were validated by succeeding research, mainly performed with *Pseudomonas* spp. Although biocontrol strains of fluorescent pseudomonads have contributed greatly to the understanding of the mechanisms that are involved in phytostimulation and disease suppression, biological preparations from spore-forming *Bacillus* spp. are preferred, because their long-term viability facilitates the development of commercial products (Haas and Defago 2005). Unfortunately, their success in agricultural application is still hampered by insufficient knowledge about basic mechanisms of interactions between PGP and plants, although some progress has been made in recent years. In order to overcome this problem, we

propose to choose *Bacillus amyloliquefaciens* FZB42 as model strain for PGP bacilli for the following reasons:

1. The strain (BGSC strain number 10A6 and DSMZ strain number DSM 23117) and its whole-genome sequence is freely available (GenBank accession: CP000560), despite the fact that the strain is commercialized by ABiTEP GmbH Berlin and successfully used in agri- and horticulture applications (http://www.abitep.de/en/Research.html).
2. In contrast to most environmental *Bacillus* strains, *B. amyloliquefaciens* FZB42 is naturally competent and amenable to genetic transformation using a modified one-step protocol, originally developed for *B. subtilis* 168 (Idris et al. 2007). In order to assign unknown gene functions, we generated numerous mutants impaired in many distinct functions, such as production of extracellular enzymes, secondary metabolites, biofilm formation, alternative factors, and PGP. Some are already deposited at Bacillus Genetic Stock Center, Ohio, OH (BGSC); the others can be obtained after request from Humboldt University, Berlin.
3. Strain derivatives of FZB42 were marked by stable chromosomal integration of genes encoding for GFP+ and other fluorescenct proteins. Those strains were found extremely useful for studying root colonization after bacterial inoculation (Fan and Borriss, unpublished).
4. A transposon library of FZB42 using the mariner transposon TnYLB-1 was generated and successfully used to screen for mutants impaired in PGP, production of small peptides, and nematocidal activity.
5. Microarrays with oligonucleotides, representing the complete set of FZB42 open reading frames and numerous intergenic sequences harboring candidate small (regulatory) RNAs for transcriptomic studies are available.
6. Even a great number of protein spots obtained after 2D gel electrophoresis of the FZB42 cytoplasmic fraction and the secretome were assigned by MALDI-TOF mass spectrometry.
7. Last but not least, *B. amyloliquefaciens* FZB42 is closely related to other known PGP bacilli (QST713, GB03, A1/3, and GA1). According to sequence homologies established on genomic and selected gene level (e.g., *gyrA* and *cheA*), FZB42 forms, together with other plant-associated bacilli, an own ecomorph, distantly related to the *B. amyloliquefaciens* type strain DSM7^{T} (Reva et al. 2004).

We hope that the community of scientists, interested on exploiting PGP bacilli, will choose FZB42 as a paradigm for research on that group of bacteria with a high potential to substitute or at least to replace, in part, such agrochemicals.

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Chapter 4
Mechanisms of Fluorescent Pseudomonads That Mediate Biological Control of Phytopathogens and Plant Growth Promotion of Crop Plants

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4.1 Introduction

Fluorescent pseudomonads are nonenteric, Gram-negative, aerobic, straight, or slightly curved rods, which are nonfermenting and motile belonging to γ-proteobacteria (Galli et al. 1992). They are pervasive bacteria which are common inhabitants of soil, water, and phyllosphere but predominant in plant rhizosphere due to the exudation of organic acids, sugars, and amino acids (Lugtenberg and Dekkers 1999). Fluorescent pseudomonads are the most promising group of plant growth-promoting rhizobacteria (PGPR) involved in biocontrol of plant diseases. This group of bacteria also includes a few pathogens and biodegraders. Saprophytic fluorescent pseudomonads are typical inhabitants of agricultural field soils and plant rhizosphere and are involved in several interactions with plants (Schroth et al. 1992). They are capable of utilizing many plant exudates as nutrient (Lugtenberg et al. 1999) and are known to possess important traits in bacterial fitness such as the ability to adhere to soil particles and to the rhizoplane, motility, and prototrophy (de Weger et al. 1994). Members of the genus *Pseudomonas* have very simple nutritional requirements and grow well under normal conditions in mixed populations with other types of microorganisms (Foster 1988). They are metabolically and functionally diverse and promote plant growth directly by phosphorus solubilization, sequestration of iron for plants by siderophores, production of phytohormones (Salisbury 1994; Ayyadurai et al. 2006, 2007; Ravindra Naik and Sakthivel 2006; Ravindra Naik et al. 2008), and lowering of plant ethylene levels (Glick 1995; Glick et al. 1999), and indirectly by suppressing pathogenic microorganisms through antibiotic production (Thomashow et al. 1990; Ayyadurai et al. 2006, 2007; Ravindra Naik and Sakthivel 2006; Ravindra Naik et al. 2008;

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Jha et al. 2009), reduction of iron available to phytopathogens in the rhizosphere (Scher and Baker 1982), synthesis of fungal cell wall-degrading enzymes, and competition with detrimental microorganisms for sites on plant roots. Additionally, fluorescent pseudomonads are capable of inducing a systemic resistance in plants against various phytopathogens (Van Loon et al. 1998; Pieterse et al. 2001). All these traits make fluorescent pseudomonads suitable for application as biological control agents (Sands and Rovira 1971).

Fluorescent pseudomonads are the extensively studied group within the genus *Pseudomonas*. They comprise *P. aeruginosa*, the type species of the genus; *P. aureofaciens*, *P. chlororaphis*, *P. putida* (two biotypes), *P. fluorescens* (four biotypes), and the plant pathogenic species *P. cichorii* and *P. syringae* (Leisinger and Margraff 1979). *Pseudomonas* strains are characterized on the basis of their nutritional features as well as by other phenotypic traits into species and species groups (Stanier et al. 1966). Conclusions of this study were supported by numerical analysis (Sneath et al. 1981) and DNA–DNA hybridization (Palleroni and Doudoroff 1972). The rRNA–DNA hybridizations that revealed five rRNA homology clusters: rRNA groups and all fluorescent pseudomonads fall into one of the five rRNA group (Palleroni et al. 1973). The Guanine-plus-Cytosine (G + C) content of their DNA ranges from 58 to 68 mol% (Palleroni 1975). *P. fluorescens* biovar III has been reported as dominant group of bacteria among fluorescent pseudomonads associated with rhizosphere of rice, the most important food crop of the world (Sakthivel and Gnanamanickam 1989). *P. fluorescens* biovar V constituted more than 75% of fluorescent pseudomonads in at least some areas of the world (Barrett et al. 1986) and represented as the predominant group in Australian soils and wheat rhizosphere (Sands and Rovira 1971). Nearly 90% of fluorescent pseudomonads in Colombia was reported to be *P. putida* (Hernandez et al. 1986). Thus, vast microbial diversity occurs among fluorescent pseudomonads in different geographic origins and this group of bacteria exhibits an array of mechanisms that mediate biocontrol of pathogens and growth promotion of crops.

4.2 Biocontrol Mechanisms

Biocontrol mechanisms such as production of fungal cell wall-degrading enzymes (Lim et al. 1991; Nielsen et al. 1998; Nielsen and Sorensen 1999; Ellis et al. 2000), antimicrobial compounds (James and Gutterson 1986; Gutterson et al. 1988; Thomashow et al. 1990; Natsch et al. 1994), namely, phenazines (Gurusiddaiah et al. 1986; Pierson and Thomashow 1992; Sunish Kumar et al. 2005), phloroglucinols (Dwivedi and Johri 2003), pyrrols (Homma and Suzui 1989; Pfender et al. 1993), polyketides (Kraus and Loper 1995), and peptides (Nielsen et al. 1999, 2000; Sorensen et al. 2001), hydrogen cyanide (Castric 1981; Bagnasco et al. 1998; Rodriguez and Fraga 1999; Siddiqui 2006), siderophores (Hamdan et al. 1991; Meyer et al. 2002), cell wall components (Meziane et al. 2005; Van Loon 2008), competition (van Loon et al. 1998), and induced resistance (van Loon et al. 1998)

also have been identified. Sigma factor genes, *rpoD* and *rpoS*, have been reported to control antibiotic production and enhance the antagonistic activities of fluorescent *Pseudomonas* (Fujita et al. 1994).

A large number of fluorescent pseudomonad species such as *P. putida* (de Freitas and Germida 1991), *P. fluorescens* (Sakthivel and Gnanamanickam 1987), *P. aeruginosa* (Anjaiah et al. 2003; Sunish Kumar et al. 2005), *P. aureofaciens* (now considered as *P. chlororaphis*) (Chin-A-Woeng et al. 1998), and *P. pyrrocinia* (de Weger et al. 1986) have been well documented for their antagonistic potential. Specific strains such as *P. fluorescens* 2-79, Pf-5, 96.578, DR 54, *P. aeruginosa* 7NSK2, PNA1, *P. aureofaciens* 30-84, and *P. chlororaphis* PCL1391 suppress disease severities caused by plant pathogens in cotton (Howell and Stipanovic 1980), rice (Mew and Rossales 1986; Sakthivel and Gnanamanickam 1987; Rosales et al. 1995), wheat (Weller and Cook 1983; Weller et al. 1985; Rovira and McDonald 1986), barley (Rovira and McDonald 1986; Iswandi et al. 1987), tomato (Buysens et al. 1996; Chin-A-Woeng et al. 1998), potato (Burr et al. 1978; Kloepper et al. 1980; Bakker and Schippers 1987), radish (Kloepper and Schroth 1978; Homma and Suzui 1989), sugar beet (Suslow and Schroth 1982; Nielsen et al. 2000; Thrane et al. 2000, 2001), cassava (Hernandez et al. 1986), chickpea (Anjaiah et al. 2003), soybean (Cattelan et al. 1999), tobacco (Keel et al. 1990), and apple and pears (Jamisiewicz et al. 1991). Antimicrobial metabolites and enzymes produced by fluorescent pseudomonads are listed in Table 4.1.

4.2.1 Fungal Cell Wall-Degrading Enzymes

Production of fungal cell wall-degrading enzymes by microorganisms is frequently involved in the attack of phytopathogenic fungi (Martin and Loper 1999; Nielsen and Sorensen 1999; Picard et al. 2000). Lysis by cell wall-degrading enzymes excreted by microorganisms is a well-known feature of mycoparasitism. Chitinase, β-1,3 glucanase, and cellulase are especially important fungus controlling enzymes due to their ability to degrade the fungal cell wall components such as chitin, β-1,3 glucan, and glucosidic bonds (Potgieter and Alexander 1966; Bartnicki-Garcia and Lippman 1973; Schroth and Hancock 1981; Chet 1987; Lorito et al. 1996). Chitinase excreting microorganisms have been reported as efficient biocontrol agents (Sneh 1981; Ordentlich et al. 1988; Inbar and Chet 1991). Role of chitinase in biological control as well as in plant defense mechanisms has been documented well (Shapira et al. 1989). Nielsen et al. (1998) reported that in the sugar beet rhizosphere fluorescent pseudomonads inhibit plant pathogenic fungi *Rhizoctonia solani* by production of cell wall-degrading endochitinase. Biological control of *Fusarium solani*, mainly via laminarinase and chitinase activities of *P. stutzeri* YPL-1, has been reported (Lim et al. 1991). Fridlender et al. (1993) reported that β-1,3 glucanase-producing *P. cepacia* decreased the incidence of diseases caused by *R. solani*, *S. rolfsii*, and *P. ultimum*.

Table 4.1 Antimicrobial metabolites and enzymes produced by fluorescent pseudomonads

Metabolite/enzymes	Producer strain	References
Fungal cell wall-degrading enzymes		
	P. stutzeri YPL-1	Lim et al. (1991)
Chitinase	*P. aeruginosa* P10	Ayyadurai et al. (2007)
β-1,3 Glucanase	*P. cepacia*	Fridlender et al. (1993)
Laminarinase	*P. stutzeri* YPL-1	Lim et al. (1991)
Phenazines		
	P. fluorescens 2-79	Thomashow and Weller (1988)
Phenazine-1-carboxylic acid	*P. aureofaciens* 30-84	Pierson and Thomashow (1992)
Phenazine-1-carboxamide	*P. aeruginosa* PUPa3	Sunish Kumar et al. (2005)
Dimer of phenazine-1-carboxylic acid	*P. fluorescens* Pf23	Sakthivel and Sunish Kumar (2008)
Pyocyanin	*P. aeruginosa* PAO1	Baron et al. (1997)
Phloroglucinols		
	P. fluorescens Pf-5	Howell and Stipanovic (1979)
	P. fluorescens Q2-87	Vincent et al. (1991)
	P. fluorescens CHAO	Keel et al. (1992)
	P. fluorescens PFM2	Levy et al. (1992)
	P. fluorescens Q8r1-96	Shanahan et al. (1992); Flaishman et al. (1990)
2,4-Diacetyl phloroglucinol	*P. fluorescens* F113	Raaijmakers and Weller (2001)
Pyrrols		
	P. fluorescens BL914	Kirner et al. (1998)
	P. aureofaciens A10338.7	Elander et al. (1968)
	P. cepacia 5.5B	Cartwright et al. (1995)
Pyrrolnitrin	*P. fluorescens* BL915	Ligon et al. (2000)
Polyketides		
	P. fluorescens Pf-5	Howell and Stipanovic (1979)
Pyoluteorin	*P. fluorescens* CHA0	Keel et al. (1992)
Mupirocin	*P. fluorescens* NCIMB10586	El-Sayed et al. (2003)
2,3-Deepoxy-2,3-didehydro rhizoxin	*P. borealis* MA342	Tombolini et al. (1999)
Rhizoxin analogues	*P. fluorescens* Pf-5	Loper et al. (2008)
Peptides		
Tensin	*P. fluorescens* 96.578	Nielsen et al. (2000)
Viscosinamide	*P. fluorescens* DR54	Nielsen et al. (1998)
Amphisin	*Pseudomonas* sp. DSS73	Sorensen et al. (2001)
Masstolides	*P. fluorescens* SS101	de Bruijn et al. (2008)
Volatiles		
	P. fluorescens Pf-5	Voisard et al. (1981)
	P. fluorescens P5, P7, P8, P21	Ayyadurai et al. (2007)
Hydrogen cyanide	*P. pseudoalcaligenes* P4	Ayyadurai et al. (2007)

4.2.2 *Antifungal Metabolites*

Production of an array of antifungal metabolites (James and Gutterson 1986; Gutterson et al. 1988; Thomashow et al. 1990) by fluorescent pseudomonads is considered one of the most important biocontrol traits. With the advent of recombinant DNA technologies, the importance of antibiotic production in biocontrol of fluorescent pseudomonads has been demonstrated using antibiotic-deficient mutants and concerted efforts have been made to study the antifungal metabolites such as phenazines (Gurusiddaiah et al. 1986; Thomashow and Weller 1988; Pierson and Thomashow 1992; Chin-A-Woeng et al. 1998), phenolics (Keel et al. 1990, 1992; Vincent et al. 1991), pyrrole-type compounds (Homma and Suzui 1989; Pfender et al. 1993), polyketides (Nowak-Thompson et al. 1994; Kraus and Loper 1995), and peptides (Nielsen et al. 1999, 2000; Sorensen et al. 2001). Antibiotic producing fluorescent pseudomonads, *P. fluorescens* 2-79, Pf-5, CHA0, 96.578, DR 54 (Thomashow et al. 1990; Keel et al. 1992; Nowak-Thompson et al. 1994; Nielsen et al. 1999, 2000), *P. aureofaciens* 30-84 (Pierson and Thomashow 1992), and *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 1998) with varying degree of biocontrol ability have been reported.

4.2.2.1 Phenazines

Phenazines are intensely colored N-containing heterocyclic pigments synthesized by different bacterial strains (Leisinger and Margraff 1979; Budzikiewicz 1993; Stevans et al. 1994). Phenazines exhibit broad-spectrum activity against bacteria and fungi (Sunish Kumar et al. 2005; Ayyadurai et al. 2006, 2007; Ravindra Naik and Sakthivel 2006; Ravindra Naik et al. 2008). Phenazines also play an important role of microbial competition in rhizosphere, including survival and competence (Mazzola et al. 1992). Phenazine-1-carboxylic acid (PCA) has been reported from fluorescent pseudomonads such as *P. fluorescens* (Gurusiddaiah et al. 1986), *P. chlororaphis* (Pierson and Thomashow 1992), and *P. aeruginosa* (Anjaiah et al. 1998). PCA was demonstrated to be effective against various fungal pathogens such as *Gaeumannomyces graminis* var. *tritici*, *Pythium* sp., *Polyporus* sp., and *R. solani* and bacterial pathogens such as *Actinomyces viscosus*, *Bacillus subtilis*, and *Erwinia amylovora* (Gurusiddaiah et al. 1986; Thomashow et al. 1990). Production of phenazine-1-carboxamide (PCN) had been reported in fluorescent pseudomonads such as *P. aeruginosa* and *P. chlororaphis* (Chin-A-Woeng et al. 1998; Mavrodi et al. 2001; Sunish Kumar et al. 2005). PCN differs from PCA with a carboxamide ($CONH_2$) group replacing the carboxyl (COOH) group at the first position of the phenazine core. PCN is more stable than PCA and exhibits antifungal activities even in alkaline pH (Chin-A-Woeng et al. 1998). Pyocyanin is bluish colored (1-hydroxy-5-methyl-phenazine) and predominantly produced by *P. aeruginosa* (Demange et al. 1987). The antibiotic cyanomycin, from *Streptomyces cyanoflavus* (Funaki et al. 1958), is also known as pyocyanin (Turner and Messenger

1986). Pyocyanin is toxic to a wide range of fungi including *Septoria tritici* and bacteria (Baron et al. 1989; Baron and Rowe 1981; Flaishman et al. 1990; Hassan and Fridovich 1980). Broad-spectrum antifungal activity of PCN has been shown toward the phytopathogens *Pythium*, *Fusarium oxysporum* f.sp. *radiciopersici*, *Sarocladium oryzae*, and *R. solani* (Chin-A-Woeng et al. 1998; Sunish Kumar et al. 2005).

4.2.2.2 Phloroglucinols

Phloroglucinols are broad-spectrum antibiotics produced by a variety of bacterial strains. Phloroglucinols are known to induce systemic resistance in plants, thus serving as a specific elicitor of phytoalexins and other similar molecules (Dwivedi and Johri 2003). Production of phenolic antibiotic, 2,4-diacetylphloroglucinol (DAPG), has been reported from *P. fluorescens* Pf-5, *P. fluorescens* CHA0, and *P. fluorescens* Q2-87 (Fenton et al. 1992; Rosales et al. 1995). The broad-spectrum antimicrobial activity of DAPG produced by fluorescent pseudomonads has drawn great attention in agriculture due to its ability to suppress plant pathogens and play a key role in the biological control (Keel et al. 1992; Thomashow and Weller 1988; Duffy and Defago 1997; Duffy et al. 2004). DAPG-producing strains such as *P. fluorescens* Pf5, CHA0, F113, Q2-87, and Q8r1-96 (Howell and Stipanovic 1980; Vincent et al. 1991; Harrison et al. 1993; Pierson and Weller 1994; Cronin et al. 1997; Raaijmakers and Weller 1998; Duffy and Defago 1999) have been used to suppress the black root rot of tobacco, root rot of tomato, *Pythium* damping-off of cucumber and sugar beet, cyst nematode and soft rot of potato, and take-all disease of wheat. The antagonistic fluorescent pseudomonads producing DAPG that inhabit in banana rhizosphere have been studied earlier (Banerjee and Langhe 1985; Sutra et al. 2000). DAPG is found to exhibit antifungal, antibacterial, and antihelminthic activities (Vincent et al. 1991; Keel et al. 1992; Levy et al. 1992; Harrison et al. 1993; Nowak-Thompson et al. 1994; Bangera and Thomashow 1996; Abbas et al. 2002) as well as phytotoxic properties (Reddi et al. 1969). The compound also exhibits herbicidal activity resembling 2,4-dichlorophenoxyacetic acid (2,4-D), a commonly used post-emergence herbicide for the control of many annual broad leaf weeds of cereals, sugarcane, and plantation crops.

4.2.2.3 Pyrrols

Pyrrolnitrin (PRN) (3-chloro-4-(2′-nitro-3′-chlorophenyl) pyrrole) is a broad-spectrum antifungal metabolite first described by Arima et al. (1964). PRN is a secondary metabolite derived from L-tryptophan. It is produced by fluorescent pseudomonads such as *P. fluorescens* (Kirner et al. 1998) and *P. aureofaciens* (Elander et al. 1968). Other variants of PRN such as isopyrrolnitrin, oxypyrrolnitrin, and monodechloropyrrolnitrin have lower antifungal activity. PRN is active against a wide range of deuteromycete, ascomycete, and basidiomycete fungi. PRN has found

its applications in agricultural fungicide as a clinical compound. *P. fluorescens* BL915 has been reported as biocontrol agent in cotton for the suppression of *R. solani* (Ligon et al. 2000). *P. cepacia* 5.5B showed a broad-spectrum antifungal activity toward phytopathogenic fungi including *R. solani* (Cartwright et al. 1995). PRN was also reported as a topical antimycotic for human use and served as a lead molecule for pharmaceutical research.

4.2.2.4 Polyketides

Pyoluteorin (PLT) is the chlorinated antifungal metabolite of mixed polyketide/amino acid origin produced by certain strains of *Pseudomonas* spp. including soil bacterium, *P. fluorescens* Pf-5 (Maurhofer et al. 1992, 1994a; Kraus and Loper 1995; Nowak-Thompson et al. 1997). Strains producing PLT suppress several soil-borne plant diseases (Howell and Stipanovic 1980; Defago et al. 1990; Maurhofer et al. 1994b). PLT is found to be more effective against the damping-off disease causing oomycete, *P. ultimum* (Maurhofer et al. 1992). The mode of its antimicrobial activity is by the selective inhibition of bacterial isoleucyl-tRNA synthetase (Bennett et al. 1999). Mupirocin, the naturally occurring polyketide antibiotic of fluorescent pseudomonads, is also known as pseudomonic acid. Mupirocin produced by *P. fluorescens* NCIMB 10586 is highly active against *Staphylococcus aureus* and a variety of Gram-positive organisms (El-sayed et al. 2003). Mupirocin is also used as a tropical and intranasal antibiotic (Carcanague 1997). The metabolite 2,3-deepoxy-2,3-didehydrorhizoxin (DDR) produced by *P. chlororaphis* MA342 is effective against several phytopathogenic fungi, including net blotch of barley caused by the fungus *Drechslera teres* (Tombolini et al. 1999). Through the insertional mutagenesis and subsequent metabolite profiling in *P. fluorescens* Pf-5, five analogues of rhizoxin, a 16-member macrolides with antifungal, phytotoxic, and antitumor activities were identified as products synthesized from a hybrid polyketide synthase or nonribosomal peptide synthetase gene clusters. The five rhizoxin analogues were reported to show differential toxicity toward two agriculturally important plant pathogens: *Botrytis cinerea* and *Phytophthora ramorum*. The rhizoxin analogues were also reported to cause swelling of rice roots, a symptom characteristic of rhizoxin itself, but were less toxic to pea and cucumber roots. The predominant compound WF-1360 F rhizoxin analogue and the newly described compound 22*Z*-WF-1360 F were reported as most toxic against the two plant pathogens and three plant species. Upon further testing against a panel of human cancer lines, rhizoxin analogues reported to exhibit potent but nonselective cytotoxicity (Loper et al. 2008).

4.2.2.5 Peptide Antibiotics

Peptide antibiotics are predominately produced in both Gram-positive (Katz and Demain 1977) and Gram-negative bacteria (Dowling and O'Gara 1994) by

a nonribosomal multienzymatic peptide synthesis (Kleinkauf and von Dohren 1990). Recently, it has been observed that fluorescent pseudomonads produce a number of different cyclic lipopeptides (CLPs), which are useful in biological control. It is also found that CLP production is a common trait among fluorescent pseudomonads isolated from sugar beet rhizosphere (Nielsen et al. 2002). Cyclic lipodecapeptide, tensin, is produced by *P. fluorescens* 96.578 (Nielsen et al. 2000). Tensin showed potent antagonistic activity against the basidiomycete fungus, *R. solani*. Significant reduction of *R. solani* infection was found in sugar beet seeds treated with tensin-producing strain *P. fluorescens* 96.578 (Nielsen et al. 2000). The mode of action of tensin on *R. solani* is still not clearly perceived. However, it is proposed that the activity might be in synergism with chitinolytic or cell wall-degrading enzymes produced by *P. fluorescens* 96.578 (Nielsen and Sorensen 1999; Nielsen et al. 2000). Viscosinamide is a cyclic lipopeptide produced by *P. fluorescens* DR54 (Nielsen et al. 1999). This compound shows prominent antifungal and biosurfactant properties (Nielsen et al. 2000, 2002; Thrane et al. 2000). It is highly effective against *R. solani*. When *R. solani* mycelium was challenged with purified viscosinamide under in vitro conditions, a number of growth modifications at hyphal tips such as increased branching, swelling, and septation leading to inhibition in radial growth were observed (Thrane et al. 2000). In soil conditions, viscosinamide-producing *P. fluorescens* DR54 is found to reduce the mycelial biomass and sclerotia formation by *R. solani* close to the seed or seedling root surfaces, thus making the fungal biomass inadequate for infection (Thrane et al. 2001). Amphisin is a lipoundecapeptide originating from the nonribosomal biosynthesis by *Pseudomonas* sp. DSS73 (Sorensen et al. 2001). The primary structure is β-hydroxydecanoyl-D-Leu-D-Asp-D-*allo*-Thr-D-Leu-D-Leu-D-Ser-L-Leu-D-Gln-L-Leu-L-Ile-L-Asp.The peptide is a lactone, linking Thr4 Oγ to the C-terminal. It is a close analogue of the cyclic lipopeptides tensin and polypeptin produced by *P. fluorescens* (Nielsen et al. 2000; Sorensen et al. 2001). The antifungal activity of amphisin is found to be more than that of other fluorescent pseudomonad peptide antibiotics such as tensin and viscosinamide (Nielsen et al. 2002). Massetolides are cyclic lipopeptide antibiotics produced by various *Pseudomonas* strains. Three genes were found to be involved in the massetolide A biosynthesis in *P. fluorescens* strain SS101. Massetolide A production is essential in swarming motility of *P. fluorescens* SS101 and plays an important role in biofilm formation (de Bruijn et al. 2008).

4.2.3 Hydrogen Cyanide

Certain strains such as pseudomonads produce hydrogen cyanide (HCN), a volatile antibiotic (Castric 1981), which helps in disease suppression (Bagnasco et al. 1998; Rodriguez and Fraga 1999; Siddiqui 2006; Voisard et al. 1981; Sacherer et al. 1994). HCN and CO_2 are formed from glycine and catalyzed by HCN synthase (Castric 1994). HCN was recognized as the biocontrol factor of *P. fluorescens*

CHA0 as it played an indispensible role in suppression of black root rot of tobacco caused by the fungus *Thielaviopsis basicola* (Voisard et al. 1981) and take-all disease of wheat caused by *G. graminis var. tritici*. However, few reports indicated that the HCN production was detrimental to growth of certain plants and it resulted in reduction of potato yields (Bakker and Schippers 1987). Few strains of fluorescent pseudomonads which failed to exhibit any antagonism in vitro toward *Pythium* and *Rhizoctonia* established successful biological control toward them in field (Kloepper et al. 1991; Lifshitz et al. 1987). Thus, disease suppression by HCN-producing fluorescent pseudomonads may be in part due to the induction of plant resistance (O'Sullivan and O'Gara 1992). Nearly a total of 800 plant species have been reported to liberate cyanide when wounded or attacked by a phytopathogen and thus suppress the cyanide-sensitive pathogens (Mansfield 1983). However, few fungal pathogens of cyanogenic plants are tolerant to cyanide because they can detoxify it by converting it to formamide (Van Etten and Kistler 1984).

4.2.4 Siderophores and Pathogen Suppression

Several species of fluorescent pseudomonads produce a range of iron complexing agents, the siderophores, under iron-limiting conditions, which have a very high affinity for ferric iron. Siderophores reported from pseudomonads so far include pyoverdines, pyochelin, quinolobactin, and ornicorrugatin. Several pyoverdines consisting of a shared dihydroxy-quinoline chromophore joined to an acyl (carboxylic acid or amide) group and a 6–12 amino acid type-specific peptide have been characterized (Budzikiewicz 1993; Meyer 2000; Lamont and Martin 2003). Pyoverdines and pseudobactins produced by a single strain have the same peptide but differ in the nature of acyl group. Fe^{3+} binding sites of pyoverdine are present in the quinoline chromophore and the peptide chain (Budzikiewicz 1993). Strains of pseudomonads utilize heterologous pyoverdines and pseudobactins for iron acquisition and the spectrum of ferrisiderophores used forms the basis of strain identification method termed siderotyping (Meyer et al. 2002; Lamont and Martin 2003). Pyoverdines can be identified by their UV Visible absorption spectrum. The Fe^{3+} complexes show absorption maximum at 400, 320, and 280 nm in correlation with the quinoline system (Poppe et al. 1987). Pyochelins frequently accompany pyoverdines and seem to be responsible for second iron transport system. Pyochelins are implicated to have similar antifungal activities to that of pyoverdines through minimizing availability of iron to other plant deleterious microorganisms. The abundant production of pyochelins was reported in *P. aeruginosa* (Cox et al. 1981). *P. fluorescens* ATCC 17400 has shown to produce quinolobactin siderophore in addition to pyoverdine, which itself results from the hydrolysis of the unstable molecule thioquinolobactin.

P. fluorescens ATCC 17400 was identified as antagonist against the oomycete, *Pythium* sp., which is repressed by iron, suggesting the involvement of siderophores

(Matthijs et al. 2007). A new class of lipopeptidic siderophore, ornicorrugatin, was known to be produced by a pyoverdine-negative mutant of *P. fluorescens* AF76. It is structurally related to the siderophore of *P. corrugata* differing in the replacement of one Dab unit by Orn (Matthijs et al. 2008). Siderophores are thought to sequester the limited iron supply available in the rhizosphere making it unavailable to harmful pathogenic fungi and thereby suppressing fungal growth (Keel et al. 1992). Siderophores such as pyoverdine and pyochelin have been documented well for suppression of *Pythium*-induced damping-off disease of tomato (Buysens et al. 1996). However, it is generally suggested that siderophores of fluorescent pseudomonads do not play a role in biocontrol in iron-rich soils (Campbell et al. 1986). The bacterial iron complexing agents, pyoverdine and salicylate may also act as elicitors for inducing systemic resistance against pathogens in tobacco (Maurhofer et al. 1998; van Loon et al. 1998).

4.2.5 Competition

Biocontrol competition refers to the ability of the beneficial organisms to compete with other harmful organisms and effectively scavenge and utilize the available nutrients and suitable niches, so that it constitutes a significant proportion of the rhizosphere–rhizoplane population (Hattori 1988). Competition for nutrients varies between different rhizospheres depending on the available sources of carbon, nitrogen, sulfur, phosphate, and micronutrients. Competitive exclusion of deleterious organisms in the plant rhizosphere by fluorescent pseudomonads is considered as a significant pathogen-suppressive biocontrol trait (O'Sullivan and O'Gara 1992). Biocontrol of *Fusarium* (Elad and Baker 1985) and *Pythium* (Elad and Chet 1987) species by fluorescent *Pseudomonas* spp. involves competition. Variations occur in the ability of different fluorescent pseudomonads to colonize a particular root niche (de Weger et al. 1987; Kloepper et al. 1980; Loper et al. 1985; Stephens et al. 1987; van Peer and Schippers 1988). Better root-colonizing ability of the strain is an integration of various factors (de Weger and Lugtenberg 1990; Lam 1990; Weller 1988). Also, various environmental factors such as water content, temperature, pH, soil types, composition of root exudates, mineral content, and other microorganisms confront an organism when it colonizes the root. Fluorescent pseudomonad strain in potato rhizosphere was greater by tenfold in a sandy loam soil than in a clay loam soil (Bahme and Schroth 1987). Variations in colonization potential of different strains of fluorescent pseudomonads can be due in part to specific cell killing by other biological entities such as predaceous protozoans (Acea and Alexander 1988), bacteriophages (Stephens et al. 1987), and *Bdellovibrio* sp., an obligate parasite of some soil Gram-negative bacteria (Scherff 1973). Flagellation is another important requirement for good colonization under certain circumstances (O'Sullivan and O'Gara 1992).

4.2.6 Cell Wall Components

Cell surface components, namely, lipopolysaccharides (LPS) and flagellins have been reported as potent determinants of ISR in plants (Meziane et al. 2005; Van Loon 2008). LPS are indispensable cell wall components of Gram-negative bacteria and have been implicated in the induction of systemic resistance (Dow et al. 2000; Leeman et al. 1995b; Van Loon et al. 1998). Flagellins, the main components of bacterial flagella, are reported to elicit defense responses in plants (Gomez-Gomez and Boller 2000; Zipfel et al. 2004). The outer membrane LPS of biocontrol strains *P. putida* WCS358 (Bakker et al. 2003; Meziane et al. 2005), *P. fluorescens* WCS374 (Leeman et al. 1995b, 1996), and *P. fluorescens* WCS417 (van Peer and Schippers 1992; Leeman et al. 1995a, 1996) and the flagella of *P. putida* WCS358 (Bakker et al. 2003; Meziane et al. 2005) are reported as important components involved in elicitation of ISR. *P. putida* WCS358 and *P. fluorescens* WCS417 and WCS374 were reported to show differential resistance-inducing activities on arabidopsis, tomato, and bean (Bakker et al. 2007). LPS of *P. fluorescens* WCS374 apart from inducing systemic resistance against *Fusarium* wilt diseases of radish (Leeman et al. 1995b) also enhanced its colonization in tomato (Dekkers et al. 1998). The O-antigen of LPS has been shown to be involved in induced systemic resistance of plants, thereby enhancing defense activities against pathogen attack (Van Peer and Schippers 1992). Also, LPS and flagellin are stronger inducers of the oxidative burst, which in turn elicits plants defense mechanisms (Van Loon 2008).

4.2.7 Induced Systemic Resistance

Colonization of the rhizosphere by certain bacteria such as nonpathogenic *Pseudomonas* can trigger a systemic resistance in plants by activating the plants' defense mechanisms phenotypically similar to pathogen-induced systemic acquired resistance (SAR) and this form of induced resistance is referred as rhizobacteria-mediated induced systemic resistance (ISR). Bacterial LPS, siderophores, and salicylic acid (SA) are found to be determinants of ISR. Similar to SAR, ISR is effective against different types of pathogens but differs from SAR in that the inducing PGPRs do not cause visible symptoms on the host plant (van Loon et al. 1998). Scientific studies shows that when PGPRs are applied to seeds or roots, their defense mechanisms are activated (Van Peer et al. 1991). Fluorescent pseudomonads are also involved in the induction of plant resistance against insects and nematodes (Renwick et al. 1991; Zahnder et al. 2001) and specific metabolites produced by these bacteria have been involved in elicitation of defense reactions of the host plants (Van Loon et al. 1998). Similar to classical induced resistance, PGPR-induced resistance is known to produce broad-spectrum resistance, which is correlated with increased amounts of pathogenesis-related (PR) proteins, peroxidases, chitinases, and β-1,3-glucanase in plant tissue. Root colonization by

P. fluorescens CHAO and its derivatives correlates with increased amounts of salicylic acid, a putative resistance signal in leaves (Maurhofer et al. 1994a). ISR is effective under field conditions and offers a natural mechanism for biological control of plant disease.

Research findings revealed that *P. fluorescens* WCS417r-mediated ISR has been found effective against a wide range of pathogens, namely, *F. oxysporum* causing vascular wilts in arabidopsis (Pieterse et al. 1996), carnation (Van Peer et al. 1991), and radish (Leeman et al. 1996), *Alternaria brassicicola* and *Pseudomonas syringae pv. tomato* causing necrotic lesions in radish (Hoffland et al. 1996), and *P. syringae pv. tomato* causing bacterial speck in *Arabidopsis* (Pieterse et al. 1996). *P. putida* 89B–27 offered resistance against *Colletotrichum orbiculare* (Wei et al. 1991), Cucumber mosaic virus (Raupach et al. 1996), *Erwinia tracheiphila* (Kloepper et al. 1993), *F. oxysporum* f.sp. *cucumerinum* (Liu et al. 1995a), and *F. oxysporum* f.sp. *lachrymans* (Liu et al. 1995b) in cucumber. *P. fluorescens* CHAO induced systemic resistance against disease caused by *Thielaviopsis basicola* (Troxler et al. 1997) and Tobacco necrosis virus (Maurhofer et al. 1994a) in tobacco. *P. aeruginosa* 7NSK2 provided resistance against Tobacco mosaic virus (De Meyer and Hofte 1997), while *P. corrugata* 13 and *P. fluorescens* C15 induced resistance in cucumber to crown rot caused by *Pythium aphanidermatum* (Zhou and Paulitz 1994).

4.3 Plant Growth Promotion Mechanisms

Plant growth-promoting ability of these bacteria is due to the production of siderophore (Neilands and Leong 1986; Becker and Cook 1988; Loper 1988), phosphatase (Katznelson and Bose 1959), phytohormones (Keel et al. 1992; O'Sullivan and O'Gara 1992; Salisbury 1994; Patten and Glick 2002), 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Preisfeld et al. 2001; Penrose and Glick 2003), and cytokinins (Garcia de Salamone et al. 2001). A number of different fluorescent pseudomonad species such as *P. putida* (Scher and Baker 1982), *P. aeruginosa* (Bano and Musarrat 2003; Sunish Kumar et al. 2005), *P. chlororaphis* (Chin-A-Woeng et al. 1998), and *P. cepacia* (Cattelan et al. 1999) have been reported as PGPR as well as biocontrol strains against phytopathogenic fungi (Garcia de Salamone et al. 2001). Pigments, enzymes, and hormones produced by fluorescent pseudomonads that promote plant growth are listed in Table 4.2.

4.3.1 Phosphate Solubilization

Phosphate solubilizing bacteria are common in the rhizosphere. Secretion of organic acids and phosphatase are common methods of facilitating the conversion of insoluble forms of phosphorus to plant-available forms (Kim et al. 1998; Richardson et al. 2001). Fluorescent pseudomonad species such as *P. chlororaphis*,

Table 4.2 Pigments, enzymes and hormones by fluorescent pseudomonads

Pigments/enzymes/hormones	Producer strain	References
Siderophores		
	P. fluorescens 3551	Loper (1988)
	P. fluorescens CHAO	Maurhofer et al. (1994b)
Pyoverdine	*P. putida* WCS358	Van Wees et al. (1997)
	P. aeruginosa PAO-1	Cox et al. (1981)
	P. fluorescens CHAO	Buysens et al. (1996)
Pyochelin	*P. aeruginosa* 7NSK2	Buysens et al. (1996)
	P. stutzeri KC	Lewis et al. (2000)
	P. fluorescens ATCC 17400	Mossialos et al. (2000)
Pseudomonine	*P. fluorescens* WCS374	Mercado-Blanco et al. (2001)
	P. chlororaphis GN212	Cattelan et al. (1999)
	P. aeruginosa NJ-15	Bano and Musarrat (2003)
	P. aeruginosa PUPa3	Sunish Kumar et al. (2005)
	P. aeruginosa FP10	Ayyadurai et al. (2006)
	P. monteilii FPB59, FPB63	Ravindra Naik et al. (2008)
	P. plecoglossicida FPB31	Ravindra Naik et al. (2008)
	P. fluoresens FP7, FP14	Ravindra Naik et al. (2008)
	P. fulva FP23	Ravindra Naik et al. (2008)
	P. putida FP2, FP3	Ravindra Naik et al. (2008)
Phosphatase	*P. mosselii* FP13	Jha et al. (2009)
	P. stutzeri	Gamble et al. (1977)
Denitrifying enzymes	*P. fluorescens* YT101, RTC01	Philippot et al. (1995)
	P. putida GR12-2	Patten and Glick (2002)
	P. aeruginosa PUPa3	Sunish Kumar et al. (2005)
	P. aeruginosa FP10	Ayyadurai et al. (2006)
	P. aeruginosa FPB9, FPB15	Ravindra Naik et al. (2008)
	P. mosselli FP13	Ravindra Naik et al. (2008)
Indole-3-acetic acid	*P. monteilii* FPB21	Ravindra Naik et al. (2008)
Cytokinins	*P. fluorescens* G20-18	Garcia de Salamone et al. (2001)
1-Aminocyclopropane-1-carboxylate deaminase	*P. putida* GR12-2	Glick et al. (1994)
	P. aeruginosa Pw60, Pw61	Ravindra Naik et al. (2008)

P. putida, *P. aeruginosa*, *P. monteilii*, *P. plecoglossicida*, *P. fluorescens*, *P. fulva*, and *P. mosselii* have been identified as phosphate solubilizing rhizobacteria (Gaur 1990; Cattelan et al. 1999; Bano and Musarrat 2003; Sunish Kumar et al. 2005; Ravindra Naik et al. 2008; Jha et al. 2009).

4.3.2 Denitrification

Denitrification is an important microbial process in which oxidized nitrogen compounds are used as alternative electron acceptors for energy production when oxygen is limited. Denitrification consists of four reactions by which nitrates are

reduced to dinitrogen by the metalloenzymes such as nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase. Fluorescent pseudomonads are the most common denitrifiers isolated from temperate soils (Gamble et al. 1977). Fluorescent pseudomonads are able to adapt to limited oxygen conditions by using nitrogen oxides as alternative electron acceptors (Stewart 1988). Respiratory nitrate and nitrite reductase have been described to implicate in the competitiveness of model strains of fluorescent pseudomonad in soil (Philippot et al. 1995; Ghiglione et al. 2000).

4.3.3 Siderophores and Growth Promotion

As described in the earlier section, siderophores are low-molecular-weight ferric-specific chelation agent synthesized and secreted to solubilize iron (Neilands 1981; Abd-Alla 1998). These microbial siderophores stimulate plant growth directly by increasing the iron availability in rhizosphere soil and indirectly by competitive inhibition of growth of phytopathogen with less efficient iron uptake system (Marek-Kozaczuk et al. 1996). Microbial siderophores play an important role in Fe nutrition of plants in neutral and basic soils (Shenker et al. 1992, 1995). Several species of fluorescent pseudomonads produce siderophores and there is evidence that a number of plant species can absorb these bacterial Fe^{3+} siderophore complexes (Becker and Cook 1988; Loper 1988; Bitter et al. 1991). Fluorescent yellow green siderophores have been named as pyoverdines (PVDs) or pseudobactins (Budzikiewicz 1993, 1997). Besides PVD, *P. aeruginosa* produces another siderophore called pyochelin with a lower affinity for iron (III) (Cox et al. 1981). Fluorescent pseudomonad species such as *P. fluorescens*, *P. stutzeri*, and *P. putida* produce pseudomonine (isoxazolidone) (Lewis et al. 2000; Mossialos et al. 2000; Mercado-Blanco et al. 2001).

4.3.4 Phytohormones and Enzymes

Direct promotion of plant growth entails either providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment. Fluorescent pseudomonads also produce phytohormone, indole-3-acetic acid (IAA) cytokinins, and vitamins (Salisbury 1994; Streit et al. 1996; Patten and Glick 2002). Fluorescent pseudomonad bacteria also produce ACC deaminase that sequesters the ethylene precursor ACC (Belimov et al. 2001; Penrose and Glick 2003). Due to their potential to enhance plant growth, and participate in carbon, nitrogen, and phosphorous cycling in nature (O'Sullivan and O'Gara 1992), the role of fluorescent pseudomonads in agriculture and environment has been a matter of interest.

4.3.4.1 Indole-3-Acetic Acid

The phytohormone, IAA, is known to be involved in root initiation, cell division, and cell enlargement (Salisbury 1994). This hormone is commonly produced by specific strains of PGPR type of fluorescent pseudomonads (Barazani and Friedman 1999; Sunish Kumar et al. 2005; Ravindra Naik et al. 2008). IAA-producing rhizobacteria have been known to increase root growth and root length, resulting in greater root surface area, which enables the plant to access more nutrients from soil. Patten and Glick (2002) reported the role of IAA-producing *P. putida* in development of the host plant root system.

4.3.4.2 Cytokinins

Cytokinins are a class of phytohormones, which are known to promote cell divisions, cell enlargement, and tissue expansion (Salisbury 1994). Cytokinins are believed to be the signals involved in mediating environmental stress from roots to shoots (Jackson 1993). Production of cytokinins and enhancement of plant growth by strains *P. fluorescens* have been reported (Garcia de Salamone et al. 2001).

4.3.4.3 1-Aminocyclopropane-1-Carboxylate Deaminase

Ethylene is the only gaseous phytohormone. It is also known as the "wounding hormone" because its production in the plant can be induced by physical or chemical perturbation of plant tissues (Salisbury 1994). Among its myriad of effects on plant growth and development, ethylene production can cause an inhibition of root growth. Glick et al. (1998) put forward the theory that the mode of action of some PGPR was the production of ACC deaminase, an enzyme that could cleave ACC, the immediate precursor to ethylene in the biosynthetic pathway for ethylene in plants. ACC deaminase activity would decrease ethylene production in the roots of host plants and result in root lengthening. Wild-type and genetically modified fluorescent pseudomonads were reported as ACC deaminase producers (Glick et al. 1994; Ravindra Naik et al. 2008). Transforming *Pseudomonas* spp. strains with a cloned ACC deaminase gene enabled the bacteria to grow on ACC as a sole source of nitrogen and to promote the elongation of seedling roots (Shah et al. 1998). The growth promotion effects also expressed in stressful situations such as flooded (Grichko and Glick 2001) or heavy metal-contaminated soils (Burd et al. 1998; Belimov et al. 2001).

4.3.5 Vitamins

Vitamins are organic nutritional factors that influence the growth of living organisms. Plants synthesize vitamins (Arrigoni et al. 1992; De Gara et al. 1993)

and their root exudates contain B-group vitamins that enhance the growth of rhizosphere bacteria (Rovira and Harris 1961; Mozafar and Oertli 1993; Azaizeh et al. 1996; Streit et al. 1996). In addition, most soil bacteria and fungi also produce the B-group vitamins (Dahm et al. 1993; Deryło and Skorupska 1993; Rodelas et al. 1993; Sierra et al. 1999; Strzelczyk and Leniarska 1985) that are beneficial for plant growth (Azaizeh et al. 1996; Mozafar and Oertli 1993; Rovira and Harris 1961). These water-soluble vitamins are known to act synergistically with other biologically active substances stimulating growth of plants and microbes (Oertli 1987). Bacterial vitamin production might be one of the several factors affecting microbial competition for root colonization (Streit et al. 1996), bacterial growth stimulation (Marek-Kozaczuk and Skorupska 2001), and symbiotic nitrogen fixation (Deryło and Skorupska 1993). *P. fluorescens* 267 was reported to produce several B-group vitamins, i.e., biotin, thiamine, cobalamine, pantothenic acid, and niacin, which influenced its colonization on clover roots (Marek-Kozaczuk and Skorupska 2001). Several reports demonstrating the beneficial effect of rhizosphere pseudomonad strains on leguminous plants are available (De Freitas et al. 1993; Deryło and Skorupska 1993; Grimes and Mount 1987; Polonenko et al. 1987). Coinoculation of legumes with plant growth-promoting rhizobacteria increased the green and dry matter per plant, the number and weight of nodules, and symbiotic N fixation (Deryło and Skorupska 1993). Under controlled conditions, coinoculation of clover with biotin auxotroph *Rhizobium leguminosarum* bv. *trifolii* and vitamin-producing *P. fluorescens* strain 267 stimulated the growth and symbiotic N fixation of clover (Deryło and Skorupska 1993; Marek-Kozaczuk et al. 1996).

4.4 Concluding Remarks

Flourescent pseudomonads are the predominant group of rhizobacteria. This group of bacteria is metabolically and functionally diverse and exhibits multiple mechanisms that mediate their ability to suppress phytopathogens and to promote crop growth and yield. Specific strains of fluorescent pseudomonads could be used as biocontrol agents and biofertilizers in sustainable agriculture. Beneficial effects of fluorescent pseudomonads on plant growth and yield result from competition, root-colonizing potential, phosphate solubilization, sequestration of iron, production of plant growth regulators, vitamins, enhancement of plant nutrient uptake, production of antibiotics, synthesis of fungal cell wall-degrading enzymes, suppression of pathogenic or deleterious microorganisms, and induction of systemic resistance against phytopathogens. Strains that exhibit biocontrol mechanisms and plant growth promotion traits could be used to achieve biological control of phytopathogens. Knowledge of different mechanisms involved in plant growth promotion and disease suppression is essential for the selection and utilization of appropriate biocontrol strains for sustainable agriculture.

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Chapter 5
Role of *Pseudomonas aurantiaca* in Crop Improvement

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5.1 Introduction

Alfalfa (*Medicago sativa* L.) is worldwide in distribution. It is the most important forage legume grown in the semiarid Argentinean Pampas because of the quality of the nutrients that it provides (Viglizzo 1995). Furthermore, its root system improves and conserves soil structure and consequently promotes soil fertility (Vance 1997). Soybean (*Glycine max* L.) cultivation is also continuously expanding in Argentina. In fact, its production has been accelerating during the last decade, making this country one of the main world exporters (Lattanzi 2002).

Interactions between plants and microorganisms and among rhizosphere microorganisms are largely unknown and available literature revealed that these interactions are complex and depend on multiple traits (Lugtenberg and Dekkers 1999). Stimulation of growth by plant growth-promoting rhizobacteria (PGPR) (Kloepper et al. 1989) has been attributed to an increase in the mobilization of insoluble nutrients and subsequent enhancement of nutrient uptake by plants (de Freitas et al. 1997; El-Komy 2005), production of plant growth regulators (Derylo and Skorupska 1993; Dubeikovsky et al. 1993) and suppression of deleterious soil bacteria and phytopathogenic fungi (Fridlender et al. 1993; Weller

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and Thomashow 1993; Liu et al. 1995). Several *Pseudomonas* strains display plant growth-promoting (PGP) activities and have been investigated due to their widespread distribution in soil, ability to colonize the rhizospheres of host plants, and capacity to produce a range of compounds that are antagonistic to a number of plant pathogens (Rodríguez and Pfender 1997; Ross et al. 2000).

Diversity studies of *Pseudomonas* spp. and their PGP potential is important not only for understanding their ecological role in the rhizosphere and the interaction with plants, but also for biotechnological applications (Berg et al. 2002).

Pseudomonas chlororaphis, *Pseudomonas aureofaciens* and *Pseudomonas aurantiaca* were considered as separate species until 1989, when *P. aureofaciens* was proposed as a later heterotypic synonym of *P. chlororaphis* with *P. aurantiaca* remaining as a separate species. More recently, Peix et al. (2007) reclassified *P. aurantiaca* as a synonym of *P. chlororaphis* based on analysis of the almost complete 16 S rRNA gene. These authors suggested the location of *P. aurantiaca* as a novel subspecies of *P. chlororaphis*.

P. aurantiaca SR1 (or *P. chlororaphis subsps. aurantiaca* SR1) strain was isolated from soybean rhizosphere in the area of Río Cuarto, Córdoba, Argentina, at the Universidad Nacional de Río Cuarto (UNRC) experimental field (33°07′ latitude S; 64°14′ longitude W; 421 m above sea level), from a typical sandy loam Hapludoll soil. This strain was initially classified as *Pseudomonas aurantiaca* by the BIOLOG (Biolog Inc., Hayward, CA) system (Rosas et al. 2001) and, more recently, by amplification and sequencing of a partial fragment from the 16 S rRNA gene.

5.2 Rhizosphere Colonization

The beneficial effect of the introduction in soil of a bacterium capable of promoting plant growth or exercising biological control of pathogens is related to its root colonization capacity (Benizri et al. 2001). It has been shown that *P. chlororaphis* mutants affected in root colonization are unable to eliminate the causal agent of the tomato foot and root rot disease (Chin-A-Woeng et al. 2000). On the other hand, coinoculation of *P. fluorescens* and *Bradyrhizobium japonicum* increased the colonization of rhizobia on soybean roots, nodule number and acetylene reduction activity (ARA) as reported by Chebotar et al. (2001). Moreover, addition of sterile spent medium of *P. fluorescens* increased the growth of *B. japonicum* in yeast mannitol broth (YMB, Vincent 1970), indicating that *P. fluorescens* may have released substances that increased the rhizobial populations.

The colonization of the soybean and alfalfa root system by *P. aurantiaca* SR1 is shown in Table 5.1. Mean comparisons were conducted using the least significant difference (LSD) test ($P < 0.05$). The density of adhering bacteria on soybean and alfalfa seeds was 5.8×10^5 CFU/seed and 1.2×10^5 CFU/seed, respectively. The tracking of the population density of *P. aurantiaca* SR1 was carried out by counting those colonies that expressed SR1's characteristic orange pigment.

Table 5.1 Root colonization by *Pseudomonas aurantiaca* in soil at different days after planting

	Bacterial densities in root (CFU/g root wet weight)	
Days after planting	Soybean	Alfalfa
10	4.8×10^5	7.1×10^4
15	3.0×10^5	3.0×10^4
25	2.0×10^4	4.0×10^3

Values within the same column followed by different letters were significantly different ($p < 0.05$)

Twenty-five days after seedling emergence, bacterial populations declined in both soybean and alfalfa root systems; however, the lower counts of *P. aurantiaca* SR1 suggested its establishment in the tested legume rhizosphere. The level of alfalfa roots colonization by *P. aurantiaca* SR1 was slightly lower than that observed in the same crop plant by Villacieros et al. (2003), who used *P. fluorescens* strain F113.

In addition, *P. aurantiaca* SR1 possesses the capacity to colonize the root system of wheat and it behaves as an endophyte strain in several crops (Rosas et al. 2005; Carlier et al. 2008; Rosas et al. 2009).

5.3 Growth Promotion Mechanisms

Several direct and indirect mechanisms to promote plant growth are present in *P. aurantiaca* SR1 as follows:

5.3.1 Indole Acetic Acid Production

Phytohormones synthesis is the most important direct PGP trait, besides biological nitrogen fixation. There are reports that suggest that PGPR synthesize several different phytohormones that enhance various developmental stages of plants and most of the attention has been focused on the role of auxins. Auxins are a class of plant hormones; the most common and well characterized is indole-3-acetic acid (IAA), which is known to stimulate both rapid and long term responses in plants.

IAA produced by microbes colonizing the seed or root surfaces is proposed to act in conjunction with endogenous IAA to stimulate cell proliferation and/or elongation and enhance the host's uptake of minerals and nutrients from the soil (Patten and Glick 2002; Suzuki et al. 2003). The growth of plants treated with IAA-secreting PGPR is affected by the amount of IAA that the bacterium produces. Thus, PGPR facilitate plant growth by altering the hormonal balance within the affected plant (Glick 1995; Vessey 2003; Asghar et al. 2004; Kang et al. 2006).

Figure 5.1 shows the growth curve and IAA production by *P. aurantiaca* SR1. The concentration of IAA in the supernatant began to increase consistently after

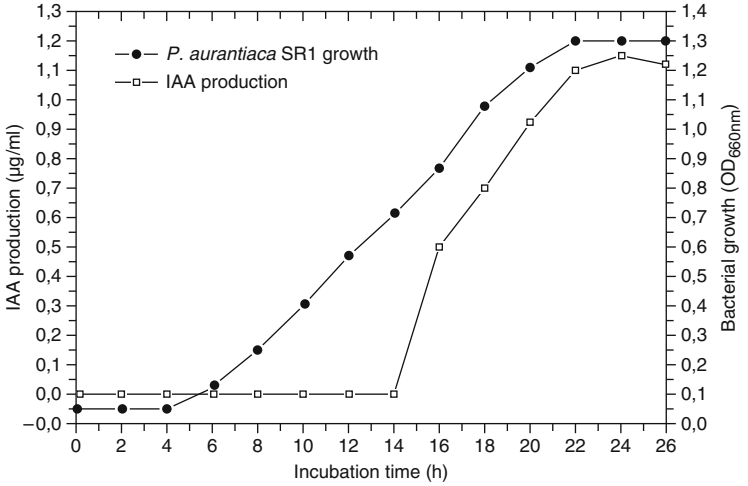

Fig. 5.1 Bacterial growth and IAA production by *P. aurantiaca* SR1 in 20% TSB medium. Culture was stopped after reaching the stationary phase

14 h of incubation and reached its highest level (11.7 µg/ml) at the end of the exponential growth phase or early stationary phase (24 h). Thereafter, there was a decrease in IAA synthesis by *P. aurantiaca* SR1.

In a recent study, Mehnaz et al. (2009) did not detect IAA production by *P. aurantiaca* PB-St2, a PGPR isolated from sugarcane stem. Our observations are in good agreement with other studies that suggested induction of IAA production during the stationary phase of culture, probably because of the induction of key enzymes involved in IAA biosynthesis (Oberhansli et al. 1990; Patten and Glick 2002; Ayyadurai et al. 2006).

5.3.2 Biocontrol Activity

Certain fluorescent pseudomonads are known to promote plant growth by producing metabolites able to inhibit bacteria and fungi that are deleterious to plants (Keel et al. 1990; Maurhofer et al. 1991; Hill et al. 1994). Some of these disease-suppressive antibiotic compounds have been chemically characterized and include phenazine-1-carboxylic acid (PCA), pyrrolnitrin, pyoluteorin, 2,5-dialkylresorcinol and 2,4-diacetylphloroglucinol (DAPG) (Thomashow et al. 1990; Keel et al. 1992; Kraus and Loper 1995; Nielsen et al. 1998; Dwivedi and Johri 2003). The results obtained by both the application of molecular techniques and direct isolation methods have demonstrated unequivocally that these antibiotics are produced in the spermosphere and the rhizosphere and are very important in suppressing soilborne plant pathogens (Bonsall et al. 1997; Ross et al. 2000; de Leij et al. 2002). DAPG produced by fluorescent pseudomonads is considered as a major

determinant in the biocontrol activity of PGPR. The broad-spectrum antibiotic DAPG has wide antifungal, antibacterial, antihelminthic, nematicidal and phytotoxic activity (Cronin et al. 1997; Raaijmakers et al. 2002). Information of the production of antifungal metabolites by *P. aurantiaca* strains are scarce. Esipov et al. (1975) describe the production of "a new antibiotically active fluoroglucide" in *P. aurantiaca*. Then, a possible route of synthesis of 2,5-Dialkylresorcinol in *P. aurantiaca* was proposed by Nowak-Thompson et al. (2003). Mandryk et al. (2007) reported new antimicrobial compounds produced by *P. aurantiaca*, having molecular formulas of $C_{18}H_{36}NO$ (molecular mass 282.8; antibacterial activity) and $C_{20}H_{31}O_3$ (molecular mass 319.3; antifungal activity). Reports about PCA production by *P. aurantiaca* could not be found, although Peix et al. (2007) used the production of PCA as a characteristic feature of *P. aureofaciens* and *P. aurantiaca* when they reclassified these species. A new method for obtaining regulatory mutants of *P. aurantiaca* capable of overproduction of phenazine antibiotics was described by Feklistova and Maksimova (2008).

Quorum sensing is the major way by which many bacteria regulate production of antifungal factors and N-hexanoyl homoserine lactone (HHL) is a compound that indicates the presence of a quorum sensing mechanism. Feklistova and Maksimova (2008) and Mehnaz et al. (2009) reported the production of N-HHL by *P. aurantiaca* B-162 and *P. aurantiaca* PB-St2, respectively.

P. aurantiaca SR1 produces an orange pigment associated with a strong in vitro antifungal activity against different pathogenic fungi such as *Macrophomina phaseolina, Rhizoctonia solani, Pythium* spp., *Sclerotinia sclerotiorum, Sclerotium rolfsii, Fusarium* spp. and *Alternaria* spp. (Rosas et al. 2001). The antifungal compound is secreted by the bacterium when culture media such as tryptic soy agar, nutrient agar or minimum media supplemented with tryptone or peptone are used. Among the tested carbon sources, mannitol and saccharose have been found to induce pigment production while glucose acts as a repressor (Rovera et al. 2000).

In our study, an antifungal compound was isolated from *P. aurantiaca* SR1 grown on plates containing 25% tryptic soy agar (TSA-Britania Laboratory, Argentina), incubated at 30°C for 5 days. The antibiotic produced by *P. aurantiaca* SR1 was confirmed as DAPG using thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and spectrometric techniques, and tested for antibiosis toward the phytopathogenic fungi *M. phaseolina*.

To purify the antifungal compound, a 0.5 ml volume of crude extract was spotted on thin-layer plates coated with silica gel 60, 20 by 20 cm (Merck) and developed in chloroform/methanol (98:2 vol/vol). The thin-layer chromatography of the isolated compound was compared with standard DAPG (Toronto Research Chemicals Cat. No. 10365500). The fluorescing and absorbing bands developed on plates were observed under long- and short-wavelength UV light. All bands and blank areas were separately removed from the plates and eluted with 100% acetone (70 ml). The silica residue was removed by centrifugation and the supernatant was transferred to a second set of microcentrifuge tubes. Each fraction was concentrated by evaporating the acetone and tested in vitro for antifungal activity.

Aliquots of 0.1 ml of the acetone elutes were placed directly onto TSA plates and were maintained under a laminar air flow until the acetone evaporated. Potato dextrose agar (PDA) plugs (5 mm) of *M. phaseolina* were placed in the center of the plates and incubated at 28°C for 5–7 days. The control consisted of a *M. phaseolina* PDA plug onto the surface of plates containing TSA medium after 0.1 ml of acetone had evaporated. The spots were identified by irradiating plates with UV light (350 and 245 nm). Only one spot showed antifungal activity (Rf: 0.35 in chloroform/methanol 98:2 vol/vol) (Table 5.2). A similar behavior was observed using chloroform:acetone (2:1) as eluant, with a Rf of 0.54. The active fraction (dissolved in methanol) was further analyzed by HPLC (Fig. 5.2). The isolated active compound was also analyzed by spectrometric techniques. UV–visible absorption spectra were recorded on a Shimadzu UV-2401PC spectrometer. ^{1}HNMR spectra were recorded on a Varian Gemini spectrometer at 300 MHz.

Table 5.2 Relative mobility (Rf) and antifungal activity against *Macrophomina phaseolina* of each band obtained from the TLC from *Pseudomonas aurantiaca* SR1 culture media

Band number	Relative mobility (Rf)	Antifungal activity
1	0.03	(−)
2	0.06	(−)
3	0.13	(−)
4	0.22	(−)
5	0.25	(−)
6	0.35	(+)
7	0.39	(−)
8	0.60	(−)

TLC in chloroform/methanol (98:2 vol/vol). The bands were observed by illumination at 254 and 350 nm. (+) inhibition. (−) no inhibition

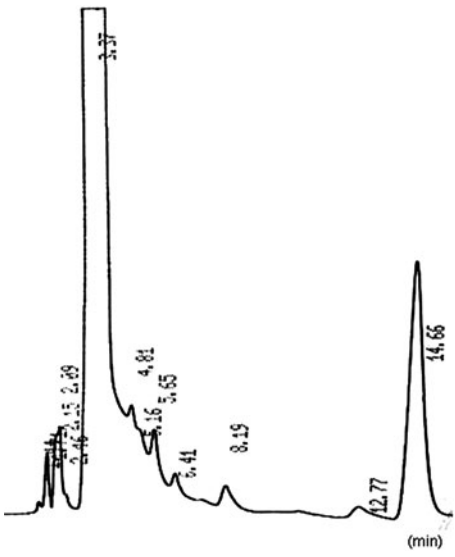

Fig. 5.2 HPLC chromatogram of DAPG (retention time 14.66 min) produced by *P. aurantiaca* SR1

Mass spectra were taken with a Varian Matt 312 operating in E.I. mode at 70 eV. IR spectra were recorded with a Nicolet Impact 400 FT-IR.

The UV–visible absorption spectrum of the active compound in methanol showed two bands at λ 267 and 328 nm. These bands are characteristic of $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions of aromatic rings bearing a carbonyl chromophore, which is characteristic for DAPG (Bonsall et al. 1997).

The Fourier transform infrared (FTIR) spectrum showed the presence of a broad absorption at 3,421 cm^{-1}, typically corresponding to a bound OH stretching, and bands at 2,923 and 2,852 cm^{-1}, which can be attributed to the C–H stretching. A strong absorption band was observed at 1,689 cm^{-1} that is recognized as a stretching of the carbonyl group. Also, medium sharp bands were shown at 1,463 and 1,384 cm^{-1} and a strong band centered at 1,074 cm^{-1}. Thus, the IR spectrum of the active sample was in agreement with the DAPG spectrum (Fig. 5.3). The mass spectrum and fragmentation of the isolated compound showed a match with that reported for DAPG, with peaks at 177, 195 (100%), 210 (M). Studies of ^{1}HNMR in acetone confirmed the DAPG structure.

P. aurantiaca SR1, formulated as an inoculant that contains 2.4×10^9 CFU/g peat, was able to produce the antifungal compound in rhizosphere soil. Genetic studies demonstrated the role of DAPG in biological control, which is complemented by direct isolation of DAPG from the rhizosphere (Bonsall et al. 1997). This effect was deduced from the antifungal activity of the residue resulting from the extraction made with organic solvents from soybean and alfalfa root systems inoculated. The TLC that was performed with the extract showed the presence of an active "band" with a value of Rf: 0.35, visualized under an UV light illuminator. The chromatography revealed the same profile as that obtained in the in vitro assays. The purification of the compound by HPLC revealed a similar profile to

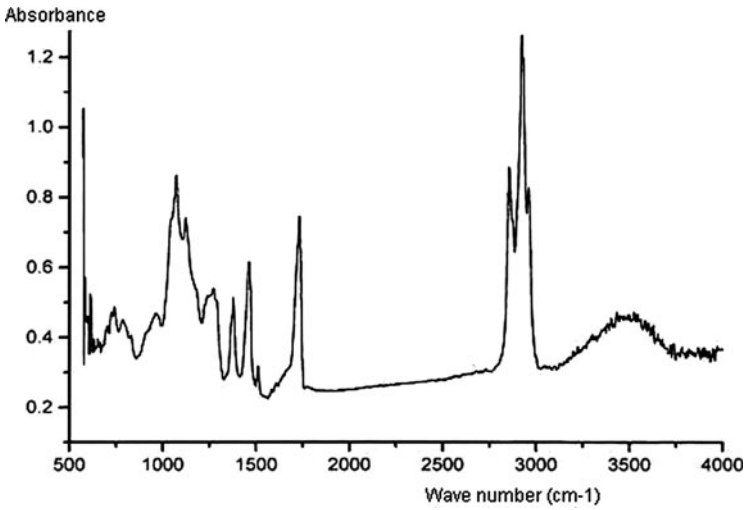

Fig. 5.3 FT-IR spectrum of DAPG produced by *P. aurantiaca* SR1

that of the compound extracted from the bacterial growth in culture medium and it is comparable with the pure standard. The TLC control (soybean and alfalfa roots from uninoculated seeds) did not present the "band" with an Rf of 0.35, indicating that other native microorganisms did not produce the compound. The antifungal compound was still detected 25 days after seedling. This detection had correlation with *P. aurantiaca* SR1 viability in the rhizosphere.

In other studies, we showed that *P. aurantiaca* SR1 produces siderophores and hydrogen cyanide (HCN). One way that microorganisms can prevent the proliferation of phytopathogens, and thereby facilitate plant growth, is through the production and secretion of low molecular mass iron-binding molecules (siderophores) with a very high affinity for iron. These bind most of the Fe^{+3} that is available in the rhizosphere, and, as a result, effectively prevent any pathogens in the immediate vicinity from proliferating because of lack of iron (Glick 1995; Rachid and Ahmed 2005; Viswanathan and Samiyappan 2007). The bacterium that originally synthesized the siderophores takes up the iron-siderophore complex by using a receptor that is specific for the complex (O'Sullivan and O'Gara 1992). Furthermore, some plants have mechanisms for binding the bacterial iron-siderophore complex and then releasing the iron so that it can be used by the plant (Wang et al. 1993). We tested the production of siderophores by *P. aurantiaca* SR1 using the Chrome azurol S method described by Alexander and Zuberer (1991). Plates were incubated at 28°C for 5 days and colonies exhibiting an orange halo were considered to be siderophore producers.

Certain PGPR strains produce volatile antibiotics, of which HCN is the most important; this compound inhibits the cytochrome oxidase of many organisms. The producing strains possess an alternate cyanide-resistant cytochrome oxidase and are relatively insensitive to HCN (Voisard et al. 1989; Rudrappa and Baiss 2008). HCN production by *P. aurantiaca* SR1 was determined as described by Bakker and Schippers (1987) in 10% TSA amended with glycine (4.4 g/l). A change in color, from yellow to orange-brown, of filter paper impregnated with 0.5% picric acid – 2% sodium carbonate indicated production of cyanide.

5.4 Legume Responses to Inoculation with *P. aurantiaca* SR1

Bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Azorhizobium* can interact with leguminous plant roots to form nodules, which function as sites for atmospheric nitrogen fixation (Relic et al. 1994). In addition, bacteria that promote nodulation of legumes by rhizobia are referred to as nodulation-promoting rhizobacteria (NPR) (Kloepper et al. 1988). However, these NPR include diverse groups of microorganisms such as *Azospirillum* (Hamaoui et al. 2001), *Azotobacter* (Tilak et al. 2006), *Pseudomonas* (Villacieros et al. 2003), *Bacillus* (Bai et al. 2003), *Paenibacillus* (Figueiredo et al. 2008), *Streptomyces* (Samac et al. 2003) and *Serratia* (Lucas García et al. 2004). Coinoculation studies with PGPR and *Rhizobium*/*Bradyrhizobium* spp. have been shown to increase root

and shoot weight, plant vigor, N_2 fixation and grain yield in various legumes including common bean (Grimes and Mount 1984), green gram (Sindhu et al. 1999), pea (Bolton et al. 1990) and soybean (Dashti et al. 1998; Rosas et al. 2006). Knight and Langston-Unkefer (1988) found that inoculation of nodulating alfalfa roots by a toxin-releasing *Pseudomonas syringae pv. tabaci* significantly increased plant growth, nitrogenase activity, nodule number, total nodule weight and nitrogen yield under controlled growth conditions. In other study, Pandey and Maheshwari (2007) reported cooperation between two rhizobacteria belonging to two distant genera, *Burkholderia* sp. and *S. meliloti*. They are rhizospheric isolates with the ability to produce IAA and solubilize inorganic phosphate. The two strains were tested on *Cajanus cajan* in sterile soil. Single inoculation of either resulted in significant increase in seedling length and weight, but an exceptional increase in seedling growth was recorded when coinoculating.

The *P. aurantiaca* and *S. meliloti* strains were maintained on 20% TSA medium. The coexistence of *P. aurantiaca* SR1 with *S. meliloti* 3Doh13 (Rosas et al. 2006) and *B. japonicum* E109 (Instituto Nacional de Tecnología Agropecuaria, Argentina) was tested. For this, *P. aurantiaca* SR1 was streaked along one side of Petri plates containing 20% TSA and parallel streaks of the respective rhizobia strains were made on the other side. Both strains were separated by a distance of 5 mm. Rhizobia was inoculated at the same time with *P. aurantiaca* and when *P. aurantiaca* growth was evident (normally after 48 h). Inhibition of rhizobial growth was measured after 5 days at 28°C. In a further experiment, we assessed the effects of the *P. aurantiaca* SR1 culture supernatant on *S. meliloti* 3Doh13 and *B. japonicum* E109 growth. *P. aurantiaca* SR1 was grown for 21 h in 20% TSB and the liquid culture was centrifuged at 6,000 rpm for 10 min at 4°C. The supernatant was filter-sterilized through a 0.22-μm Whatman filter and aliquots (0.2 ml) were placed directly onto 20% TSA agar plates. Then, *S. meliloti* 3Doh13 was streaked on this medium and it was incubated for 5 days at 28°C.

P. aurantiaca SR1 did not show any inhibitory effect neither on *S. meliloti* 3Doh13 nor on *B. japonicum* E109 in the coexistence assays performed at different times. Moreover, the exposure to the culture filtrate of strain SR1 did not affect the rhizobial growth.

Also, the combined effect of *P. aurantiaca* SR1 and *S. meliloti* 3Doh13 on growth and nodulation of alfalfa and *P. aurantiaca* SR1 and *B. japonicum* E109 on soybean growth under aseptic conditions was determined in this study. Rhizobia were routinely grown on yeast extract mannitol (YEM) solid medium (Vincent 1970) and *P. aurantiaca* SR1 on TSB medium. Bacterial cultures were raised to a level of 7×10^8 cfu ml^{-1} for *S. meliloti* 3Doh13, 1×10^9 cfu ml^{-1} for *B. japonicum* E109 and 4.5×10^8 cfu ml^{-1} for *P. aurantiaca* SR1.

First, soybean seeds were disinfected for 20 min with 0.4% calcium hypochlorite solution and alfalfa seeds were scarified by shaking for 15 min in concentrated sulfuric acid; then, seeds were disinfected with 70% ethanol for 3 min and washed with repeated changes of sterile, distilled water (Andrés et al. 1998; Rosas et al. 2006).

Second, seeds were treated with a culture of each rhizobial strain and *P. aurantiaca* by mixing culture broths of both the inoculant strains in a 1:1 ratio (v/v). One gram of

alfalfa seeds and ten grams of soybean seeds were inoculated with 2 ml of the single or mixed bacterial suspension, and average populations of bacteria on inoculated seeds were in the order of 1×10^5 cfu alfalfa seed^{-1} and 3×10^5 cfu soybean seed^{-1}, determined by dilution plate technique. Seeds (at least five seeds per treatment) were placed in tubes containing 10 ml of 0.1 M phosphate buffer (pH 7.4). Tubes were vortexed for 2 min and serial dilutions plated on 20% TSA and incubated at 28°C. Colonies were counted after 72 h.

Eight alfalfa or four soybean bacterized seeds were sown in plastic pots (15 cm diameter) filled with autoclaved perlite/sand (2:1). Plants were kept in a chamber under controlled conditions: 16 h light at 28°C and 8 h dark at 16°C, and a light intensity of 220 μE m^{-2} s^{-1}. Pots were watered alternately with sterile, distilled water and an N-free Jensen solution (Vincent 1970). N-control was watered in the same manner, but with the addition of 0.5% KNO_3 l^{-1} to the original Jensen solution. Each independent treatment was replicated three times.

Forty-five days after sowing, plants were harvested in order to evaluate shoot length, root length, shoot dry weight, root dry weight, nodule number and N content (only in alfalfa shoot). All samples were oven dried (at 70–80°C) for 48 h and weighed. The total N content was determined by a modified Kjeldahl method (Baker and Thompson 1992) by treating 100 mg sample with 1.25 g of catalytic mix (potassium sulfate:mercuric oxide, in a 24:1 relation) and 2.5 ml of conc. H_2SO_4. The mixture was digested for 40 min. Then, the ammonia liberated by alkalinization with NaOH solution was separated by distillation and collectical (as ammonium borate) in a 4% boric acid. Ammonium was determined by titration with a 0.02 N HCl. An automatic analyzer of N (Kjeltec Auto 1030, Tecator, Sweden) was used.

The composite inoculation of *P. aurantiaca* with *S. meliloti* increased the plant length, shoot and root dry weights and total N content in comparison to inoculation with *S. meliloti* alone or uninoculated control (Table 5.3). The dry matter accumulation in root and shoot and N-content in aerial parts increased significantly ($p \leq 0.05$) with respect to the N-control.

Nodulation on root systems of alfalfa plants following coinoculation with *S. meliloti* 3Doh13 and *P. aurantiaca* SR1 was increased by 34% compared to single inoculation. However, this did not correlate with the dry weight of nodules. The shoot and root dry weights of coinoculated plants were 1.09 and 1.24 times higher, respectively, than those observed for *S. meliloti*-inoculated alfalfa plants and 1.64 and 1.7 times with respect to uninoculated controls. The coinoculation of *P. aurantiaca* SR1 and *S. meliloti* 3Doh13 significantly enhanced nodulation at 45 days of growth.

The coinoculation of *B. japonicum* E109 – *P. aurantiaca* SR1 promoted an increase in shoot and root dry biomass, but not in the number of nodules, compared with single inoculation with *B. japonicum* (Table 5.4). The shoot and root dry weight of coinoculated plants were 1.24 and 1.35 times, respectively, greater than that of *B. japonicum*-inoculated soybean and 1.85 and 1.93 times with respect to uninoculated controls. The coinoculation of seeds with antibiotic-producing bacteria and rhizobia strains resistant to these compounds were proposed as a

Table 5.3 Effect of coinoculation of alfalfa with *Pseudomonas aurantiaca* SR1 and *Sinorhizobium meliloti* 3Doh13 on seedling growth and symbiotic parameters under sterile conditions

Microbial treatment	Shoot length ($cm.plant^{-1}$)	Shoot dry weight ($mg.plant^{-1}$)	Root length ($cm.plant^{-1}$)	Root dry weight ($mg.plant^{-1}$)	Number of nodules ($no.plant^{-1}$)	Dry weight of nodules ($mg.plant^{-1}$)	Shoot N ($mg.g^{-1}$)
Uninoculated control	5.68 ± 0.51	52 ± 3.4	6.72 ± 0.43	20 ± 0.8	_	_	41.9
Control + N	7.51 ± 0.6	76.2 ± 4.3	12.61 ± 0.33	25.8 ± 0.8	_	_	48.2
S. meliloti 3DOh13	7.08 ± 0.43	78.2 ± 5.1	10.28 ± 0.52	27.3 ± 1.4	7.5 ± 0.9	5.6 ± 0.5	47.6
S. meliloti 3DOh13 + *P. aurantiaca* SR1	8.20 ± 0.29	85.8 ± 4.5	13.78 ± 0.47	34 ± 5.1	10.1 ± 0.6	5.8 ± 0.7	50.7

Values within the same column followed by the same letter are not significantly different according to LSD ($p \leq 0.05$)
Experiments were repeated three times and results represent the mean per treatment of 25 plants for each treatment

Table 5.4 Effect of coinoculation of soybean with *Pseudomonas aurantiaca* SR1 and *Bradyrhizobium japonicum* E109 on seedling growth and symbiotic parameters under sterile conditions

Microbial treatment	Shoot length ($cm.plant^{-1}$)	Shoot dry weight ($mg.plant^{-1}$)	Root length ($cm.plant^{-1}$)	Root dry weight ($mg.plant^{-1}$)	Number of nodules ($no.plant^{-1}$)	Dry weight of nodules ($mg.plant^{-1}$)
Uninoculated control	49.1 ± 7.2	531 ± 86	22.6 ± 3.2	180 ± 25	_	_
Control + N	58.3 ± 4	1,002 ± 33	27.7 ± 2.5	332 ± 11	_	_
B. japonicum E109	54.6 ± 6.1	790 ± 25	26.3 ± 3.4	256 ± 9.1	28.8 ± 5.4	32 ± 4
B. japonicum E109 + *P. aurantiaca* SR1	57.3 ± 2.1	983 ± 21	24.9 ± 5.1	347 ± 13	25.5 ± 6.5	48 ± 6

Values within the same column followed by the same letter are not significantly different according to LSD ($p \leq 0.05$)
Experiments were repeated three times and results represent the mean per treatment of 25 plants for each treatment

way of reducing the detrimental interactions in the rhizosphere and thereby enhancing the colonization and nodulation of legume roots by rhizobia (Li and Alexander 1988).

5.5 Conclusion

Extensive studies have demonstrated that PGPRs could have an important role in agriculture, improving crop productivity. In recent years, emphasis on the use of two or more microorganisms has been made with the aim of maximizing beneficial

plant growth responses. It is important to identify the best strains of beneficial microbes, elucidate the mechanisms involved in growth promotion, to verify their compatibility and combined efficiency, both in vitro and in vivo, and employ such a combined inoculum in a real agricultural situation.

In this chapter, we describe the indole-3-acetic acid production by *P. aurantiaca* SR1 as well as the isolation and purification of a metabolite involved in the antibiosis capacity of this strain. The chemical characterization revealed that the cited compound is DAPG and its production was qualitatively detected in the in vitro assays and in the rhizosphere.

The *P. aurantiaca* strain was able to establish in the rhizosphere environment of the crop from which it was isolated (soybean) and also showed the capacity of colonizing the root system of alfalfa. It is interesting to note that although *P. aurantiaca* SR1 exerts a biocontrol effect on plant pathogenic fungi, probably due to the production of the secondary metabolites DAPG, HCN and siderophores, it does not interfere with rhizobial strains.

To summarize, we have shown that *P. aurantiaca* SR1 strain acted synergistically with *S. meliloti* 3Doh13 and *B. japonicum* E109 in promoting growth and symbiotic parameters of alfalfa and soybean under sterile conditions. It suggests a potential use of combinations of this strain and rhizobia in improving growth of these legumes. Our results are a contribution in the selection of bacterial strains for the formulation of new inoculants.

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Chapter 6
What Is Expected from the Genus *Azospirillum* as a Plant Growth-Promoting Bacteria?

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6.1 The Genus *Azospirillum*

The discovery of the first species of microaerophilic nitrogen-fixing bacteria of the genus *Azospirillum* was due to the introduction of nitrogen-free semisolid media. Under the conditions of oxygen gradient formed in semisolid media, the organisms, attracted by their characteristic aerotaxis, move to the region within this medium where their respiration rate is in equilibrium with the oxygen diffusion rate. They form typical veil-like pellicles approximately 5 mm below the surface, and then they move up close to the surface when, due to their N_2-dependent growth, more and more cells accumulate. After this discovery, several other diazotrophs with the same microaerophilic behavior were also isolated, belonging to other bacterial genera but using the same strategy.

At least 15 *Azospirillum* species have been described, but in terms of physiology and genetics the most studied ones are *A. lipoferum* and *A. brasilense* described by Tarrand et al. (1978). Both are abundant, mainly in tropical areas, associated with forage grasses, maize, wheat, rice, sorghum, sugarcane, and several other plants that also harbor this bacterial genus (Hartman and Baldani 2006; Zambrano et al. 2007). However, besides its association with plants, *Azospirillum* species have also been associated with other environments under extreme conditions of temperature or contamination (Nosko et al. 1994; Eckford et al. 2002; Young et al. 2008). The third species *A. amazonense* was isolated and described in the year 1983 from forage grasses planted in Amazonian region but is also associated with rice,

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maize and sorghum rhizosphere, and other grasses in the central-south of Brazil (Magalhães et al. 1983; Reis Jr et al. 2006). A group in Germany classified *A. halopraeferans*. It was isolated from kallar grass (*Leptochloa fusca*) growing under saline conditions in Pakistan and seems to be specific to that plant, since attempts to isolate *A. halopraeferans* from other plants growing in saline soil in Brazil failed (Reinhold et al. 1987, 1988). Further on, a new species isolated from rice plants in Iraq was described and named *A. irakense* (Khammas et al. 1989), although subsequently this species has not been reported as isolated from other grasses or any other country its name was validated as *Azospirillum* new species (List No. 39, 1991). In 1997, studies performed in Australia, using analysis of the 16S rDNA sequence of *Conglomeromonas largomobilis* subsp. *largomobilis*, showed that this bacterium had close relation to the species *A. lipoferum* and *A. brasilense*, but sufficiently distant to guarantee separate species status. On the basis of the phylogenetic evidence, Ben Dekhil et al. (1997) proposed the transfer of the subspecies *C. largomobilis* ssp. *largomobilis* to the genus *Azospirillum* as *Azospirillum largomobile* that was then corrected to *A. largimobile* (Sly and Stackebrandt 1999). New group of *Azospirillum* species continued to be described all around the world. In 2001, it was described and named *A. dobereinerae* in honor to the Brazilian scientist Johanna Döbereiner who initiated the studies of this genus in Brazil (Eckert et al. 2001). Another species was isolated from the paddy soil of a rice plant in 1982 and named as *A. oryzae* in China (Xie and Yokota 2005). Then, using a forage grass planted in China, another species was also described using surface sterilized roots and stems of *Melinis minutiflora* Beauv, and in this case, several modified semisolid media were used to collect 15 new strains of *A. melinis* (Peng et al. 2006). In 2007, using the same medium at pH 7.2–7.4 described by Xie and Yokota (2005), two new strains were identified by a research scientist group from Canada. These isolates were obtained from the rhizosphere of corn planted in Ontario and were named *A. canadense* (Mehnaz et al. 2007a) and *A. zeae* (Mehnaz et al. 2007b). *A. canadense* utilizes other carbon sources not used as the discriminative ones among previously described species as presented in Table 6.1. It uses many organic acids such as malate, pyruvic, acetic, succinic, citric, and formic acids. A newly described *Azospirillum* species was isolated from oil-contaminated soil by a group in Taiwan using nutrient agar. It was named *A. rugosum* as they form colonies that changed to wrinkled appearance on solid agar-medium and could be differentiated from its close phylogenetic relatives (*A. canadense, A. brasilense*, and *A. doebereinerae*) on the basis of carbon source utilization, gelatin hydrolysis, nitrate reduction, and arginine dihydrolase activity (Young et al. 2008). In 2009, two new species were described: *A. palatum* (Zhou et al. 2009) and *A. picis* (Lin et al. 2009). The first one was isolated from soil in China and according to the description is negative for acetylene reduction, do not produce indoles or reduce nitrate and/or nitrite. The second one was from Taiwan from discarded road tar, which generally contains, among other things, polycyclic aromatic hydrocarbons (PAH) and other poisonous or carcinogenic compounds. The *A. picis* fix nitrogen and is also positive for nitrate reductase, but indoles production has not been observed. Most recently another new species have been proposed, *Azospirillum*

Table 6.1 *Azospirillum* species and their pattern of carbon sources utilization

Azospirillum	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A. lipoferum	+	+	−	+	v	+	−	v	+	+	v	−	−	+	v	−	+	+	−	−
A. brasilense	−	v	−	+	−	v	−	+	v	+	−	−	−	−	−	−	−	−	−	−
A. amazonense	v	+	+	+	+	+	+	−	+	−	+	v	+	−	+	v	+	−	+	+
A. halopraeferans	nd	v	nd	+	nd	−	nd	nd	−	+	nd	nd	nd	+	+	nd	+	−	−	nd
A. irakense	+	+	+	v	+	+	+	−	+	−	−	+	+	−	+	+	v	−	+	+
A. largimobile	+	+	−	+	−	+	+	−	+	+	−	−	−	+	−	−	+	+	nd	−
A. dobereinerae	−	nd	−	+	nd	v	−	v	v	+	−	−	−	+	−	−	−	+	−	−
A. oryzae	nd	+	−	+	nd	+	nd	nd	+	nd	−	−	−	−	nd	+	+	−	−	nd
A. melinis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	nd	+	+	+
A. canadense	−	−	−	−	−	−	−	nd	−	−	−	−	−	−	−	−	nd	−	−	−
A. zeae	v	+	−	+	+	+	−	nd	v	+	−	−	−	+	−	−	nd	+	−	−
A. rugosum	−	−	nd	+	nd	nd	nd	+	+	nd	nd	−	−	−	nd	nd	nd	nd	nd	nd
A. palatum	v	v	+	+	nd	nd	nd	nd	+	−	nd	nd	+	+	nd	nd	nd	−	+	+
A. picis	+	+	−	nd	−	+	−	+	+	nd	nd	nd	−	+	−	−	+	+	−	−
A. thiophilum	nd	+	nd	+	nd	+	nd	nd	+	+	nd	−	nd	+	−	−	nd	+	−	−

Carbon sources: 1. N-acetylglucosamine, 2. L-arabinose, 3. D-cellobiose, 4. D-fructose, 5. L-fucose, 6. D-galactose, 7. Gentiobiose, 8. D-gluconate, 9. D-glucose, 10. Glycerol, 11. Myo-inositol, 12. Lactose, 13. Maltose, 14. D-mannitol, 15. D-mannose, 16. L-rhamnose, 17. D-ribose, 18. D-sorbitol, 19. Sucrose, 20. D-trehalose

Data from Eckert et al. (2001), Sly and Stackebrandt (1999), Ben Dekhil et al. (1997), Khammas et al. (1989), Reinhold et al. (1987), Xie and Yokota (2005), Peng et al. (2006), Mehnaz et al. (2007a, b) and Lavrinenko et al. (2010)

Symbols: +, positive; −, negative; v, variable or inconsistent; nd, not determined

thiophilum, isolated from sulfur bacterial mat collected from a sulfide spring in Russia (Lavrinenko et al. 2010). Although it has close relationship with several *Azospirillum* species, this new isolated strain BV-ST is capable of mixotrophic growth under microaerobic conditions with the simultaneous utilization of organic substrates and thiosulfate as electron donor for energy conservation. To summarize the knowledge accumulated about the genus up to now, we can assume that *Azospirillum* spp. are Gram-negative bacteria that belong to the alphaproteobacteria phylum. On the basis of the newly discovered species, they are present in a wide diversity of environments and plants, including not only those of agronomic importance such as cereals, sugarcane, and forage grasses, but also from other plant species such as coffee and fruit plants and orchids. They are aerobic nonfermentative chemoorganotrophs, vibroid and produce several phytohormones, mainly auxins (not described for all species yet). They utilize several carbon sources mainly sugars and sugar alcohols (Table 6.1) and the pattern of carbon utilization has been used for discriminatory purpose among species of the genus.

6.2 Genetics and Biochemistry

Data acquired from more than 30 years of the *Azospirillum* genetic and biochemical traits have been extensively reviewed by Steenhoudt and Vanderleyden (2000). Most structural and regulatory genes related to nitrogen-fixation genes including

nod genes have been identified in *Azospirillum* spp.; however, the mechanism of their regulation at transcriptional and translational levels is still a matter of study. Several research groups have dedicated to study the mechanisms of action of Ntr system, NifA, PII, and several other genes involved in the central nitrogen metabolism of *Azospirillum* (Araújo et al. 2004, 2008; Huergo et al. 2008).

Genes involved in chemotaxis signal transduction in other bacterial species (c*heA,W, Y, B*, and *R*) were identified in *Azospirillum* and most recently a *chsA* -Tn5 mutant strain (74031) isogenic to the *A. brasilense* wild type Sp7 was not impaired in its growth rate but showed reduced motility in soft-agar plates (Carreño-López et al. 2009). The authors observed that the impairment of motility during chemotaxis was not related to alteration of flagella synthesis but to different responses to chemotactic compounds. The deduced protein ChsA contains a sensory PAS domain and an EAL domain suggesting it may be part of the signaling and regulation system of another pathway controlling chemotaxis in *Azospirillum*. This data may reinforce the presence of multiple chemotaxis systems in this genus as previously reported in the literature.

The genome of *Azospirillum* sp B510, an isolate from inner tissue or rice plants, consisted of a single chromosome (~3.311 Mbp) and six plasmids designated as: pAB510a, pAB510b, pAB510c, pAB510d, pAB510e, and pAB510f. The total of 3,416 protein-encoding genes is distributed among the genome and most of the plasmids, but 25% (1,559) have been assigned as hypothetical genes. The high number of rRNA genes, nine sets of *rrns* genes, is also a peculiar characteristic of this species that is not typical of alphaproteobacteria genome, studied so far (Kaneko et al. 2010). Genes other than the *nif* gene cluster that are involved in N_2 fixation and are homologs of *Bradyrhizobium japonicum* USDA110 include *fixABCX, fixNOQP, fixHIS, fixG*, and *fixLJK,* present in the genome of this bacterium. In *A. brasilense*, IAA is generally synthesized from tryptophan via indole-3-pyruvic acid (IPyA), and genes coding enzymes related to this pathway are cotranscribed in this species. Interestingly, no homolog of the *ipdC*, which codes for the key enzyme indole-3-pyruvate decarboxylase, or *iaaC* was found in the B510 genome. Otherwise, two putative plant hormone-related genes encoding tryptophan 2-monooxytenase (*iaaM*) and indole-3-acetaldehyde hydrolase (*iaaH*), homologous to genes of *A. tumefaciens* and *P. syringae*, could play this role in IAA biosynthesis via indole-3-acetamide (IAM) pathway. Another relevant data is that in *Azospirillum* sp. B510 genome a homolog of *acdS* gene was identified, although it was previously reported that plant growth promotion (PGP) trait related to ACC (amino cyclopropene carboxylic acid) deaminase activity was absent in *A. brasilense* (Holguin and Glick 2003). Comparison between the *Azospirillum brasilense* Sp7 pRhico (plasmid that is responsible for the interaction with plant roots) and pAB510f suggests an evolutionary link and some functional relationship between both. However, remaining portions of pRhico, such as those containing exoC and tRNA genes, were scattered throughout the B510 genome and most of the sequences were poorly conserved, indicating that drastic genome rearrangements had taken place since the two species diverged as posed by Kaneko et al. (2010). During this century the description of new species and the recent publication of the

genome of an *Azospirillum* sp. associated with rice brought new insights into the genetic potential of this genus, not only to agricultural application but also to bioremediation and biocontrol (Bashan et al. 2004; Kaneko et al. 2010).

6.3 Bacterial Behavior in Plants

The associated bacteria capable of stimulating plant growth are generally known as PGPR mainly because of their ability to reduce other bacterial population and competence to establish the association to plant roots (Kloepper and Schroth 1981). However, this term can introduce some bias in its application since not only root-associated bacteria can contribute to PGP mechanisms as also posed by the authors. Thus, in order to include other beneficial plant–bacteria associations besides the root-associated we adopt in this chapter the term plant growth-promoting bacteria (PGPB) according to the definition found in the study by Bashan and Bashan (2005).

Bacterial colonization is well described for *Azospirillum*, especially *A. brasilense*. Attachment to the root system is mediated by the polar flagellum and is followed by an irreversible anchoring of the bacteria after a period of time (Steenhoudt and Vanderleyden 2000). Figure 6.1 shows the biphasic model of the colonization proposed by Steenhoudt and Vanderleyden (2000), but in this case other phenomenon exists with a homologous local strain of *A. brasilense* isolated in Argentina, associated with strawberry roots (Pedraza et al. 2007).

The lateral flagella are not essential during the adsorption phase for the colonization process. But how is the behavior of a population on the root system? Is quorum sensing (QS) involved in this coordinative process? The QS was previously shown to regulate swarming motility in diverse bacteria, notably in *Serratia*

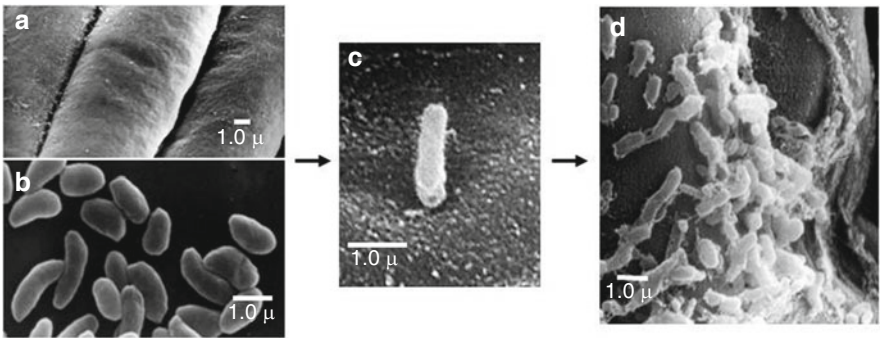

Fig. 6.1 Biphasic attachment process of *A. brasilense* to the root surface. (**a**) Strawberry root surface without inoculation; (**b**) Pure culture of *A. brasilense* REC3; (**c**) First step, *Azospirillum* as a single cell attached to the root surface; (**d**) Second step, bacterial cells forming aggregates and imbibed within the fibrillar material

marcescens (Eberl et al. 1996) and *Yersinia enterocolitica* (Atkinson et al. 2006). Boyer et al. (2008) tested two *A. lipoferum* strains, TVV3 and B518, isolated respectively from the rice rhizosphere (cultivar IR64) in Vietnam and from disinfected stems of rice (cultivar Kasalath) in Japan. In strain TVV3 *luxI* and *luxR* homologs have been identified but could not be inactivated, while strain B518 (isolated as a rice endophyte) produced larger amount of N-acylhomoserine lactones (AHLs) than did the strain TVV3. In order to quench AHLs accumulation in *A. lipoferum*, the *attM* gene from *Agrobacterium tumefaciens*, encoding an AHL-lactonase, was cloned under the constitutive PnptII promoter and conjugated into *A. lipoferum*. The results showed that in *A. lipoferum*, swarming and swimming motilities on semisolid agar plates were not affected by AHL inactivation. However, synthesis of Laf, the main structural component of the lateral flagella responsible for swarming motility in *A. lipoferum*, is impaired in B518 (pBBR1-*attM*), as revealed by proteomic analysis (Boyer et al. 2008). Thus unlike what occurs in a *laf*1 mutant of *A. brasilense* Sp7 that has lost its ability to swarm (Moens et al. 1995), B518 retains its ability to swarm despite the absence of Laf; this could be explained by the putative QS-independent production of biosurfactants implicated in bacterial swarming, as serrawetin in *S. marcescens* (Lindum et al. 1998).

But, is QS a common feature in the *Azospirillum* genus? Vial et al. (2006) used several strains of *Azospirillum* in order to verify if the common molecule of QS is present in this genus. AHL-mediated quorum-sensing in *Azospirillum* appears to be an exception rather than a rule. The authors tested 40 *Azospirillum* strains for their ability to synthesize AHLs. The AHL production was detected in four strains belonging to the *A. lipoferum* species isolated from rice rhizosphere. The authors also imply that other molecules not yet discovered can play a role in this genus, such as 3-hydroxypalmitic acid methyl ester (Clough et al. 1997) as observed in *Ralstonia*, or cis-11-methyl-2-dodecenoic acid described in *Xanthomonas* (Wang et al. 2004), two genera belonging to the alphaproteobacteria phylum as well.

The fact that AHLs are not implicated in regulation of phytostimulatory effects of *A. lipoferum* is an interesting result, as many bacteria possessing AHL-deactivating enzymes are rhizosphere inhabitants (Uroz et al. 2003). Moreover, QS in the rhizosphere can also be disrupted by abiotic factors such as alkaline pH, and by biotic factors, such as AHL mimics produced by some plants (Teplitski et al. 2000).

Bacteria colonize the rhizoplane and are found in high number upon emergence of lateral roots and also near the root cap (de Oliveira et al. 2002). These sites are normally colonized by several bacteria as they exude more carbon sources than other root areas, as they are growing regions. *Azospirillum* as a motile bacterium is capable of navigating in gradients of oxygen, redox molecules and nutrients by constantly monitoring its environmental changes in order to inhabit where it is optimal for surviving and growing. Although there is no strict host specificity in *Azospirillum*–plant associations, a strain-specific chemotaxis was reported; strains isolated from the rhizosphere of a particular plant showed preferential chemotaxis toward chemical compounds found in root exudates of that plant (Bacilio-Jimenéz et al. 2003; Pedraza et al. 2010). These results suggested that chemotaxis may contribute to host-plant specificity and could largely be determined by metabolism.

Furthermore, the specificity of bacterial strains probably reflects the adaptation of the bacteria to the nutritional conditions provided by the plant and can, thus, play an important role in the establishment of *Azospirillum* in the rhizosphere of the host, as previously suggested by Reinhold et al. (1985).

6.4 Plant Growth Promotion: Mechanisms

Azospirillum species influence plant growth through versatile mechanisms; they include N_2 fixation, phytohormone production (e.g., auxins, cytokinins, and gibberellins), increased nutrient uptake, enhanced stress resistance, vitamin production, siderophores and biocontrol, and some of them do P solubilization (Steenhoudt and Vanderleyden 2000; Dobbelaere et al. 2003; Seshadri et al. 2000; Rodriguez et al. 2004). However, the PGP trait ACC (amino cyclopropene carboxylic acid) deaminase activity was absent in *A. brasilense* (Holguin and Glick 2003). Some of these features are discussed as follows:

6.4.1 Phytohormone Production

Hormones exist in nature as molecules that regulate growth, development and differentiation of cells and tissues. They act as chemical messengers and are present in small amounts. In plants we call them phytohormones and they have been, in classical terms, divided into five classes: auxins mostly indole-3-acetic acid – IAA, cytokinins (CK), giberellins (GA), abscisic acid (ABA) and ethylene (ET). However, recently new molecules are also included as regulatory compounds, such as jasmonates, brassinolides, salicylic acid, polyamines, nitrous oxide, and strigolactones that are also related to regulation of the defense reactions of plants against pathogens (Santner et al. 2009). But bacteria also produce a wide variety of these signaling molecules and influence plant growth. *Azospirillum* is a well-known bacterium that produces high amounts of auxins "in vitro" and different pathways are involved in this production. The IAA is the best characterized and the most abundant member of the auxins family (Woodward and Bartel 2005). The best characterized pathways in *Azospirillum* auxin production is via indole-3-acetamide (IAM) and indole-3-pyruvate (IPyA) intermediates, both well described by Spaepen et al. (2007).

The root morphology is altered by the inoculation of *A. brasilense*, and the knowledgement is accumulated on data based on this species as it is one of the oldest ones of the genus. Several mutants of the IAA biosynthetic genes were developed, describing the action of these mutants on the development of the root system (Dobbelaere et al. 1999). Also the environmental conditions regulate the action of the mutants and affect root colonization. For example, IAA production and expression of the key gene *ipcC* have shown to be increased under carbon limitation, during reduction in growth rate and at acidic pH values (Ona et al. 2003,

2005; Vande Broek et al. 2005). This strategy is important when plant exudates become limited, so the signaling molecule is produced by the bacteria and the root senses to continue emitting lateral roots and root hairs, which are sources of exudates to maintain the bacterial population on the roots. Unfortunately, all these measurements are performed under experimentally controlled conditions, with a single bacterial species colonizing the plant. But how these signaling molecules act in nature, with a diverse population of microorganisms competing for a single source of carbon and are influenced by the environment? It is still a gap to solve. The use of model plants such as *Arabidopsis* colonized by PGPB together with quantitative imaging of roots can contribute to a new view of this question.

Azospirillum also produced CK, a phytohormone with a broader group of molecules and derived from ^{6}N-substituted aminopurines. They are widespread among other PGPB and their spectrums do not differ from the molecules produced by plants. CKs are produced in the root tips and developing seeds, and then they are transported to the shoot where they regulate several processes such as cell division, leaf expansion, and delaying of senescence (Spaepen et al. 2009). But in the opposite site of auxins, the insights into the role with plant–microbe interaction are related only to the measurements of bacterial production on bioassays as the development of mutants is not easy to obtain. The effect is based on the balance between auxins and CKs produced by the partners.

Another class of phytohormones that *Azospirillum* also produces is the GA; these compounds are involved in division and elongation of plant cells and influence almost all stages of plant growth. Several species of the genus produce different GAs and, in addition, they metabolize exogenously applied GA. The exact mechanism of production is not described, but Lucangeli and Bottini (1996, 1997) showed that a maize line dwarf-1 (dwarfism induced by inhibitors of the GA biosynthesis) could be reverted by the inoculation with *A. brasilense* and *A. lipoferum*. The species *A. brasilense* also produces ABA, but the mechanism has not yet been proved (Cohen et al. 2008). As all hormones interact with each other, ABA can interfere with the CK pool, as it interferes with its synthesis as cited by Spaepen et al. (2009).

After all these years and with some many new species described, it will be necessary several more years to cover the complexity of *Azospirillum* phytohormone interaction with plants. Furthermore, most of these data are based on "in vitro" tests using cultivated bacteria and measuring the production in a vial. Hence, they cannot be directly correlated with plant exudates (carbon source variable), population size (lower than in vials), and influence of the environment (including biotic and abiotic factors) as it happens in natural environmental conditions.

6.4.2 Siderophore Production

Siderophores are low-molecular-mass (<1,500 Da) compounds with high iron affinity that allows soil microorganisms to sequester and solubilize ferric iron in iron-poor environments, preventing any soil-borne pathogens to proliferate due to iron

limitation (Chaiharn et al. 2009). As other soil bacteria that have to compete with microorganisms for the limited available iron, *A. lipoferum* express siderophore-mediated iron transport systems. QS seems to negatively regulate siderophore synthesis in the strain used by Boyer et al. (2008), the B518, whereas no regulation was observed in TVV3 (the other strains tested by the same authors isolated from the rice rhizosphere). In soil, where the AHL concentration is low due to the small population density, high diffusion or AHL inactivation allows B518 to produce siderophores and thus being more competitive in acquiring iron. Different *A. brasilense* strains, isolated from strawberry plants, produce siderophores (Pedraza et al. 2007), and it was found that the amount produced is related to the origin of the bacterial strain (rhizospheric or endophytic), the endophytic being the best producers.

6.4.3 P-Solubilization

Phosphorus (P) is one of the major elements in plants, but especially in tropical soils, its availability is very low (<5% of the total P pool). Microorganisms can solubilize insoluble mineral P by releasing phosphatases (organic-P) or producing organic acids (release inorganic-P). This process is common for soil bacteria (>40% of culturable ones). About the genus *Azospirillum*, this feature is not entirely known yet as many attempts to determine its P-solubilizing capacities failed due to the experimental conditions (e.g., growth culture media). However, Seshadri et al. (2000) reported in vitro inorganic P-solubilization by *A. halopraeferans*. In addition, in vitro gluconic acid formation and P-solubilization from sparingly soluble phosphorus sources by two strains of *A. brasilense* (Cd and 8-I) and one strain of *A. lipoferum* JA4 were reported by Rodriguez et al. (2004). Strains of *A. brasilense* were capable of producing gluconic acid growing in soluble calcium phosphate medium when their usual fructose carbon source was amended with glucose. At the same time, there was a reduction in pH of the medium and released of soluble phosphate. To a greater extent, gluconic acid production and pH reduction were also observed for *A. lipoferum* JA4. These data add to the very broad spectrum of PGP abilities of this genus.

6.4.4 Nitrogen Fixation

Biological nitrogen fixation (BNF) is a well-described process especially for legume in association with rhizobia. As they possess a nodule that can be counted and plants without nodule or nitrogen fertilizer are complete nitrogen deficient, it was easy to propose the mode of action, describe the amount produced by BNF, and understand the several steps of colonization and fixation.

For other plants that do not possess nodules to fix nitrogen, the contribution is not 100% of the total N accumulated in tissues. The best known results of grasses

are related to sugarcane where it was proved that in controlled condition, 70% of the nitrogen accumulated came from the biological process (Urquiaga et al. 1992) and in field assays it was reduced to 30% (Yoneyama et al. 1997). Then another problem arises, who is the responsible for the BNF observed? As we already know, only 2–3% of the total bacteria are described, and every year new genus and lots of species are described. It is not easy to follow the same for the ecology of a new strain, plant localization, and experiments performed in order to calculate the impact of a single species in the BNF process. So we are describing bacteria without knowing its significance to plant nutrition.

A lot of field assays were performed in order to evaluate the effect of its inoculation on several grasses, and some authors have described them in detail. Okon and Labandera-González (1994) concluded that a significant increase in yields from 5 to 30% could be achieved by the inoculation with *Azospirillum*, especially with lower doses of nitrogen fertilizers. Also the effect was attributed to phytohormone production rather than nitrogen fixation. Fages (1994) showed that the success of using *Azospirillum* is around 50–75%, which is more than the normal measure expected for leguminous inoculation that is around 50%. Sumner (1990) described positive results to *Azospirillum* inoculation and most of the experiments on wheat were performed between 1983 and 1985. Recently, rice cropped under rain-fed conditions in Tucumán, AR, showed clear effects of *Azospirillum* inoculation when compared to N-fertilized noninoculated controls (Pedraza et al. 2009).

6.5 Agricultural Application

The utilization of bacteria as an inoculant product is the ideal goal based on the performance of rhizobial inoculants, especially in Brazil, where 100% of the soybean production use the bacteria and not the fertilizers to obtain 100% of the N necessary for the plant nutrition. After so many years of testing, isolating and describing *Azospirillum*, some efforts were also carried out in relation to have a commercial product using this bacterium. The technology is also based on the rhizobial product that is applied on the seed cover in a mixture with peat or using different kinds of liquid formulations.

At the beginning, only *A. brasilense* was the elected one as inoculant. In the United States, a product called Azo-Green™, produced by a company called Genesis Turfs Forages, was recommended to be applied on the seeds to improve germination, root system, drought resistance, and plant health. In Italy, Germany, and Belgium another product containing a mixture of *A. brasilense* (strain Cd) and *A. lipoferum* (strain Br17) was formulated in a mixture of vermiculite or in liquid formulation. The commercial name was Zea-Nit™ and was produced by Heligenetics and they recommended the reduction of 30–40% of the N fertilization of the plants. In France another AzoGreen™ was used in a maize application in the Agbasar Station, at Northeast of Tongo, Africa, with the increment of 100% in yield using a strain isolated by Fages and Mulard (1988), the CRT-1. In Mexico,

a product called "Fertilizer for Maize" was developed by the University of Puebla and applied in 5,000 ha in 1993 (Okon and Labareda-González 1994). More recently, in 2008, another inoculant product based on *Azospirillum* was developed to coffee plants in Mexico, and its application showed reduction of time during the three phenological plant cycle (Jímenez Salgado – http://www.proyectocinco.com/notas/reportaje04abr08.htm). Uruguay also had a product called Graminante™ that was commercialized as a powder mixed with calcium carbonate.

But regarding these bacteria, which are different for each country, why are they the best ones? Perrig et al. (2007) evaluated phytohormone and polyamine biosynthesis, siderophore production, and phosphate solubilization in two strains (Cd and Az39) of *A. brasilense* used for inoculant formulation in Argentina during the last 20 years. Siderophore production and phosphate solubilization were evaluated in a chemically defined medium, with negative results. Phytohormones IAA, cytokinins called zeatin, GA3, ABA, ethylene, and growth regulators putrescine, spermine, spermidine, and cadaverine (CAD) were found in culture supernatants of both strains. IAA, zeatin, ethylene, and polyamine CAD were found in all two strains. Az39 and Cd showed differential capability to produce the five major phytohormones and CAD in chemically defined medium. This fact has important technological implications for inoculant formulation as different concentrations of growth regulators are produced by different strains or culture conditions.

It is also important to maintain the good quality of the inoculant in order to provide efficient root colonization or invasion. It is necessary to adjust the cell density (10^9 minimum per gram) shelf life, free of contaminants and agronomically proved that the strain applied is capable to maintain the yield in the absence of a reduced dose of nitrogen or improve crop yield, in the presence of nitrogen application (PGPB action). In 2009, a company in Brazil began to sell a product based on *Azospirillum* strains for maize and rice application. In Argentina, there are several companies producing and selling inoculants based on *A. brasilense* that are being applied as solid (powder) or liquid formulations in different commercial crops (e.g., rice, maize, wheat, sunflower, sorghum, etc.).

Presently, with the reality of having to produce more food at a lower cost, without environmental pollution, biological fertilization with PGPB is an alternative for a sustainable agriculture. Although beneficial effects of inoculating with *Azospirillum* sp. has been widely described, the efforts in isolating new strains and assessing their PGP characteristics in natural environmental situations must continue in order to support their agricultural use as inoculant or biofertilizer.

6.6 Final Considerations

From the numerous scientific literature available today, it is clear that the *Azospirillum*–plant interaction is a natural and ubiquitous phenomenon. With the increasing understanding and knowledge of the benefits conferred to the host by this

association, we are one step forward to developing and improving an environment-friendly nutrient source and biocontrol agent for agronomically important crops. But for this, the efforts should be focused not only on describing new species, sometimes based on few bacterial isolates, but also on assessing their PGPB capacities, including their exploitation within the interspecies biodiversity.

Despite the recent advances in its biotechnological use as inoculants or biofertilizers, commercialization of this technology based on *Azospirillum* still demands extensive optimization and comprehensive study of the effects of the application. However, the prospects of this technology are promising if we take into consideration the rising cost and declining reserves of fossil fuels in the world, as well as pollution problems. In this scenario, the progressive installation of this biotechnology would mitigate environmental concerns arising from the use of nitrogenous fertilizers and its costs of acquisition, especially in developing countries, in order to support a sustainable agriculture.

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Chapter 7
Plasmid Plasticity in the Plant-Associated Bacteria of the Genus *Azospirillum*

Elena I. Katsy

7.1 Introduction

The genus *Azospirillum* is a member of the class Alphaproteobacteria and the family Rhodospirillaceae. This genus was commonly described as gram-negative, nitrogen-fixing, oxidase-positive motile aerobic/microaerophilic bacteria with a DNA G + C content of about 70 mol% and with vibrioid, S-shaped, or helical cells which grow best on salts of organic acids (Tarrand et al. 1978). On the basis of phylogenetic, chemotaxonomic, physiological, and biochemical data, 15 *Azospirillum* species have been validly described to date (Table 7.1). Comparative analyses of the 16S rRNA gene sequences showed that *A. brasilense*, *A. canadense*, *A. rugosum*, and *A. palatum*; *A. lipoferum*, *A. halopraeferens*, *A. largimobile*, *A. doebereinerae*, *A. oryzae*, *A. melinis*, *A. zeae*, *A. picis*, and *A. thiophilum*; and *A. amazonense* and *A. irakense* form three groups of the most closely related *Azospirillum* species (Mehnaz et al. 2007a, b; Lin et al. 2009; Zhou et al. 2009; Lavrinenko et al. 2010).

Azospirilla occur worldwide in a broad range of complex and heterogeneous environments, including soil and the roots, stems, and leaves of cereals, forage grasses, vegetables, and many other plants. These bacteria have been widely used as models for studying the mechanisms of mutually beneficial associative plant–microbial interactions for the past 30 years.

All *Azospirillum* species are motile owing to the presence of flagella. Several species have a mixed type of flagellation. For example, in *A. brasilense*, *A. lipoferum*, and *A. irakense*, a single polar flagellum (Fla) is produced in liquid environments, and numerous lateral flagella (Laf) are induced in addition to Fla on viscous and solid media (Tarrand et al. 1978; Hall and Krieg 1983; Khammas et al. 1989). Fla is responsible for swimming, whereas Laf are used

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Table 7.1 List of validly described *Azospirillum* species

Azospirillum species	Type strain	Source	References
A. brasilense	Sp7	Rhizosphere of pangola grass (*Digitaria decumbens*), Brazil	Tarrand et al. (1978)
A. lipoferum	Sp59b	Roots of wheat (*Triticum aestivum*), Brazil	Tarrand et al. (1978)
A. amazonense	Y1	Roots of pangola grass (*Digitaria decumbens*), Brazil	Magalhães et al. (1983)
A. halopraeferens	Au4	Roots of Kallar grass (*Leptochloa fusca*), Pakistan	Reinhold et al. (1987)
A. irakense	KBC1	Roots of rice (*Oryza sativa*), Iraq	Khammas et al. (1989)
A. largimobile	ACM 2041	Water sample from a lake, Australia	Skerman et al. (1983), Ben Dekhil et al. (1997), Sly and Stackebrandt (1999)
A. doebereinerae	GSF71	Roots of Chinese silver grass (*Miscanthus sinensis*), Germany	Eckert et al. (2001)
A. oryzae	COC8	Rhizosphere of rice (*Oryza sativa*), Japan	Xie and Yokota (2005)
A. melinis	TMCY 0552	Stems and roots of molasses grass (*Melinis minutiflora*), China	Peng et al. (2006)
A. canadense	DS2	Rhizosphere of corn (*Zea mays*), Canada	Mehnaz et al. (2007a)
A. zeae	N7	Rhizosphere of corn (*Zea mays*), Canada	Mehnaz et al. (2007b)
A. rugosum	IMMIB AFH-6	Oil-contaminated soil, Taiwan	Young et al. (2008)
A. palatum	ww 10	Forest soil, China	Zhou et al. (2009)
A. picis	IMMIB TAR-3	Discarded road tar, Taiwan	Lin et al. (2009)
A. thiophilum	BV-S	Sulfur bacterial mat from a sulfide spring, Russia	Lavrinenko et al. (2010)

for swarming, i.e., the population's movement across wet surfaces (Hall and Krieg 1983). Chemotactic responses of motile *Azospirillum* strains toward seed and root exudates, a major source of nutrients and signals for rhizobacteria increase the probability that the bacteria find the plant in the soil. Binding of azospirilla to plant surfaces is a next essential phase in the interaction of these bacteria with their hosts. Polar flagella seem to have some adhesive properties and mediate relatively rapid and reversible adsorption of azospirilla to plant roots. Lipopolysaccharides (LPS), exopolysaccharides (EPS), and capsular polysaccharides (CPS) play a role in the subsequent firm anchoring of azospirilla to the plant root surface and in proliferation of these bacteria on the roots. The association of azospirilla with plants generally does not require invasion of plant tissues. However, some strains enter the plant and colonize the root and stem interior (recently reviewed by Barassi et al. 2007).

The well-known plant-growth-promoting activity of azospirilla (Okon and Labandera-Gonzalez 1994) is attributed to the synthesis of plant hormones

(auxins, cytokinins, and gibberellins), deamination of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), production of nitrite and nitric oxide, nitrogen fixation, phosphate solubilization, biocontrol of phytopathogens, and other activities (for a recent review, see Bashan and de Bashan 2010). The use of a great number of *Azospirillum* strains (including *A. brasilense* Sp7, Cd, and Sp245; *A. lipoferum* Sp59b and 4B; and *A. irakense* KBC1) as plant inoculants under laboratory, greenhouse, and field conditions frequently resulted in acceleration of seed germination; enhancement of root, shoot, and leaf growth; increase in plant nitrogen and protein content and plant disease resistance; and several other beneficial effects (reviewed by Okon and Labandera-Gonzalez 1994; Lucy et al. 2004; Bashan and de Bashan 2010). However, the outcome of inoculation with the same *Azospirillum* strain varied significantly depending on the inoculant delivery system, soil type, climatic conditions, plant species, and many other parameters (Okon and Labandera-Gonzalez 1994; Lucy et al. 2004). Thus, a better understanding of the factors that influence the survival, ecological fitness, and plant-growth-promoting activities of azospirilla would be very useful.

It is accepted that the bacterial lifestyle influences genome size, content, and architecture (Bentley and Parkhill 2004). Accordingly, azospirilla have rather complex genomes. Although the *Azospirillum* genomes vary considerably in size, from 4.8 megabase pairs (Mb) in *A. irakense* to 9.7 Mb in *A. lipoferum* (Martin-Didonet et al. 2000), all of them consist of multiple replicons. Multipartite genomes are also typical of many other alphaproteobacteria, including those interacting with plants (Batut et al. 2004; MacLean et al. 2007). It was supposed that the presence of additional replicons may increase the adaptive potential of bacteria (González et al. 2006).

In *A. brasilense*, *A. lipoferum*, and *Azospirillum* sp. B510, a great number of genetic loci involved in the production of major cell-surface polymers (LPS, EPS, and CPS), components of the motility apparatus, and secondary metabolites important for the *Azospirillum*–plant interactions have been assigned to plasmids (Katzy et al. 1990, 1998; Vanbleu et al. 2004, 2005; Pothier et al. 2008; Prigent-Combaret et al. 2008; Lerner et al. 2009a, b; Kaneko et al. 2010; Katsy et al. 2010; Petrova et al. 2010b).

Several studies have recently shown that the genomes of *A. brasilense*, *A. lipoferum*, and *A. irakense* are quite dynamic and that some plasmids are involved in major genomic rearrangements (Katsy et al. 2002; Petrova et al. 2005a, b, 2010a; Vial et al. 2006; Pothier et al. 2008; Katsy and Prilipov 2009). In this review, I discuss what is currently known about plasmid plasticity in *Azospirillum* and how this genetic plasticity influences the bacterial phenotype.

7.2 General Features of Multipartite *Azospirillum* Genomes

A genomic resource for *Azospirillum* includes the complete genome sequence of the rice endophyte *Azospirillum* sp. B510 (Kaneko et al. 2010). Strain B510, isolated from the surface-sterilized stems of *Oryza sativa* cv. Nipponbare

(Elbeltagy et al. 2001) is closely related to *A. oryzae* COC8 (Xie and Yokota 2005; Kaneko et al. 2010). Inoculation of rice seedlings with *Azospirillum* sp. B510 significantly increased the growth of newly generated leaves and shoot biomass in a greenhouse, as well as tiller numbers and seed yield under paddy field conditions (Isawa et al. 2010). Inoculated rice plants became more resistant to diseases caused by the phytopathogenic fungus *Magnaporthe grisea* and the bacterium *Xanthomonas oryzae* via an unclear mechanism (Yasuda et al. 2009). It should be noted, however, that in field experiments, the effects of *Azospirillum* sp. strain B510 inoculation on the growth of different rice cultivars varied depending on the rice genotype and nitrogen level (Sasaki et al. 2010).

The results of two other projects, focused on the genomes of *A. brasilense* Sp245 (http://genome.ornl.gov/microbial/abra/), a strain isolated from surface-sterilized wheat roots (Baldani et al. 1983), and *A. lipoferum* 4B (http://www.genoscope.cns.fr/), isolated from the rhizosphere of rice (Thomas-Bauzon et al. 1982), have not been published yet.

7.2.1 Plasmids

Plasmids are extrachromosomal replicons that often facilitate adaptations of their hosts to new and hostile environments (Kado 1998). Practically all analyzed *Azospirillum* strains possessed plasmids, ranging in size from 6 kilobase pairs (kb) to more than 1.8 Mb (Martin-Didonet et al. 2000; Kaneko et al. 2010). The plasmid complement seems to play an important role in the beneficial *Azospirillum*–plant associations (Table 7.2).

The genome of *Azospirillum* sp. B510 is arranged into seven replicons: a chromosome of 3.31 Mb and six plasmids, pAB510a–pAB510f, of 0.26–1.46 Mb (Kaneko et al. 2010). Notably, the plasmid constituent of the *Azospirillum* sp. B510 genome encodes more proteins than the chromosome (Table 7.3).

The presence of ribosomal RNA genes on several high-molecular-weight replicons (Caballero-Mellado et al. 1999; Martin-Didonet et al. 2000; Kaneko et al. 2010) supports the idea that at least some *Azospirillum* plasmids should be regarded as "minichromosomes" (Wood et al. 1982). Even a not very large, ~90-MDa (~159-kb) plasmid (p90, or pRhico) (Vanbleu et al. 2004) could not be eliminated from *A. brasilense* Sp7 cells, suggesting that it is essential for the viability of the host strain (Onyeocha et al. 1990).

Plasmids of *A. brasilense*, *A. lipoferum*, and *A. irakense* are prone to frequent rearrangements, leading to the appearance of bacteria with individual plasmid profiles (Matveev et al. 1987; Katsy et al. 1990, 2002; Petrova et al. 2005a, 2010a; Vial et al. 2006). Differences in plasmid contents of the same *Azospirillum* strain kept in different laboratories (Caballero-Mellado et al. 1999; Martin-Didonet et al. 2000; Pothier et al. 2008) could also be attributed (at least in part) to plasmid plasticity. For example, an 85-MDa plasmid (p85) of *A. brasilense* Sp245 and

Table 7.2 Known plasmid-encoded traits potentially important for beneficial *Azospirillum*–plant interactions

Bacterial phenotype	*Azospirillum* strain	Plasmid involved	References	Possible role in *Azospirillum*–plant interaction
Flagellation, motility, and chemotaxis	*A. brasilense* Sp7	pRhico (p90)	Vanbleu et al. (2004)	Bacterial movement toward the plant
	A. brasilense Sp245	p85 and p120	Katzy et al. (1998, 2001), Borisov et al. (2009)	
	Azospirillum sp. B510	pAB510a, pAB510b, pAB510c, pAB510d, and pAB510e	Kaneko et al. (2010)	
	A. brasilense Sp7	pRhico	Vanbleu et al. (2004, 2005), Lerner et al. (2009a, b)	
	A. brasilense Sp245	p85 and p120	Katzy et al. (1998), Borisov et al. (2009), Katsy et al. (2010)	
Cell-envelope and motility apparatus components	*Azospirillum* sp. B510	pAB510a, pAB510d, pAB510e, and pAB510f	Kaneko et al. (2010)	Bacterial establishment on plant surfaces
	A. brasilense Sp245	p85	Katzy et al. (1990)	
Indole-3-acetic acid production	*Azospirillum* sp. B510	pAB510b	Kaneko et al. (2010)	Auxin stimulation of plant growth
	Azospirillum sp. B510	pAB510b	Kaneko et al. (2010)	
ACC-deaminase activity	*A. lipoferum* 4B	a 750-kb plasmid	Prigent-Combaret et al. (2008)	Lowering of plant ethylene levels
	A. brasilense Sp7	p115	Petrova et al. (2010b)	
	A. brasilense Sp245	p85	Petrova et al. (2010b)	
		250-kb (a probable cointegrate of p85 with another plasmid) and ~750-kb		
Nitrite reduction and NO formation	*A. brasilense* Sp245	plasmids	Pothier et al. (2008)	Plant root growth promotion by NO
	Azospirillum sp. B510	pAB510c	Kaneko et al. (2010)	
Uptake of siderophores	*Azospirillum* sp. B510	pAB510a, pAB510d, and pAB510e	Kaneko et al. (2010)	Reduction of iron available to phytopathogens

a 115-MDa plasmid (p115) of *A. brasilense* Sp7 are frequently absent from the plasmid profiles of Sp245 and Sp7 clones. These low-copy-number plasmids (Katsy 1992; Holguin et al. 1999) are incompatible (Katsy 1992) suggesting a common plasmid maintenance mechanism. Preliminary data presume that p85 and p115 could reversibly integrate into other *A. brasilense* replicons (Petrova et al. 2005a, b; Katsy and Prilipov 2009).

Table 7.3 Some features of the sequenced *Azospirillum* sp. B510 genome[a]

GenBank accession number	Circular DNA molecule	Length (kb)	GC content (%)	Coding DNA (%)	Predicted protein encoding genes	IS elements[b]	Prophages	Genomic islands
AP010946	Chromosome	3,311.4	67.8	86	2,893	77	2	6
AP010947	pAB510a	1,455.1	67.6	88	1,131	57	–	–
AP010948	pAB510b	723.8	67.5	89	631	78	–	–
AP010949	pAB510c	681.7	67.4	89	533	20	–	–
AP010950	pAB510d	628.8	68.0	87	519	7	–	1
AP010951	pAB510e	537.3	67.5	87	415	11	–	1
AP010952	pAB510f	261.6	65.9	90	187	30	–	–

[a]Compiled from GenBank records AP010946–AP010952 and from Kaneko et al. (2010)
[b]Total number of complete and partial IS elements

7.2.2 Insertion Sequences

As mentioned above, *Azospirillum* genomes seem to be highly dynamic entities. A significant source of genomic plasticity could be the transposition of insertion sequence (IS) elements, which are widespread in bacteria (Mahillon and Chandler 1998). Transpositions of IS elements may cause gene inactivation or activation of silent genes. Recombination between homologous copies of IS elements provokes DNA inversions or deletions (Mahillon and Chandler 1998). Besides, IS elements can mediate horizontal gene transfer (Heuer and Smalla 2007).

Two IS elements of the *A. brasilense* Sp245 plasmid p85, IS*Azba1* (distantly related to the IS*256* family) and IS*Azba3* (from the IS*5* family/IS*903* group), mediate fusions of p85 with foreign plasmids (Katsy and Prilipov 2009). These IS elements seem to be powerful natural tools useful for the enrichment of the *A. brasilense* genome with novel genetic material. As other factors potentially contributing to the known genetic plasticity of p85, truncated IS*Azba2* of the IS*L3* family and a phage integrase gene were also identified in this plasmid (Katsy and Prilipov 2009).

Two IS elements found on pRhico of *A. brasilense* Sp7 are homologous to the *Bradyrhizobium japonicum* IS elements ID145 and ID270, encoding a transposase and a DNA invertase (Vanbleu et al. 2004).

A striking feature of the *Azospirillum* sp. B510 genome is the abundance of multiple copies of IS elements (Table 7.3). The B510 genome contains 139 complete and 141 truncated copies of IS elements from at least 12 families (IS*3*, IS*5*, IS*21*, IS*66*, IS*110*, IS*256*, IS*630*, IS*701*, IS*1380*, IS*As1*, IS*L3*, and ISNCY). Many of these IS elements are present in more than one copy on the same or on different replicons (Kaneko et al. 2010) and thus represent potential sites for DNA recombination. The presence of redundant IS elements suggests their important role in the *Azospirillum* genome rearrangements.

7.2.3 Prophages

Many bacteriophages contribute to bacterial diversity owing to transduction of new genes and lysogenic conversion (Chibani-Chennoufi et al. 2004).

Several temperate strain-specific *Azospirillum* bacteriophages are known (Elmerich et al. 1982; Germida 1984; Boyer et al. 2008). Interestingly, in stable lysogens of *A. lipoferum* SpBr17, prophage Al-1 was supported as a 22-MDa (~37-kb) plasmid (Elmerich et al. 1982).

Phages isolated from cell lysates after mitomycin C treatment of *A. brasilense* cultures have 62–65-kb genomes (Boyer et al. 2008). In the closely related *A. brasilense* strains Cd and Sp7, practically identical 62.3-kb prophages reside in ~376-MDa (570-kb) plasmids; in addition, the 27.3-kb fragments of these prophages are duplicated in the 90-MDa plasmids (pRhico) (Boyer et al. 2008). In an almost complete nucleotide sequence of pRhico from *A. brasilense* Sp7, six putative prophage genes were identified (Vanbleu et al. 2004).

In *Azospirillum* sp. B510, two diverse prophages span 66.7 and 31.8 kb of the chromosome; an altered copy of the 66.7-kb prophage also resides in the chromosomal DNA (Kaneko et al. 2010).

Since the phages induced from *A. lipoferum*, *A. doebereinerae*, and *Azospirillum* sp. B510 (Boyer et al. 2008; Kaneko et al. 2010) had very small (ca. 10-kb) genomes, they were suggested to be the phage-like gene-transfer elements (GTE) (Boyer et al. 2008). However, no transducing activity has been recorded in any *Azospirillum* phage or GTE yet.

7.2.4 Genomic Islands

The contents of bacterial genomes can be changed quickly and significantly because of horizontal gene transfer and subsequent integration of foreign genetic elements into resident DNAs, where they form "genomic islands" (Dobrindt et al. 2004).

Apart from selfish mobility loci, genomic islands harbor other genes with specific functions. Expression of those genes can change bacterial behavior, increase bacterial fitness, competence in interacting with higher organisms, and adaptability to new environments ("fitness," "ecological," "saprophytic," "symbiosis," "pathogenicity," and other islands) (Dobrindt et al. 2004; Heuer and Smalla 2007).

Sequence analysis of the *Azospirillum* sp. B510 genome revealed six chromosomal and two plasmid (located in pAB510d and pAB510e) regions with typical signs of genomic islands (Kaneko et al. 2010). All the eight regions were located between duplicated portions of tRNA genes; possessed putative genes for integrases, site-specific recombinases, and transposases; and differed from the core genome in G + C content. The sizes of those genomic regions varied from 6.7 kb (chromosomal B510GI03) to 71 kb (B510GIe8 in pAB510e) (Kaneko et al. 2010).

Besides several loci coding for an integrase, transposases, and hypothetical proteins, a 22.6-kb genomic island (B510GId7) from pAB510d includes predicted genes for a transcriptional regulator and two-component sensor histidine kinase and response regulator (GenBank accession number AP010950).

In addition to numerous mobility and recombination loci, the 71-kb genomic island B510GIe8 possesses genes encoding a transcriptional regulator, two-component sensor histidine kinases and response regulators, a hemolysin-type calcium-binding protein, H^+-transporting and Mg^{2+}-importing ATPases, the chemotaxis protein methyltransferase, glycosyltransferases, and other predicted proteins (GenBank accession number AP010951). The genomic islands probably provide *Azospirillum* sp. B510 with some selective advantages.

7.3 Plasmid Dynamics and Phenotypic Variations in Several *Azospirillum* Species

Soil bacteria are supposed to use genetic and phenotypic diversification for survival in harsh and fluctuating environments. Various genomic rearrangements, often involving mobile genetic elements, may be induced in bacteria under specific growth conditions, such as prolonged starvation and oxidative and temperature stresses (Rajeshwari and Sonti 2000; Faure et al. 2004; Foster 2007). The presence of numerous plasmids, IS elements, phage-associated genes, and genomic islands in the *Azospirillum* genomes strongly suggests that these mobile arsenals could often be responsible for the phenotypic and genomic plasticity observed in these bacteria (see below).

7.3.1 Azospirillum brasilense

Azospirilla produce various cell-surface glycopolymers: LPS, EPS, and CPS. In most gram-negative bacteria, LPS consists of the hydrophobic lipid A, a core oligosaccharide, and an O-specific polysaccharide (OPS). A "smooth-type" LPS (S-LPS) possesses all the three structural entities; a "rough-type" LPS (R-LPS) is devoid of OPS; and SR-LPS is formed by lipid A-core capped with a single OPS unit (Raetz and Whitfield 2002).

Multiple open reading frames apparently responsible for the synthesis of LPS, EPS, CPS, and other cell-surface biopolymers were revealed in pRhico of *A. brasilense* strain Sp7 (Vanbleu et al. 2004, 2005; Lerner et al. 2009a, b). These findings may explain why it was not possible to cure the Sp7 cells from pRhico (Onyeocha et al. 1990).

Almost nothing is known about the functions of other *A. brasilense* Sp7 plasmids. However, plasmid rearrangements were found that were accompanied

by alterations in a number of Sp7 traits (Matveev et al. 1987; Petrova et al. 2005a, b, 2010a).

For example, indirect data on the possible involvement of the p115 plasmid in the regulation of bacterial R–S dissociation were obtained (Matveev et al. 1987). After prolonged incubation of *A. brasilense* Sp7 at 40–42°C, a fairly stable variant, Sp7-S, was identified, that had lost the 115-MDa replicon. The replicon loss correlated with persistence of the S phenotype in Sp7-S, i.e., the formation of smooth colonies (Matveev et al. 1987).

The differences between the R- and S-variants of Sp7 presumably resulted from the age-dependent changes in the contributions of two full-length LPS to the architecture of the colony surface rather than from partial or complete loss of OPS (Matora et al. 2003).

After several years of storage of *A. brasilense* Sp7-S at −70°C, its R-, SR-, and S-variants Sp7.1–Sp7.9 were isolated that contained derivatives of pRhico with changed molecular weights (94, 107, 121, 124, and 131 MDa) and structures (Petrova et al. 2005a, 2010a). For example, in *A. brasilense* Sp7.2 and Sp7.4, the new 131-MDa plasmid showed homology to both pRhico and p115 and seemed to be a hybrid of pRhico and a segment of p115 (Petrova et al. 2005a).

It was suggested that at least part of p115 has integrated into the chromosome of the variant Sp7-S, making the genome unstable and prone to DNA rearrangements (Petrova et al. 2005a). The genetic instability of *A. brasilense* Sp7-S may also be due to the specific method of its derivation (incubation at elevated temperature) (Matveev et al. 1987). It should be noted that soil bacteria are often exposed to elevated temperatures in their natural habitats. Upon multiple freezing–thawing of the *A. brasilense* Sp7 culture (which is also quite probable in soil ecosystems), an S-variant, Sp7.K2, was isolated that had also lost p115 (Petrova et al. 2005a).

A close relative of Sp7, *A. brasilense* strain Cd, lacking p115 (Petrova et al. 2005b) was isolated from the roots of bermuda grass after inoculation with Sp7 (Eskew et al. 1977). Both strains were characterized by nearly identical two-dimensional protein maps and DNA restriction fragment length polymorphisms (RFLP) (De Mot and Vanderleyden 1989; Gündisch et al. 1993; Petrova et al. 2005b). However, subtle differences were revealed in the LPS antigenic structures of Sp7 and Cd (Kirchhof et al 1997; Petrova et al. 2005b). Unlike in *A. brasilense* Sp7-S, further plasmid rearrangements were not detected in *A. brasilense* Cd. In contrast to *A. brasilense* Sp7, Sp7-S, and other *Azospirillum* strains, colonies of strain Cd do not dissociate on minimal synthetic media and retain their S-phenotype over a long time (Petrova et al. 2005b). The genetic causes for the differences in colony morphology and LPS structure in the closely related strains Sp7 and Cd, characterized by distinct plant-associated backgrounds, are of interest.

All the studied phenotypic variants of *A. brasilense* Sp7 retained the ability to swim and swarm. Moreover, the rates of their spreading in soft media were 1.7–2.7 times higher than that in the parent strain Sp7. Several variants of Sp7 have increased resistance to ampicillin and to cationic (cetyltrimethylammonium bromide) and anionic (sodium dodecyl sulfate) surface-active agents. The capability of a

number of Sp7 variants to grow on a minimal medium with cetyltrimethylammonium bromide as the sole carbon source deserves further investigation (Petrova et al. 2005a, b).

Lerner et al. (2010) isolated spontaneous derivatives of *A. brasilense* $Sp7^{-}$, a non-aggregating variant of Sp7, after 12-day starvation ($Sp7_{E}$ and two other variants) or colonization of maize roots (one variant). These derivatives differed from the parent strain in several traits. According to preliminary data, a plasmid rearrangement occurred in the variant $Sp7_{E}$. As compared to *A. brasilense* $Sp7^{-}$ and Sp7, the EPS-overproducing variant $Sp7_{E}$ has modified LPS and EPS, increased resistance to environmental stresses and to some antibiotics, and enhanced biofilm-forming capability (Lerner et al. 2010).

In natural environments, bacteria mainly exist in biofilms, i.e., structured communities embedded into a polymeric extracellular matrix and located at the interface between two media (Nikolaev and Plakunov 2007). The efficiency of biofilm formation by *A. brasilense* Sp7 and its variants was analyzed by Petrova et al. (2010a). It turned out that in Sp7, the above-described plasmid rearrangements (Petrova et al. 2005a) negatively affected biofilm formation on hydrophobic (polystyrene) and (less frequently) hydrophilic (glass) abiotic surfaces (Petrova et al. 2010a).

The *A. brasilense* Sp7 variants lacking p115 and containing the modified pRhico were also less active in plant root colonization during the first hours of interaction (Petrova et al. 2010a). Four to five hours after inoculation of wheat seedlings, the cells of Sp7 actively colonized root tips, hairs, and root fractures. On the next day, the Sp7 biofilms became more compact and multilayered, with cell aggregates starting to form. The overall picture of wheat root colonization by the variants of Sp7 was similar to that observed in the parent strain; however, at the initial steps of formation, their biofilms were thinner (especially in the cases of Sp7.K2 and Cd), single cells could be detected, and no colonization was observed in surface regions remote from the root tip. After 24 h, the wild-type Sp7 strain and all its variants formed cell aggregates on wheat roots; most of them were located on root hairs and tips and in places of root hair initiation, and visible interstrain differences disappeared (Petrova et al. 2010a). Thus, it seems that coordinated expression of the complete set of plasmid genes is important for more rapid adaptation of *A. brasilense* Sp7 to the new environments and for more successful realization of the biofilm development program.

As distinct from many other *Azospirillum* strains colonizing only plant surfaces, *A. brasilense* Sp245 is capable of invading plant roots (Baldani et al. 1983; Assmus et al. 1995). This strain has high potential for producing phytohormones and inducing the branching of roots and root hairs (Jain and Patriquin 1984).

A. brasilense Sp245 possesses LpsI and LpsII with subtle differences between their OPSs and/or core oligosaccharides recognizable with polyclonal antibodies raised against the wild-type LPS (Katzy et al. 1998) and by ion-exchange chromatography (Fedonenko et al. 2004). The OPSs of both LPSI and LPSII are composed of identical pentasaccharide D-rhamnose (D-Rha) repeating units (Fig. 7.1) (Fedonenko et al. 2002).

1
→2)-β-*D*-Rha*p*-(1→3)-α-*D*-Rha*p*-(1→3)-α-*D*-Rha*p*-(1→2)-α-*D*-Rha*p*-(1→2)-α-*D*-Rha*p*-(1→

2
→6)-α-*D*-Gal*p*NAc-(1→4)-β-*D*-Man*p*NAcA-(1→

Fig. 7.1 Structures of the repeating units of the O-specific polysaccharides of *A. brasilense* Sp245 (1) and its variant Sp245.5 (2), which spontaneously lost plasmids p85 and p120 upon the formation of a new, ~300-MDa megaplasmid (Fedonenko et al. 2002, 2010)

A 120-MDa plasmid (p120) of *A. brasilense* Sp245 encodes a number of predicted glycosyltransferases participating in the biosynthesis of LPSI, LPSII, and Calcofluor-binding polysaccharides (CBPS, Cal^+ phenotype), which include both EPS and CPS fractions (Katzy et al. 1998; Katsy et al. 2010).

Interestingly, the same penta-D-rhamnan OPSs were identified in *A. brasilense* SR75 (Fedonenko et al. 2005), Sp107, and S27 and in *A. lipoferum* RG20a (Boiko et al. 2010). At least in two of these strains (*A. brasilense* SR75 and Sp107), 120-MDa plasmids contain a region that is highly homologous to a 14-kb segment of p120 coding for the LPS biosynthesis enzymes. On the other hand, in the genomes of *A. brasilense* SR75 and Sp107, no homology to another (~8.3-kb) segment of p120 was found (Fedonenko et al. 2005; Katsy et al. 2010).

The p85 plasmid of *A. brasilense* Sp245 is known to be involved in tryptophan metabolism, related to auxin production (Katzy et al. 1990), and to encode predicted glycosyltransferases (Katsy et al. 2010), denitrification enzymes (Petrova et al. 2010b), and some other proteins (Katsy and Prilipov 2009; Petrova et al. 2010b).

After an analysis of the plasmid profiles of *A. brasilense* Sp245 strains obtained from different laboratories, Pothier et al. (2008) concluded that the smallest replicon (i.e., p85) probably underwent various independent rearrangements: a deletion of ~10 MDa, a cointegration with another plasmid, and a complete loss by the strain used in the Sp245 genome-sequencing project.

A variant of *A. brasilense* Sp245, named Sp245.5, was characterized that had spontaneously lost p85 and p120 upon the formation of a new ~300-MDa megaplasmid after long-term storage of the bacteria in a rich medium (Katsy et al. 1994, 2002). The wild-type (Sp245) and derivative (Sp245.5) strains have the same acetylene reduction activity; nearly identical SDS-PAGE profiles of the outer membrane proteins, except for an additional 44-kDa protein in Sp245.5 (Katsy et al. 1994); and similar RFLP patterns, except for the loci probably involved in plasmid rearrangement (Katsy et al. 2002). As distinct from the parent *A. brasilense* strain Sp245, Sp245.5 is unable to reduce nitrite and produces about two times less indole-3-acetic acid in the presence of tryptophan (Katzy et al. 1995).

Furthermore, strain Sp245.5 has drastic alterations in the biosynthesis of CBPS (Cal^- phenotype) and possesses a highly heterogeneous LPS, which could not be recognized by polyclonal antibodies raised against the LPS of Sp245 (Katsy et al. 2002). The macromolecular organization of the Sp245.5 LPS differs distinctly from that of the Sp245 LPS. Whereas the core oligosaccharide of the wild-type strain shows a very high degree of substitution with polysaccharide chains, attesting to the

prevalence of S-LPS in the LPS pool (Fedonenko et al. 2002), Sp245.5 displays both S- and R-LPS at an approximate ratio of 2:1 (Fedonenko et al. 2010).

The repeating unit of the OPS of *A. brasilense* Sp245.5 is a disaccharide consisting of residues of *N*-acetyl-D-galactosamine and *N*-acetyl-D-mannosaminuronic acid (Fig. 7.1), which has not been hitherto found in *Azospirillum* (Fedonenko et al. 2010).

Thus, the OPS of *A. brasilense* Sp245.5 differs from the D-rhamnan OPS of the parent strain Sp245 (Fedonenko et al. 2002) in both composition and structure (Fedonenko et al. 2010). It should be noted that *N*-acetyl-D-mannosaminuronic acid is a rare sugar, which, for instance, is present in the enterobacterial common antigen, a cyclic or linear heteropolysaccharide (Lugowski et al. 1983).

The loss of the D-rhamnan OPS by *A. brasilense* Sp245.5 could result from the adaptation of this bacterium to the conditions of several years' storage in a rich medium at room temperature. Probable activation of mobile elements and genetic rearrangements under the above storage conditions could lead to the deletion and/or inactivation of the D-rhamnan LPS biosynthesis genes with simultaneous activation of silent genes and a pathway for the production of OPS with novel monosaccharide composition and structure of the repeating unit.

The dramatic change in the LPS structure correlates with the activation of biofilm formation by *A. brasilense* Sp245.5 on hydrophilic and hydrophobic surfaces. Biofilms formed by its cultures are significantly more pronounced than those of the parent strain Sp245 (Fig. 7.2) (Sheludko et al. 2008).

A. brasilense Sp245.5 retained the flagellation and swimming motility pattern of the wild-type strain. However, its cells swim slower (at a speed of 17.1 ± 0.8 μm/s) than the cells of Sp245 (29.3 ± 0.9 μm/s). On semisolid media, populations of the Sp245.5 cells form "diffuse" spreading zones, whereas the wild-type bacteria form distinct swarming rings. Most probably, the altered spreading phenotype of Sp245.5 is caused by the principal change in the structure of cell-surface polysaccharides, which unbalances important carbohydrate–carbohydrate and protein–carbohydrate interactions (Shelud'ko et al. 2009).

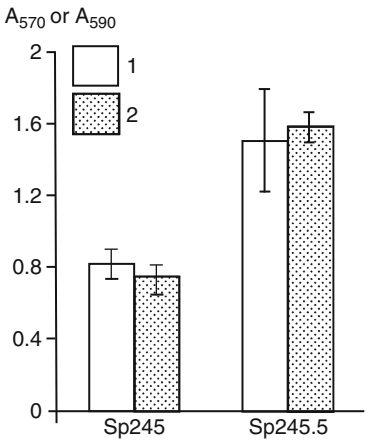

Fig. 7.2 Relative amount of biomass in the *A. brasilense* Sp245 and Sp245.5 biofilms formed on the surfaces of polystyrene (A_{570}) (1) and glass (A_{590}) (2) upon 96-h incubation at 28°C. Crystal violet staining of the biofilms was used. Details of the experiments can be found in Sheludko et al. (2008)

Thus, the spontaneous change in the content and structure of the *A. brasilense* Sp245 plasmids profoundly affects the metabolism, cell-surface polymers, and social activities of this bacterium.

A. brasilense WN1, a strain associated with wheat (Blaha et al. 2006), harbors plasmids of approximately 190, 260, 500, 570, and 650 kb (Vial et al. 2006). This strain showed the capacity for production of stable immotile variants (at a frequency of 3.6×10^{-3} per cell per generation), a process concurrent with a loss of the 260-kb replicon (Vial et al. 2006).

7.3.2 *Azospirillum lipoferum*

Under laboratory conditions, the strain 4B of *A. lipoferum* frequently produces a stable phase variant, named $4V_I$ (Alexandre and Bally 1999). In distinction to the wild-type strain, the $4V_I$ variant assimilates other carbohydrates; lacks a polar flagellum and does not swim; constitutively produces lateral flagella and has enhanced swarming capacity; and does not reduce triphenyltetrazolium chloride (Alexandre and Bally 1999; Alexandre et al. 1999) or nitrous oxide (Vial et al. 2006). When grown at extremely low oxygen concentrations, *A. lipoferum* $4V_I$ produces a stable laccase-positive melanin-producing atypical variant named $4V_{II}$. Neither *A. lipoferum* $4V_I$ nor $4V_{II}$ is able to revert to the wild-type phenotype (Alexandre and Bally 1999).

A. lipoferum 4B was isolated from the rice rhizosphere simultaneously with the immotile laccase-positive strain *A. lipoferum* 4T (Bally et al. 1983), which turned out to be very similar to the variant $4V_{II}$. Proven to be closely related to *A. lipoferum* 4B (Haurat et al. 1994; Vial et al. 2006), *A. lipoferum* 4T was supposed to be a spontaneous variant of 4B (the motile, laccase-negative strain) generated via a two-step phenotypic switching event within the rice rhizosphere (Alexandre and Bally 1999). *A. lipoferum* 4T retained the ability of 4B to efficiently colonize rice roots, but unlike the motile 4B, it needs the roots to be stabilized in sterile soil (Alexandre et al. 1996). Both *A. lipoferum* 4B and 4T coexist in sterile soil and the rice rhizosphere. In the rhizosphere of rice, the frequency of the appearance of stable immotile variants of *A. lipoferum* 4B (similar in other traits to the parent strain) seemed elevated (Alexandre et al. 1996).

In *A. lipoferum* 4B, replicons of 40, 310, 460, 700, 750, ~1,000, ~1,600, and ~2,200 kb were identified (Vial et al. 2006). Both *A. lipoferum* 4T and V_I retained all the replicons of 4B, except for the 750-kb plasmid. Interestingly, this plasmid harbors the *acdS* gene, a predicted product of which deaminates the ethylene precursor ACC and may be involved in determining the plant-growth-promoting activity of *A. lipoferum* 4B (Prigent-Combaret et al. 2008).

A new plasmid of 400 kb, nonhomologous to the lost 750-kb replicon, was detected in *A. lipoferum* 4T (Vial et al. 2006). It remains to be established whether the 400-kb plasmid was present in an integrated form in the genome of *A. lipoferum* 4B or was acquired by strain 4T via horizontal gene transfer from other rhizosphere bacteria.

7.3.3 *Azospirillum irakense*

A. irakense KBC1, a strain isolated from the rhizosphere of rice (Khammas et al. 1989), possesses megaplasmids of approximately 1,050 and 1,400 kb (Vial et al. 2006). At a frequency of up to 10^{-4} per cell per generation, KBC1 produces unstable immotile variants, the subculturing of which gives a mixture of wild-type and variant colonies (at a ratio of about 10 to 1, respectively). The phenotypic switches correlate with a supposed reversible split of the 1,400-kb replicon into 160-kb and 1,240-kb plasmids (Vial et al. 2006).

7.4 Concluding Remarks

As discussed above, in several strains of *A. brasilense*, *A. lipoferum*, and *A. irakense*, spontaneous DNA rearrangements were accompanied by changes in the structure of the major cell-surface polymers, bacterial motility, biofilm-forming capability, resistance to xenobiotics, and other traits potentially important for bacterial survival in soil and for colonization of plants. It is highly probable that the natural intrastrain diversification of azospirilla is the key to their success as ubiquitous environmental bacteria. However, the ecological consequences of the *Azospirillum* plasmid (and, on the whole, genome) plasticity have not been comprehensively studied yet. It seems important to find out how the profound genomic and phenotypic plasticities influence the adaptation of azospirilla to the complex environments such as soil and the phytosphere, as well as their plant-growth-promoting ability.

A challenge lies in determining the molecular mechanisms responsible for frequent genomic rearrangements. The accumulation of several *Azospirillum* genome sequences and the development of new experimental tools for the examination of intercellular and single-DNA-molecules variability (Medini et al. 2008; McCaughan and Dear 2010) are expected to yield more comprehensive knowledge about bacterial genomes as fluid and dynamic entities. These insights would have broader implications for understanding the natural behavior of other rhizobacteria, also prone to phenotypic switches (Achouak et al. 2004; Espinosa-Urgel 2004; Martínez-Granero et al. 2005) and for competent managing of sustainable plant–bacterial associations.

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Chapter 8
Enterobacter: Role in Plant Growth Promotion

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8.1 Introduction

Of all the variables that impact upon plant growth, soil microbial activity is arguably the very complex but plays a very important role in agricultural (or conservation) management. The importance of the microbiota to biogeochemistry has long been appreciated (Conrad 1996). Interactions between plants and microbes have long been known and we are increasingly aware of inter-kingdom communication signals across a broader range of ecological interactions than simple two-species mutualisms. Few would argue the point that the microbiota are an intimate part of the plant ecosystem and that understanding their roles will lead to new management opportunities. By describing patterns of variation in soil microbiota and explaining the basis of their ecological interactions with plants, soil microbial ecologists aim to develop new management tools for plant systems.

Plant growth promoting rhizobacteria (PGPR) can have an impact on plant growth and development in two different ways: indirectly or directly. The indirect promotion of plant growth occurs when bacteria decrease or prevent some of the deleterious effects of a phytopathogenic organism by one or more mechanisms. On the other hand, the direct promotion of plant growth by PGPR generally entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment (Glick 1995). Rhizosphere bacteria multiply to high densities on plant root surfaces where root exudates and root cell lysates provide the ample nutrients. Sometimes, they exceed 100 times to

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those densities found in the bulk soil (Campbell and Greaves 1990). Certain strains of these plant-associated bacteria stimulate plant growth in multiple ways: (1) they may fix atmospheric nitrogen, (2) reduce toxic compounds, (3) synthesize phytohormones and siderophores, or (4) suppress pathogenic organisms (Bloemberg and Lugtenberg 2001). Research on the "biocontrol" activity of rhizobacteria has seen considerable progress in the recent years. Disease suppression of soilborne pathogens includes competition for nutrients and production of antimicrobial compounds or lytic enzymes for fungal cell walls or nematode structures (Persello-Cartieaux 2003). By contrast, systemic resistance can also be induced by rhizosphere-colonizing *Pseudomonas* and *Bacillus* species where the inducing bacteria and the challenging pathogen remained spatially separated excluding direct interactions (Ryu et al. 2004).

Family Enterobacteriaceae, in the class Gammaproteobacteria, encompass a wide range of microorganisms including 42 genera in the last edition of Bergey's Manual of Systematic Bacteriology (Garrity 2005), but half of the isolates of new or unusual Enterobacteriaceae seem to be misidentified (Farmer 2005). To avoid misidentification of species in future, Paradis et al. (2005) propose the classification of species within Enterobacteriaceae by studying the genes encoding the elongation factor Tu and F-ATPase-β-subunit additionally to the gene encoding the 16S small ribosomal subunit. The genera within the family Enterobacteriaceae that feature members described as plant growth promoting bacteria (PGPB) are *Citrobacter*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Kluyvera*, *Pantoea* and *Serratia*, although some of these genera also contain species reported to be plant pathogens, for example *Erwinia carotovora* (Rodríguez-Díaz et al. 2008). The seven genera mentioned above have undergone changes in their taxonomy in the time elapsed between the two most recent releases of Bergey's Manual of Systematic Bacteriology. The genus *Enterobacter* was first described by Hormaeche and Edwards in 1960 (Hormaeche and Edwards 1960). Since *Enterobacter aerogenes* is considered a homotypic synonym to *Klebsiella mobilis* and *Enterobacter agglomerans* is transferred to the genus *Pantoea*, 13 species are left in the genus *Enterobacter*, i.e., *Enterobacter amnigenus*, *Enterobacter cowanii*, *Enterobacter gergoviae*, *Enterobacter intermedius*, *Enterobacter pyrinus*, *Enterobacter sakazakii*, and seven species which are combined in the so-called *Enterobacter cloacae* complex which include *Enterobacter asburiae*, *E. cloacae*, *E. dissolvens*, *E. hormaechei*, *E. kobei*, *E. nimipressuralis*, and *E. cancerogenus*, the senior synonym of *E. taylorae*. This genus contains the *Enterobacter agglomerans* group, which was extremely heterogeneous. Strains previously included in the *E. agglomerans* group have been proposed to be relocated into the genera *Erwinia*, *Leclercia* and *Pantoea*. Another species, *E. intermedius*, was first described as a senior subjective synonym for the species *Kluyvera cochleae* as shown by DNA–DNA hybridization (Brenner and Farmer 2005) but was later transferred to the genus *Kluyvera* as the species *K. intermedia* comb. nov. and *K. cochleae* was demonstrated to be a later synonym of *K. intermedia* (Pavan et al. 2005). PGPB strains must be rhizospheric competent and are able to survive and colonize in the rhizospheric soil (Cattelan et al. 1999). Unfortunately, the interaction between associative PGPR and plants can be

unstable. The good results obtained in vitro cannot always be dependably reproduced under field conditions (Chanway and Holl 1993; Zhender et al. 1999). The variability in the performance of PGPR may be due to various environmental factors that may affect their growth and exert their effects adversely (sometimes) on plant. The role of environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil can not be ruled out. To achieve the maximum growth promoting interaction between PGPR and nursery seedlings, it is important to discover how the rhizobacteria exerting their effects on plant and whether the effects are altered by various environmental factors, including the presence of other microorganisms (Bent et al. 2001). Therefore, it is necessary to develop efficient strains suitable for field conditions. One possible approach is to explore soil microbial diversity for PGPR having combination of plant growth promoting (PGP) traits and well adapted to particular soil environment. Ahmad and Khan (2010) studied *Enterobacter* for fungicide tolerance and production of PGP traits both in the presence and absence of fungicides. Strain PS2 showed PGP activities even in the presence of fungicides which, however, decreased progressively corresponding with the increase in fungicide concentration. Keeping in view the above constrains, the present chapter was designed to screen certain rhizospheric bacterial isolates belonging to the genera *Enterobacter* for their multiple PGP activities. Microorganisms have developed the mechanisms to cope with a variety of toxic metals for their survival in the environment enriched with such metals. We observed few rhizobacteria tolerant to heavy metals and exhibiting a couple of PGP activities. It was also apparent that more cultures of PGPR isolated from chickpea rhizosphere were tolerant to elevated levels of heavy metals. Burd et al. (1998) found that by decreasing heavy metal toxicity, PGPR increase plant growth. The selection of microorganisms both metal tolerant and efficient in producing PGP compounds can be useful to speed up the recolonization of the plant rhizosphere in polluted soils (Carlot et al. 2002). Heavy metals, at higher concentration, are toxic to cells and may cause cell death by interacting with nucleic acids and enzyme active sites (Ohsumi et al. 1988; Hazel and Williams 1990; Cervantes and Gutierrez-Corana 1994). On the otherhand, *Azotobacter* spp, when inoculated into heavy metal contaminated soil, inhibited N_2 fixation (Briely and Thornton 1983). Chromium-resistant *Pseudomonads*, isolated from paint industry effluents, were able to stimulate seed germination and growth of *Triticum aestivum* in the presence of potassium dichromate (Hasnain and Sabri 1996). It is expected that inoculation with rhizobacteria containing PGP characteristics consequently promote root and shoot growth as well as nodulation. Application of organic acid secreting bacteria for counteracting the toxicity of metal ions to their plant hosts has been developed. *E. asburiae* PS13 isolates from pigeon pea rhizosphere (Gyaneshwar et al. 1999) is known to produce different low molecular weight organic acid depending on the carbon source available. Kavita et al. (2008) reported that mung bean seedlings inoculated with *E. asburiae* PS13, a gluconic acid producer, enhanced plant growth in the presence of phytotoxic levels of Cd^{2+} in gnotobiotic pot experiments as compared to the uninoculated Cd-treated plants. Addition of organic acids to Cd-stressed seedlings

promoted root elongation. Deepa et al. (2010) studied PGP potential of strains NII-0907(*E. aerogenes*), NII-0929 (*E. aerogenes*), NII-0931(*E. cloacea*) and NII-0934 (*E. asburiae*) members of the genus *Enterobacter*. All the four enterobacter species are very good phosphate solubilizers (60.1–79.5 μg/ml/day after tenth day of incubation); indole acetic acid (IAA) producers (23.8–104.8 μg/ml/day after 48 h of incubation); HCN producers and siderophore producers. Studies have been carried out to investigate their influence on cowpea and recorded significant increase in higher root and shoot lengths compared with uninoculated control. Further evaluation of the isolates exhibiting multiple PGP traits on soil–plant system is needed to uncover their efficacy as effective PGPR.

8.2 Plant Growth Promoting Attributes

Enterobacter have the potential to contribute in the development of sustainable agricultural systems. Generally, *Enterobacter* function in three different ways: synthesizing particular compounds for the plants, facilitating the uptake of certain nutrients from the soil, and lessening or preventing the plants from diseases. The mechanisms of PGPR-mediated enhancement of plant growth and yield of many crops are not yet fully understood. However, the possible explanation include (a) the ability to produce a vital enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene in the root of developing plants thereby increasing the root length and growth; (b) the ability to produce hormones such as auxin, i.e., IAA; (c) a symbiotic nitrogen fixation; (d) antagonism against phytopathogenic bacteria by producing siderophores; and (e) solubilization and mineralization of nutrients, particularly mineral phosphates.

8.2.1 Nitrogen Fixation

Atmospheric N constitutes approximately 80% of the air we breathe. Although abundant and ubiquitous in the air, N is the most limiting nutrient to plant growth because the atmospheric N is not available for plant uptake. Some bacteria are capable of N_2 fixation from the atmospheric N pool. These bacteria form various associations with plants: (a) Many free-living N_2-fixing bacteria occur in soil. (b) Some have adapted to form symbioses; others have intimate endophytic associations with plants. (c) Few live in close association in the plant root zone (rhizosphere) without forming intimate endophytic symbioses. Infact, the amount of N fixed by these different systems is considerable, although variation resulting from environmental conditions or different plant–microbe combinations is vast. The close proximity of these microorganisms to their host plants allows efficient plant use of fixed N and minimizes volatilization, leaching, and denitrification. In

addition to symbiotic bacteria infecting roots, numerous taxa of less intimately associated N_2-fixing bacteria can be considered for crop growth improvement. Examples of such bacteria include *Acetobacter diazotrophicus* and *Herbaspirillum* spp. associated with sugarcane, sorghum, and maize (Triplett 1996; James et al. 1997; Boddey et al. 2000), *Azoarcus* spp. associated with kallar grass (*Leptochloa fusca*) (Malik et al. 1997), and *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Rhizobium* associated with rice and maize (James 2000).

8.2.2 Siderophore Production

Siderophores are low molecular weight bio-molecules secreted by micro-organisms in response to iron starvation for acquisition of iron from insoluble forms by mineralization and sequestration (Lankford 1973). Although some siderophores are known to chelate other ions, their specificity for iron is the most consistent feature. Lost ability to synthesize siderophores leads to loss of ability to synthesize cyanide and thus marred biocontrol abilities of strain proves the physiological importance of iron (Voisard et al. 1989). Siderophores produced by rhizosphere inhabitants have been studied well and it has been reported that the ability to produce siderophores not only improves rhizosphere colonization of producer strain but also plays an important role in iron nutrition of plant (Vansuy et al. 2007) and antagonism against phytopathogens (Chincholkar et al. 2007; Singh et al. 2008, 2010). Role of siderophores in induced systemic resistance (ISR) in plants was also well appreciated (De Meyer et al. 1999). The presence of an efficient iron uptake system can therefore contribute to protect the host plant against pathogenic infections. *E. coli* K12, *Enterobacter* sp. 638 (Taghavi et al. 2010) are able to synthesize the siderophore enterobactin (EntD, EntF, EntC, EntE, EntB and EntA), secrete (EntS), recover the iron–enterobactin complex using a ferric siderophore uptake system (ExbDB), and extract the iron using an enterobactin esterase (Fes) after internalization of the iron–enterobactin complex. The genes involved in this biosynthesis of enterobactin are grouped with genes encoding two ABC transporters involved in iron uptake (sitABCD and fepCGDB) in a large cluster of 17 genes (Ent638_1111-1128).

8.2.3 1-Aminocyclopropane-1-Carboxylase

ACC deaminase is a multimeric enzyme with a monomeric subunit molecular mass of approximately 35–42 kDa. It is a sulfhydryl enzyme that utilizes pyridoxal 5-phosphate as an essential co-factor. Pyridoxal phosphate is tightly bound to the enzyme in the amount of approximately one molecule per subunit; it displays a characteristic pyridoxaldimine visible absorbance at 418 nm (Jackson 1997). In this case, the substrate, ACC is exuded by plant tissues and is then taken up by the

ACC deaminase-containing microbe (Saraf et al. 2010). There is a wide range (>100-fold) in the level of ACC deaminase activity that is observed in nature from one organism to another so that these organisms may be conceptually divided into two groups: those with either high or low enzyme activity. High ACC deaminase expressing organisms typically bind relatively nonspecifically to a variety of plant surfaces. This group includes most, if not all, rhizosphere and phyllosphere organisms as well as endophytes, all of which can act as a sink for ACC produced as a consequence of plant stress. These organisms display little preference for one particular plant over another. On the other hand, low deaminase-expressing organisms bind only to specific plants or are expressed only in certain tissues, and they do not lower the overall level of ethylene in the plant, but rather prevent a localized rise in ethylene levels. This group includes most, if not all, rhizobia as well as plant ACC deaminases (Glick 1995). Burd et al. (2000) reported on the potential of the ACC deaminase-producing bacterium *Kluyvera ascorbata* SUD165 to protect canola (*Brassica napus*) and tomato (*Lycopersicon esculentum*) seeds from the heavy metal toxicity induced by high concentrations of nickel (Ni), lead (Pb), and zinc (Zn). Saravanakumar and Samiyappan (2007) reported that *Pseudomonas fluorescens* TDK1 possessing ACC deaminase activity enhanced the soil salinity resistance of groundnuts and observed increase yields over the groundnuts treated by *Pseudomonas* spp. that lacked ACC deaminase activity. However, the role of ACC deaminase in root colonization in bacteria is unknown (Wang et al. 2002). ACC deaminase from bacteria, *Pseudomonas putida* and *P. fluorescens*, *Enterobacter cloacae* CAL2 and UW4 (Shah et al. 1998), *Kluyvera ascorbata* SUD165 (Burd et al. 1998), and yeast, *Hansenula saturnus* (Honma and Shimomura 1978), and fungus, *Penicillium citrinum* (Jia et al. 2006) have been reported. However, work in this area represents only plant–microbe interaction.

8.2.4 Antimicrobial Compounds

Considerable progress has been made over the past two decades to elucidate the mechanisms by which PGPR suppress diseases. The primary mechanism of biocontrol by fluorescent *pseudomonads* involves production of antibiotics such as 2,4-diacetylphloroglucinol (PHL) (Bangera and Thomashaw 1996), pyoluteorin (PLT), pyrrolnitrin (PRN), phenazine-1-carboxyclic acid (PCA), 2-hydroxy phenazines and phenazine-1-carboxamide (PCN). In addition to direct antipathogenic action, antibiotics also serve as determinants in triggering ISR in the plant system and contribute to disease suppression by conferring a competitive advantage to biocontrol agents (Fravel 1988). Synergism between antibiotics and ISR may further increase host resistance to plant pathogens. Some other antibiotics produced by PGPR include pyrrolnitrin, oomycinA, viscosinamide, butyrolactones, kanosamine, zwittermycin-A, aerugine, rhamnolipids, cepaciamide A, ecomycins, pseudomonic acid, azomycin, antitumor antibiotics FR901463, cepafungins (Constantinescu 2001), and antiviral antibiotic karalicin. These antibiotics are

known to possess antiviral, antimicrobial, insect and mammalian antifeedant, antihelminthic, phytotoxic, antioxidant, cytotoxic, antitumour, and PGP activities. The *Enterobacter* sp. 638 genome (Taghavi et al. 2010) encodes a chloramphenicol acetyltransferase (cat, Ent638_1533) which provides resistance to 20 mg/ml chloramphenicol. A precursor of the important electron carrier ubiquinone, 4-hydroxybenzoate, is also known to have antimicrobial activity. In contrast to *E. coli* K12, which is not able to degrade chorismate into 4-hydroxybenzoate and pyruvate, *Enterobacter* sp. 638 possesses the ubiC (Ent638_0243) gene that codes for the putative enzyme able to perform this reaction (Siebert et al. 1996).

8.2.5 Hormonal Signals Involved in Plant Growth Promotion

Most commonly proposed signal molecules for PGP include bacterial synthesis of the auxin, cytokinin, gibberellin, and breakdown of plant produced ethylene by PGPR mediated of 1-aminocyclopropane-1-carboxylate deaminase. In the last 5 years, additional signals from microbes have been found to play a role in plant morphogenetic processes, including the *N*-acyl-L-homoserine lactones (AHLs) and volatile organic compounds (VOCs). AHLs belong to a class of bacterial quorum sensing signals from Gram-negative bacteria. These compounds enable bacterial cells to regulate gene expression depending on population density. Very recently, it was found that AHLs can be recognized by plants, alter gene expression in roots and shoots, and modulate defense and cell growth responses. Similarly, bacterial volatiles such as acetoin and 2,3-butanediol produced by certain PGPR can be used for plant–bacteria communication and as a PGP triggers. Some rhizobacteria, such as strains from *B. subtilis*, *B. amyloliquefaciens*, and *Enterobacter cloacae*, promote plant growth by releasing volatiles (Ryu et al. 2003). The highest level of growth promotion was observed with 2,3-butanediol and acetoin. Mutants of *B. amyloliquefaciens* IN937a and *B. subtilis* GB03, blocked in the biosynthesis of these compounds, are inactive in plant growth promotion. More recently, Zhang et al. (2008) observed that *B. subtilis* GB03 increases the photosynthetic efficiency and chlorophyll content of *A. thaliana* through the modulation of endogenous signaling of glucose and abscisic acid sensing; thus the bacterium plays a regulatory role in the acquisition of energy by the plant.

8.3 Molecular Biology and General Features *Enterobacter* sp.

The genome of the gamma-proteobacterium *Enterobacter* sp. 638 (Taghavi et al. 2010), a member of the Enterobacteriaceae, is composed of a single circular chromosome of 4,518,712 bp with an overall G + C content of 52.98%, and of a 157,749 bp plasmid pENT638-1 having an overall G + C content of 50.57%. The chromosome of *Enterobacter* sp. 638 displays a clear GC skew transition, which corresponds with its replication origin (oriC) and terminus. Similar to *E. coli* K12, the oriC site contains a perfect DnaA-binding box (TTATCCACA) (Weigel et al. 1997), which is located

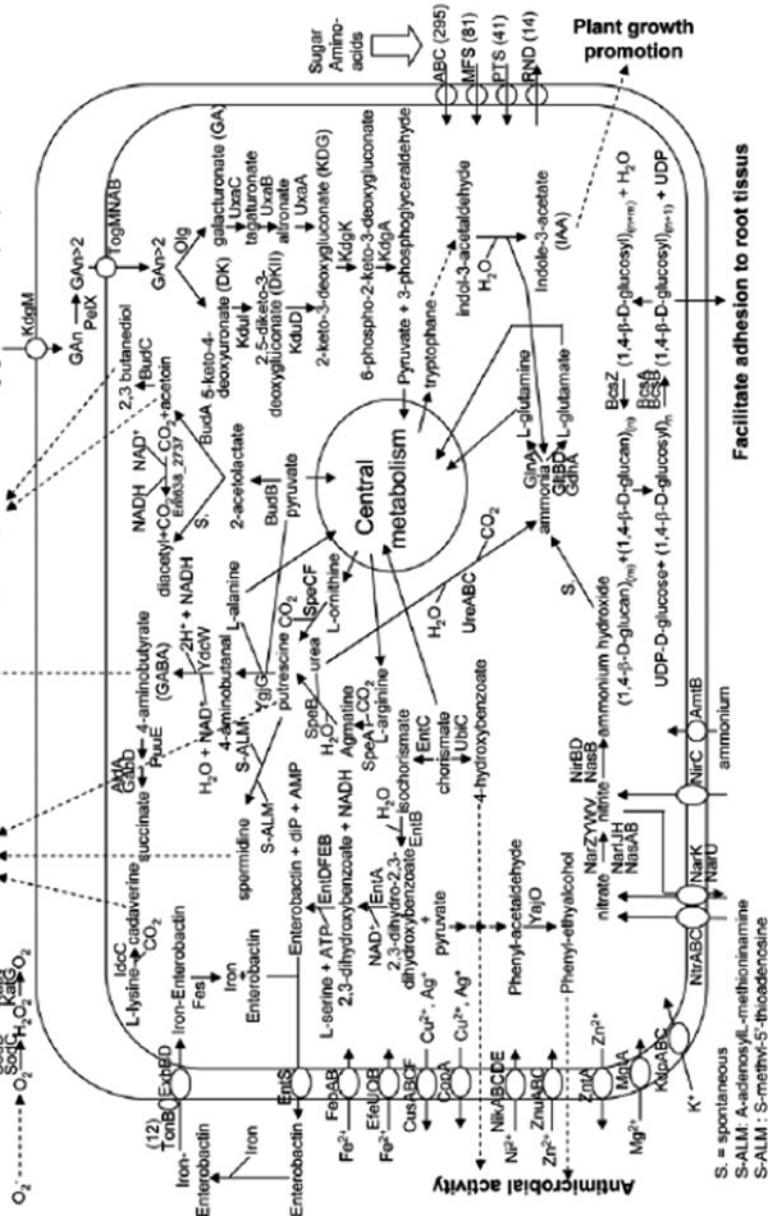

Fig. 8.1 Modified figure adapted from Taghavi et al. (2010). Overview of metabolism and transport in *Enterobacter* sp. 638. Predicted pathways for the interaction between *Enterobacter* sp. 638 and the plant are shown. Export or import of solutes is designated by the direction of the *arrow* through the transporter. The *dash arrows* show the putative function of certain molecular biosynthesized by *Enterobacter* sp. 638 in regard to its plant

31,985 bp upstream of the dnaA ATG start codon (at coordinate 4,487,245 bp). The *Enterobacter* sp. 638 genome encodes 4396 putative coding sequences (CDS), with 4247 CDS encoded on the chromosome representing a coding density of 87.9%. Plasmid pENT638-1 encodes 149 putative CDS having a coding density of 80.4%. After their manual annotation, 3561 CDS (81%) could be assigned to putative biological functions, while 836 CDS (19%) were annotated as hypothetical proteins of unknown function. For the CDS with unassigned functions, conserved hypothetical proteins are represented by 689 CDS (15.7%), while 147 CDS (3.3%) had no homology to any previously reported sequence. Using the COGnitor module from the MaGe system, 3597 CDS (81.8%) could be assigned to one or more COG functional classes. The repartition of *Enterobacter* sp. 638 CDS among the different COG classes is very similar to what is observed for *E. coli* K12 (Blattner et al. 1997). The three most abundant classes are amino acid (E), carbohydrate (G), and inorganic iron (P) transport and metabolism and represent more than 37% of all CDS, pointing to the symbiotic life styles of *Enterobacter* sp. 638 and *E. coli* K12 that require efficient uptake of host-provided nutrients. Seven sets of 5S, 16S, 23S rRNA genes, and one additional 5S rRNA gene were found. A total of 83 tRNA genes with specificities for all 20 amino acids and a single tRNA for selenocysteine have been identified. Based on the genome analysis, *Enterobacter* sp. 638 seems well adapted to survive in the plant rhizosphere because it encodes many transporters involved in carbohydrate, amino-acids, and iron uptake, as well as some heavy metal resistance genes. An overview of the metabolic properties and important transport pathways for interactions between *Enterobacter* sp. 638 and its plant host is presented in Fig. 8.1.

8.4 Selected Species of *Enterobacter*

The concept of introducing PGPR into the rhizosphere using the transplant plug is based on the hypothesis that their establishment in the relatively clean environment of planting media would afford them an opportunity to develop stable populations in the seedling rhizosphere, and that these populations would then persist in the field. This review covers the perspective of few selected species of *Enterobacter* and the role they are playing in plant growth promotion via direct and indirect mechanisms. The further elucidation of different mechanisms involved will help to make these bacteria a valuable partner in future agriculture.

8.4.1 Enterobacter cloacae UW5: Aromatic Amino Acid-Dependent Expression of Indole-3-Pyruvate Decarboxylase and Overexpression of hns Gene

Paradoxically, IAA produced by PGPR has been found to enhance host root system development. Plant roots colonize with the PGPR species *Azospirillum brasilense*

Sp6, *Enterobacter cloacae* UW5, and *Pseudomonas putida* GR12-2 displayed increase in root hair formation, the number and length of lateral roots, and/or primary root length that are dependent on bacterial IAA production.

A number of IAA biosynthetic pathways have been identified in bacteria, most requiring tryptophan as a precursor. Synthesis via the intermediates indole-3-acetamide or indole-3-pyruvate is widespread among IAA-producing bacteria. Most phytopathogens, such as *Agrobacterium tumefaciens* and *P. syringae* pv. *savastonoi*, use the indole-3-acetamide pathway to synthesize IAA (32, 54), while the indole-3-pyruvate pathway is found in many PGPR species, including *A. brasilense* and *E. cloacae*, and in the nonpathogenic epiphytic bacterium *Erwinia herbicola* 299R (Brandl and Lindow 1996; Costacurta et al. 1994). In the latter pathway, the precursor tryptophan is converted to indole-3-pyruvate by tryptophan transaminase, and indole-3-pyruvate is then converted to indole-3-acetaldehyde by indole-3-pyruvate decarboxylase (IPDC). IAA is produced after oxidation of indole-3-acetaldehyde by indole-3-acetaldehyde oxidase. The key enzyme in this pathway, IPDC, is encoded by *ipdC*, and elimination of *ipdC* abolishes IAA biosynthesis in *E. cloacae* UW5 and greatly reduces IAA production in *A. brasilense* and *E. herbicola* 299R (Brandl and Lindow 1996; Costacurta et al. 1994).

The activity of *tyrR* in *E. cloacae* UW5 was abolished by insertion of a tetracycline resistant cassette into the coding sequence, creating *E. cloacae* J35. The 2.1-kb tetracycline resistance cassette was amplified using tet-KpnI F and R primers and pJP2 as the template for PCR and then subcloned into pGEM-T Easy. A 1,177-bp *tyrR* fragment, amplified using the primers T1F and T4R, was also subcloned into pGEM-T Easy. The tetracycline resistance cassette was excised from pGEM-T Easy and inserted into a native KpnI site in *tyrR* (708 bp downstream from translation start codon) as a KpnI fragment. The interrupted *tyrR* gene fragment was excised from pGEM-T Easy and cloned into the NotI site in the suicide plasmid pJQ200SK, creating pJQ200TM. pJQ200TM was transformed into calcium chloride-competent *E. coli* S17-1 (*_pir*) cells and subsequently introduced into *E. cloacae* UW5 by conjugation. Double recombinants were identified by tetracycline resistance and gentamicin sensitivity. The site of insertion in the genome was verified by PCR amplification using the primers T1F and T1R (note that the T1R sequence is not present in pJQ200TM). The PCR amplicon generated from the genome of the *tyrR* insertional mutant (*E. cloacae* J35) was 2.1 kb larger than that from the wild-type strain, confirming the replacement of wild-type *tyrR* with the mutant *tyrR* gene fragment. Ryu and Patten (2008) described here that the transcription factor TyrR directly and positively controls *ipdC* expression and IAA production in the PGPR *E. cloacae* UW5 and that TyrR-dependent expression increases in response to exogenous tryptophan. A sequence with only a single base mismatch to the consensus sequence for the TyrR box (TGTAAA-N6-TTTACA) in *E. coli* (Pittard et al. 2005) has been identified in the promoter region of *ipdC* in this bacterium and in other closely related bacteria, even though the promoter sequences are otherwise quite dissimilar. Loss of IAA production and lower levels of *ipdC* expression following disruption of TyrR function through

insertional mutagenesis in mutant strain *E. cloacae* J35 confirmed the requirement for TyrR. The high degree of nucleotide sequence identity to the consensus sequence for the TyrR binding site suggests a strong TyrR protein-promoter DNA interaction. This is supported by the ability of purified TyrR to bind to the *ipdC* promoter fragment containing the TyrR box in vitro, and by the induction of *ipdC* expression, in the absence of an effector molecule. The observed transcription of *ipdC* in the absence of tryptophan supplements to the culture medium may be mediated by binding of endogenous aromatic amino acid cofactors to TyrR. Although TyrR can bind to strong boxes in the absence of cofactors, the addition of aromatic amino acids strengthens the interaction between TyrR and its recognition sequence (Andrews et al. 1991; Pittard et al. 2005). The increased affinity of TyrR for the promoter results in increased transcription, as was observed here by an increase in *ipdC* transcript abundance measured by real-time qRT-PCR and by an increase in *ipdC* promoter driven glucuronidase activity after addition of the TyrR cofactor tryptophan. An *E. cloaca* is an enteric, plant beneficial bacterium that suppresses damping off caused by *Pythium ultimum* on cucumber and other crops. An *Enterobacter cloaca* is a common seed associated bacterium that suppressed seed infections, protecting a number of plant species from *P. ultimum* induced damping off. Plant protection by *E. cloacae* is achieved largely by degradation of long chain unsaturated fatty acids in seed exudates, which eliminates the stimulation of *P. ultimum* sporangia. *E. cloacae* suppresses *P. ultimum* infections when applied as a seed coating of carrot, cotton, cucumber, lettuce, radish, sunflower, tomato, and wheat (Windstam and Nelson 2008).

Cloning and sequencing of the PCR fragment revealed that the transposon was inserted in the 5′ flanking region of a gene with homology to the *hns* gene encoding the small histone-like protein H-NS in *E. coli* and *Enterobacter* spp. (English et al. 2010) H-NS binds predominantly to AT-rich sequences, regions of intrinsic curvature commonly found in promoter sequences, and represses transcription of a large number of genes (Lucchini et al. 2006; Navarre et al. 2006). The expression of 5% of genes in *E. coli* is regulated by H-NS (Hommais et al. 2001), and many of these are important for adaptation to environmental changes such as alterations in temperature, pH, osmolarity, and growth phase (Stella et al. 2006). H-NS has also been noticed to repress genes that have been acquired by bacteria through horizontal gene transfer (Lucchini et al. 2006; Navarre et al. 2006). In addition to its role as a transcriptional repressor, H-NS positively regulates the production of flagellin and several outer membrane porins, lipopolysaccharide biosynthesis, and motility (Ono et al. 2005). Motility is important for plant colonization and conditions in the rhizosphere have been shown to select for motile strains (Martinez-Granero et al. 2006). Production of flagella and lipopolysaccharides has previously been shown to be required for plant colonization (Turnbull et al. 2001); therefore, it may be through the enhancement of one or more of these mechanisms that H-NS over expression in *E. cloacae* J28 promotes colonization of roots. Understanding the role of H-NS in regulating colonization has important implications for the development of robust PGPR strains for agricultural applications. It may be possible through

genetic engineering to enhance the ability of introduced inoculants to competitively colonize the root of crop plants.

8.4.2 Enterobacter ludwigii *sp.: A Novel Species of Plant Growth and Its Clinical Relevance*

This description is based on phylogenetic analyses of partial *hsp60* sequence data collected in a recent population genetic study (Hoffmann and Roggenkamp 2003) as well as on DNA–DNA-hybridization assays and phenotypic characterization performed in the frame of the present study. Phenotypic characterization was performed using API20E, Biotype 100, and a series of conventional tests. *E. ludwigii* strains are Gram-negative rods, motile, catalase positive, oxidase and DNAase negative, fermentative, and non-pigmented in nature. They exhibit the general characteristics of the family Enterobacteriaceae, the genus *Enterobacter*, and the *E. cloacae* complex. Growth occurs after 18–24 h at 15–42°C with an optimum at 36°C on all non-selective media such as Columbia agar with 5% sheep-blood, chocolate, TSA, Luria, or Brain–Heart agar as well as on semi-selective media such as MacConkey or ENDO agar in the form of non-pigmented colonies (Shoebitz et al. 2009).

A detailed biochemical profiling of the species is given in Table 8.1 showing some tests in use for differentiating *E. ludwigii* from the other species of the genus. Its identification is mainly possible by the ability to grow on 3-methyl-D-gluco-pyranose and on myo-inositol. All strains analysed produced a Bush class 1 beta-lactamase rendering resistance to ampicillin, amoxicillin plus clavulanic acid, and cefoxitin in the disk diffusion tests. About 20% of the strains displayed a resistance pattern typical for AmpC hyperproduction (resistance to piperacillin, piperacillin plus tazobactam, cefoxitin, cefotaxime, ceftazidime, and susceptibility to cefepime). All strains are susceptible to trimethoprim plus sulphamethoxazole, gentamicin, meropenem, and ciprofloxacin.

It is widely known that a variety of bacterial species, including PGPRs, can act as biocontrol agents protecting plants from bacterial and fungal diseases (Romero et al. 2007). *P. agglomerans*, a strain closely related to *E. ludwigii* as shown by its 16S rDNA sequence, is able to antagonize *Sclerotinia sclerotiorum* (Berg et al. 2002). To assess whether the strain has the capability to act as a biocontrol agent, antagonism towards *Fusarium solani* has been investigated. BNM 0357 strain depicted fungicidal activity on *F. solani* vegetative growth in mixed cultures as well as in cultures in which BNM 0357 and *F. solani* are not in contact. This behavior suggests that bacteria compete with fungal development (e.g., for nutrients in the culture) and also that a diffusible compound could be responsible for the fungal growth inhibition. As expected, *A. brasilense* showed no fungal inhibiting activity. *F. solani* spore germination was also inhibited by strain BNM 035. These results indicate that *E. ludwigii* BNM 0357 is an efficient fungal

Table 8.1 Differentiation of *Enterobacter* species

Species	Biochemical test[a]													
	D-fucose	L-fucose	α-D-melibiose	1-0-methyl-α-galacto-pyranoside	Esculin	α-L-rhamnose	D-arabitol	Dulcitol	Myo-inositol	Adonitol	3-0-Methyl-D-gluco-pyranose	Putrescine	3-Hydroxy-butyrate	Mucate
E. ludwigi sp. nov. cluster V	−	V	+	+	V	+	−	−	+	−	+	−	+	+
Type strain EN-119^T	−	−	+	+	+	+	−	−	+	−	+	−	+	+
E. absuriae	−	−	+ −	+ −	+	+ −		−	+	−	−	− +	+	V
E. kobei	−	−	+	+	−	+	−	V	+	−	−	V	+ −	V
E. hormaechie	V	+	− +	−	−	+	− +	+	−	−	V	−	−	V
E. cloacae	−	−	+	+	−	+	−	−	+	−	−	+	+	V
E. dissolvens	V	V	+	+	+	+	−	−	+	V	−	+	+	+
E. cancerogenus	+[f]	+[e]	−[b]	−[f]	+[b]	+[e]	−[e]	−[b]	−[e]	−[e]	+[e]	+[e]	−[e]	+[e]
E. nimipresuurallius	−[f]	−[e]	+[b]	−[f]	+[b]	+[e]	−[e]	−[b]	−[e]	−[e]	−[f]	−[e]	−[e]	+[e]
E. amnigenus	−[f]	−[e]	+[b]	+[f]	+[b]	+[e]	−[e]	-[b]	−[e]	−[e]	−[f]	−[e]	−[e]	+[e]
E. cowanii	−[c]	−[c]	+[c]	+[f]	+[c]	+[c]	−[c]	+[c]	−[c]	−[c]	−[f]	−[f]	−[f]	+[c]
E. georgoviae	−[f]	V[e]	+[b]	+[f]	+[b]	+[e]	+[e]	−[b]	V[e]	−[e]	−[f]	V[e]	V[e]	−[e]
E. intermedius	−[f]	V[e]	+[b]	+[f]	+[b]	+[e]	−[e]	+[b]	−[e]	−[e]	−[f]	−[e]	−[e]	+[e]
E. pyrinus	−[f]	−[e]	+[d]	−[f]	+[d]	+[d]	+[e]	−[d]	+[d]	−[d]	−[f]	+[f]	−[f]	−[e]
E. sakazakii	−[f]	−[e]	+[b]	+[f]	+[b]	+[e]	−[e]	−[b]	V[e]	−[e]	−[f]	+[e]	−[e]	−[e]

Table adapted from Hoffmanna et al. (2005)

[a]Incubation at 36°C. Symbols: −, 0–10%; − +, 10–20%; V, 20–80%; + −, 80–90%; +, 90–100%

[b]Sources of data according to O'Hara et al. (1989)

[c]Sources of data according to Inoue et al. (2000)

[d]Sources of data according to Chung et al. (1993)

[e]Sources of data according to Grimont and Grimont (1992)

[f]Type strain considered only, no information in literature

antagonist at least towards *F. solani*. Since the *Fusarium* spp. are the causative agent of wilt diseases in a variety of plants of economic interest, it would be interesting to further investigate the capability of *E. ludwigii* BNM 0357 to antagonize the fungus and extend the study to other species of *Fusarium*. BNM 0357 inoculation promoted a slight, statistically significant rise in shoot fresh weight and length, an effect which was not observed at all in plants inoculated with *A. brasilense*. A relatively more significant effect, also restricted to BNM 0357 inoculation, was observed on root fresh weight that increases by 50%. Strengthening of the root system in addition to the capability of the isolate to facilitate solubilization of mineral phosphate could be an important trait for BNM 0357 to improve plant growth depending on the nutritional resources of the soil. In this regard, it is important to emphasize that the Andisol from where BNM 0357 was isolated usually shows high phosphorus sorption capacity (Campillo et al. 2005) making the presence of phosphorus solubilizing microorganisms highly profitable for crops in such soil ecosystem.

8.4.3 *Enterobacter radicincitans* sp.: A Novel Plant Growth Promoting Species

This is based on the report of Kampfer et al. (2005) *E. radicincitans* sp. nov., a PGP species of the family Enterobacteriaceae. According to this study this organism produced acids from various sugars. *E. radicincitans* forms rod-shaped cells 0.8–1.2 mm in length and 1.0–1.6 mm in width, is Gram-negative and motile in nature. Colonies on nutrient agar are beige pigmented, 2–3 mm in diameter and mucoid. The optimum growth temperature is about 30° C, growth does not occur at 10°C and below and at 45°C and above (Kampfer et al. 1991). It is cytochrome oxidase negative and catalase positive. Strain D5/23T showed a positive arginine dihydrolyse reaction and was negative in production of urease, lysine decarboxylase, ornithine decarboxylase but positive to that of Esculin. Tryptophane deaminase, indole production, and H_2S production were negative. The Voges–Proskauer test was positive after 48 h of incubation. Citrate and malonate were positive. Acid was produced from the following compounds: glucose, lactose (after 48 h), sucrose, D-mannitol, dulcitol, salicin, sorbitol, L-arabinose, L-rhamnose, maltose, D-xylose, trehalose, cellobiose, and D-mannose. No acid production was observed for adonitol, inositol, raffinose, methyl-D-glucoside, erythritol, melibiose, and D-arabitol.

Utilization of a wide range of C and N sources that include *N*-acetylglucosamine, L-arabinose, L-arbutine, D-cellobiose, D-galactose, D-fructose, gluconate, D-glucose, D-maltose, D-mannose, L-rhamnose, D-ribose, sucrose, salicine, D-trehalose, D-xylose, D-maltitol, D-sorbitol, D-mannitol, acetate, cis-aconitate, trans-aconitate, citrate, fumarate, glutarate, DL-lactate, L-malate, pyruvate, L-aspartate, L-alanine, L-proline, and L-serine was positive. The following compounds are not utilized as sole sources

of carbon: *N*-acetylgalactosamine, α-D-melibiose, adonitol, i-inositol, putrescine, propionate, adipate, azelate, 4-aminobutyrate, DL-3 hydroxybutyrate, mesaconate, itaconate, 2-oxoglutarate, suberate, β-alanine, L-ornithine, L-phenylalanine, L-histidine, L-leucine, L-tryptophane, 3-hydroxybenzoate, 4-hydroxybenzoate, and phenylacetate. The strain also shows nitrogenase activity, auxine and cytokinine production (Kampfer et al. 2005). This bacterium shows mesophilic, chemoorganotrophic, facultatively anaerobic growth; these biochemicals support substrate utilization profile for *E. radicincitans* with their PGP potentials.

8.4.4 *Enterobacter asburiae* PSI3 *and E. asburiae* PS2: Secreting Organic Acid and Phytotoxic Effect of Cd Metal and Phosphate Solubilization Influenced by Fungicide

Several other bacteria that are known to promote plant growth in the presence of Cd have PGPR characteristics, which are the implicated mechanisms that contribute to the improved growth in presence of Cd stress in their host plants (Safronova et al. 2006; Sinha and Mukherjee 2008). In the present case, the role of organic acid secretion by *E. asburiae* PSI3 in imparting Cd tolerance to mung bean plants was explored by exogenous application of pure organic acids to seedlings cultivated under hydroponics. Under this mode of cultivation (as against in soil), the plantlets were very highly sensitive to Cd and had complete arrest of root elongation at 50 μM $CdCl_2$. This is in agreement with the observation that legume crops are highly sensitive to Cd (Inoune et al. 1994) and also with the report that the extent of the damaging effect of Cd in hydroponics differs from sand culture (Metwally et al. 2005). In soils the reduced toxicity may be due to binding of the metal ion to clay surfaces or to organic matter.

Root elongation rate is an important parameter depicting robustness of the plants, as efficient root development allows exploring for water and mineral nutrients. A decrease in root elongation due to heavy metal has often been reported (Burd et al. 1998; Metwally et al. 2005; Parker and Pedler 1998). When various organic acids were added to plant roots exposed to toxic levels of Cd, there was a partial relief of its effect on root elongation presumably due to the complexation of the metal ions with the acid anion. This was further supported by the reduction in root associated Cd as seen by hematoxylin staining of roots treated with organic acid. Nigam et al. (2000) have argued that metal:organic acid complex is less phytotoxic than the free form of Cd. Parker and Pedler (1998) who described root elongation responses of Al treated *T. aestivum* to exogenously added malate have shown that exogenous addition of organic acid to seedling roots can reduce metal toxicity to plants. Efficacy of different organic acids in the chelation of Cd^{2+}, as determined by hematoxylin competition assay, showed that among the organic

acids tested, citrate and oxalate are most efficient at releasing Cd^{2+} from its hematoxylin complex in compliance with the formation constants (Ryan et al. 2001). Of special interest is the observation that gluconate, a monocarboxylate, is also significantly proficient in binding to Cd^{2+}. Although this acid has never been reported to be secreted by plant roots, it is one of the major organic acids secreted by several rhizosphere bacteria (Goldstein 1995) and may be of significance in microbially mediated alleviation of metal toxicity (Kavita et al. 2008).

Phosphate solubilization potentials of *E. asburiae* strain PS2 was described by Ahmad and Khan (2010). In the presence of varying concentrations of fungicides, P solubilization was checked both qualitatively and quantitatively using a solid and liquid Pikovskaya medium. In general, when the concentration of each fungicide was increased from 1× to 3×, the size of the halo decreased considerably ($p < 0.05$). The effect of three times the recommended rate (3×) of each fungicide was most adverse on the halo formation compared to the recommended (1×) and two times the recommended rate (2×). The order of toxicity of the fungicides at 3× on the halo size (solubilization index) was: tebuconazole > hexaconazole = metalaxyl > kitazin. In addition, the amount of P-solubilized in the liquid medium decreased corresponded with the increasing concentrations of fungicides. Maximum reduction of the P-solubilizing activity of the *E. asburiae* strain PS2 in the broth was found to be 67, 89, and 93% over the control when tebuconazole at 100, 200, and 300 μg/L, respectively, was added to the medium.

8.4.5 *Enterobacter sakazakii* (Cronobacter): Evidence for Plant Association

The isolation of *Cronobacter* organisms has been reported from a wide spectrum of environmental sources including water, soil, dust from households, and food production lines, as well as from foods such as fruits, vegetables, herbs, cereals, and grains. In addition, *Cronobacter* spp. has been isolated from lemon root stocks (Gardner et al. 1982), wheat (Forlani et al. 1995), rice (Yang et al. 1999) and soybean plants (Kuklinsky-Sobral et al. 2004). Some physiological traits exhibited by the organisms showed the ability to produce a yellow pigment (Lehner et al. 2006), the formation of a gum-like extracellular polysaccharide (Lehner et al. 2005) as well as its ability to resist desiccation during long dry periods (Riedel and Lehner 2007) suggest that they might be of environmental origin. However, the natural habitats of *Cronobacter* spp. are still unknown. In a review article by Berg et al. (2005), the rhizosphere was described as a reservoir for several opportunistic human pathogens including species closely related to *Cronobacter* spp., such as *Enterobacter cloacae* (Berg et al. 2005). It was proposed that mechanisms involved in the interaction between plant-associated bacteria and their host plants are similar

to those responsible for pathogenicity in bacteria and that these mechanism may also be involved in colonizing the human body (Rahme et al. 1995; Cao et al. 2001). Schmid et al. (2009) stated that members of the genus *Cronobacter* can be readily isolated from plant roots and that clinical as well as plant isolates are capable of developing epiphytic and endophytic colonization of tomato and maize roots. It is interesting to note that *Cronobacter* spp. can produce factors potentially beneficial to plant growth. This provides evidence for plants as the original natural habitat of *Cronobacter* spp. Other questions such as the mode of entry, potential proliferation of bacteria within plant cells, and/or whether the cells are transported by an (active/passive) mechanism to other parts of the plants (fruits, seeds) will be subject of further investigation.

8.4.6 *Enterobacter cancerogenus*: A Novel Plant Growth Promoting Agent

Enterobacter cancerogenus (can. cer. o'ge. nus. L. n. *cancer*, crab, the disease cancer; L. v. *gigno*, to produce; L. masc. adj. *cancerogenus*, cancer inducing). The bacteria isolated by UroSeviC from poplars (*Populus* species) affected by a canker disease were described and designated *Erwinia cancerogena* in 1966 (UroSevie 1966). Although Lelliott (1974) and Lelliott and Dickey (1984) have indicated that *Erwinia cancerogena* probably is a species of *Enterobacter* because it produces positive reactions for arginine and ornithine decarboxylase, additional data have not been reported to substantiate the suggested change to *Enterobacter*. *Enterobacter cancerogenus* is Gram-negative, straight rods that are motile with peritrichous flagella and are facultatively anaerobic. This species could give positive results for following characteristics: catalase production, nitrate reduction, Voges–Proskauer reaction, KCN tolerance, esculin hydrolysis, β-galactosidase production, utilization of acetate, citrate, glutamate, DL-lactate, malate, succinate, L-alanine, DL-α-alanine, and L-serine, production of acid from L-arabinose, D-xylose, D-ribose, D-glucose, D-lactose, D-galactose, L-rhamnose, D-mannose, D-fructose, D-trehalose, D-cellobiose, D-mannitol, glycerol, salicin, mucate, pyruvate, and α-D-galacturonate. Liquefaction of gelatin at 27°C is evident at 15 days, but exhibits negative for the following characteristics: pigment production, oxidase, the methyl red test, production of deoxyribonuclease, hydrogen sulfide, lipase, lysine decarboxylase, and urease, phenylalanine deamination, reducing substances produced from sucrose, utilization of alginate, benzoate, propionate, and sodium potassium tartrate; production of acid from L-sorbose, melezitose, ethanol, adonitol, i-erythritol, inulin, glycogen, chitin, and D-tartaric acid, production of gas from D-arabinose and myo-inositol (Dickey and Zumoff 1988). *Enterobacter cancerogenes* MSA2 supplemented with 1% carboxymethylcellulose showed overall plant (*Jatropha curcas* L.) growth promotion effect resulting in enhanced root length, fresh root mass, fresh shoot mass, dry root

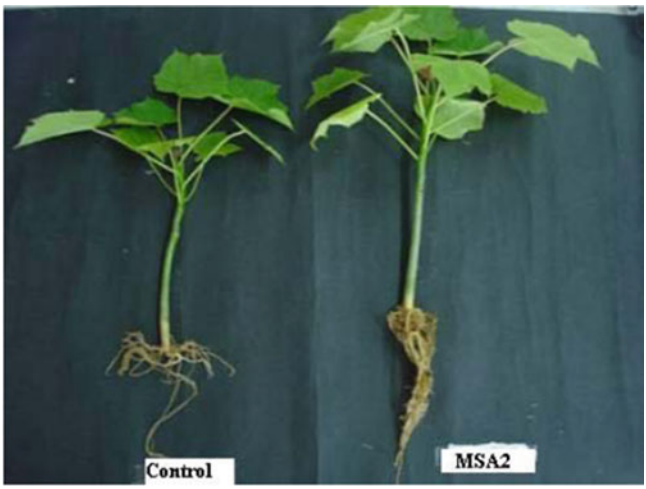

Fig. 8.2 Effect of *Enterobacter cancerogenus* MSA2 on growth of *Jatropha curcas* after 90 DAS in comparison to control

mass, dry shoot mass, number of leaf, chlorophyll content, and biomass over control under the days of experimental observation (Fig. 8.2) (Jha 2011).

8.5 Conclusion

Colonization of the rhizosphere by micro-organisms results in modifications in plant growth and development. This chapter examines the mechanisms involved in growth promotion by plant growth-promoting rhizobacteria of *Enterobacter* species, a common soil bacterium that enhances development of plant root systems and protects host plants against pathogen infection. Continued research with colonization and biofilm formation by these bacterial genera also holds potential for developing biofertilizer and biocontrol agents that may be self perpetuating within the colonizing host plants. Focusing research in these areas may also be aimed to establish *Enterobacter* spp. as promising/potential PGPR. Microbes being an integral component of any soil ecosystem provide life to the soil. Native soils minus microbes are merely dead material. It is now widely being recognized that the presence and abundance of microbial wealth provide soils richness in terms of making available slow release of mineral nutrients, continuous breaking down of complex macro-molecules and natural products into simpler ones to enrich beneficial substances, maintaining physicochemical properties of the soils and most essentially, providing support to the plants in terms of growth enhancement and protection against diseases and pests through their metabolic activities that go on in the soil along day and night.

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Chapter 9
Nitrogen-Fixing Endophytic Bacteria for Improved Plant Growth

Sharon Lafferty Doty

9.1 Introduction

Nitrogen is an essential element for life as we know it. It is a primary component of all the proteins and nucleic acids in living cells. Plants without sufficient nitrogen are stunted, have yellow chlorotic leaves, and produce low yields. Although the atmosphere contains nearly 80% dinitrogen gas, this form of nitrogen is not biologically available to most life. To produce enough food for a growing human population, global agriculture production has become more dependent on the use of chemical fertilizers at the cost of the environment, climate, and possibly human health. The Haber–Bosch process for producing ammonia for plant growth spurred the Green Revolution, allowing significant increases in crop yield. The process requires large amounts of natural gas, high pressure, and high temperature (Vance 2001). Developed at a time of inexpensive and plentiful fossil fuels, the benefits of increased crop yield at low cost were reaped without apparent thought to the environmental costs. With the rising prices of energy, the cost of fertilizer has also dramatically increased. There is, therefore, a need both financially and environmentally to improve crop growth without reliance on chemical fertilizers.

The use of synthetic nitrogen fertilizers has a number of negative effects on the environment (Cocking 2005). Less than half of the applied fertilizer is actually taken up by the crops; the remainder is lost to leaching, run-off into streams, or metabolism by soil microbes (Bhattacharjee et al. 2008). Elevated nitrogen run-off into water systems causes a rapid growth of algae from the input of nutrients, depleting the water of oxygen and resulting in widespread dead zones. The ammonia in the soil is metabolized by some bacteria into reactive nitrogen forms. These include nitric oxide (NO) and nitrogen dioxide (NO_2), collectively termed NOx.

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These reactive forms become ozone that is damaging to plants and animals, and is a significant greenhouse gas. Another form of reactive nitrogen is nitrous oxide (N_2O) that is 300 times more potent than carbon dioxide as a greenhouse gas. As a result, farmlands have a strong impact on air pollution, contributing 8.9 million tons of nitrogen annually (Peplow 2005). In addition to these environmental impacts of the use of chemical fertilizers, there may also be more direct effects on human health. A review by the US National Institutes of Health suggested that increased nitrate concentrations in drinking water could contribute to cancer, and nitrogen-related air pollution could be elevating the incidence of cardiopulmonary ailments (Townsend and Howarth 2010).

Prior to the use of chemical fertilizers, farmers relied more on biological nitrogen fixation (BNF) well known to occur in the legume–rhizobium symbiosis. In this complex interaction, leguminous plants such as peas and beans recruit specific rhizobial bacterial species, form nodules on the roots, and permit the infection of the nodules with nitrogen-fixing bacteria. The plant provides sugars that it synthesizes through photosynthesis to its symbionts as long as they provide usable nitrogen to the plant. These specific bacteria are able to biologically convert atmospheric dinitrogen gas into ammonia using the enzyme nitrogenase and high levels of ATP, the energy currency of living cells. The interaction between the legume and the rhizobium is highly regulated, and a great deal of communication occurs between the two organisms as the symbiosis is being established. The relationship benefits not only the plants and microbes but also the farmer, since good crop yields are obtained without the addition of fertilizer. Often, non-leguminous crops are rotated with legumes because there is sufficient fixed nitrogen remaining in the soil for another crop.

For decades, researchers studied the legume–rhizobium relationship with the goal of initiating such symbiosis between rhizobium and non-leguminous crop plants. Although spontaneous nodules sometimes developed, and rare infections occurred, none of the forced interactions yielded significant transfers of fixed nitrogen to the crop. A breakthrough was made in the 1980s when research into the microbes living within sugarcane, a non-leguminous tropical crop species, indicated that a significant amount of biologically fixed nitrogen was transferred to the plant from these internal symbionts. Since then, researchers began to focus more on this hitherto unstudied ecosystem within plants.

The interior of plants provides habitat for a wide range of bacteria and fungi that benefit the plant host by increasing nutrient acquisition, stress tolerance, pathogen resistance, and aiding in phytoremediation of environmental pollutants (Reis et al. 2000; Hirsch 2004; Cook et al. 1995; Siciliano et al. 2001; Vance 2001; Bhattacharjee et al. 2008). The term “endophyte” was coined by Dobereiner to describe these internal microbes that spend a significant part of their life cycle within plants and do not cause disease (Dobereiner 1992). Although endophytes can increase plant growth through a variety of mechanisms, an especially intriguing method of growth promotion is through providing fixed nitrogen. Only a subset of these endophytic microorganisms “fixes” nitrogen into the usable forms of ammonia and nitrate. Such microbes are termed diazotrophs, and have been

found in major food crops including rice, maize, sugar cane, sweet potato, and coffee. In some cases, the nitrogen-fixing bacteria from these crop plants associated not only with the plant species from which they were isolated but also with unrelated plants. Applications of nitrogen-fixing bacteria on non-leguminous crops could potentially increase growth and yield while reducing the need for chemical fertilizers (Bhattacharjee et al. 2008).

Although the study of endophytes and their hosts is relatively young compared to the study of rhizobia and legumes, some similarities and differences are beginning to be elucidated (Fig. 9.1). In both cases, plant signal molecules (organic acids, carbohydrates, and amino acids) are released in the exudate coming from the roots, and these signals act as chemo-attractants to the microorganisms. The bacteria need to come into contact with the plant root. In the case of rhizobia and legumes, a complex interaction takes place with many variations in detail but generally leading to root hair curling, invasion via an infection thread, and ultimate colonization of the root nodule (Masson-Boivin et al. 2009). By contrast, endophytic associations including the details of how endophytes enter, pass the casparian strips in the plant root endoderm, and colonize the plant interior are not yet known. Since plant genotype is a key factor in determining how much nitrogen is obtained from endophytes (Reis et al. 2000) and because different plant species in the same environment can have different endophytic species compositions, it seems clear that there is specificity as in the legume–rhizobia symbiosis. In a microarray study of the plant genes induced by inoculation with endophytic bacteria, it was revealed that certain plant signaling genes (receptors and protein kinases) were expressed exclusively in the inoculated plants (Nogueira et al. 2001). More studies of this type

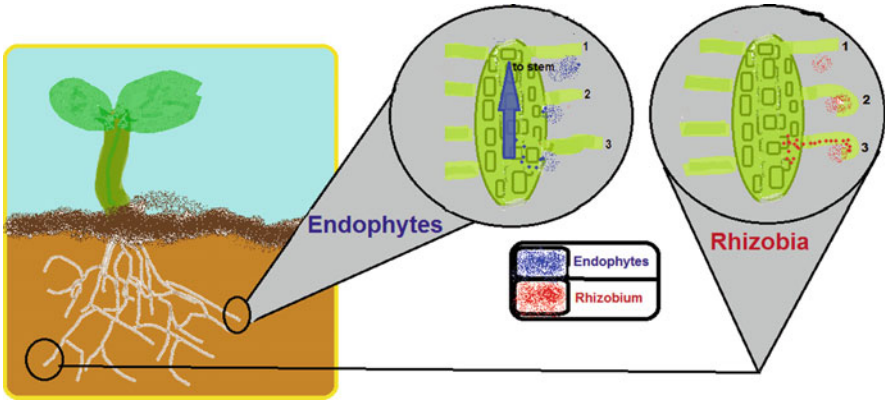

Fig. 9.1 Comparison of infection and colonization of rhizobia and endophytes. In step 1, both classes of bacteria move to the root area in response to plant signals. In step 2, the rhizobia initiate root hair curling, and then enter the plant in step 3 through an infection thread of plant origin. The rhizobia then colonize a specific region within the resulting root nodule. By contrast, endophytes generally enter at lateral root junctions (or at wound sites) in an opportunistic fashion in step 2. They colonize the plant interior and can move upward, in some cases spreading to the stems and leaves, in the vascular tissue and in intercellular spaces (Figure courtesy of Christina M. Doty)

must be conducted in order to determine which plant genes are involved generally in plant–endophyte interactions.

Unlike most rhizobial infections that enter in a plant-assisted direct manner, endophytes seem to enter more in an opportunistic way. Using fluorescently labeled bacteria, researchers have determined that the common entry points for endophytes are at lateral root junctions and at wound sites, similar to pathogen entry points. Low levels of cell wall degrading enzymes are probably released in order to enter without initiating the plant defense response. Upon successful entry, the endophytes appear to form microcolonies within the vascular tissue or in the spaces between plant cells. The initial colonization of the plant by an endophyte is largely driven by the chance opportunity that a competent endophyte that can persist within that plant (Hardoim et al. 2008) will happen to be near enough to perceive the plant signals, and be able to enter at either a wound, or at disturbed cells at lateral root junctions or root cap. Therefore, it is probably a strong advantage for plants growing in nutrient-poor environments to be able to propagate asexually through cuttings. In this way, the new plant already comes with a viable collection of appropriate endophytes (Stettler 2009).

Another difference between the rhizobium–legume and endophyte–plant partnership is in the protection of the nitrogenase enzyme from oxygen. In order for nitrogenase to reduce dinitrogen gas, the immediate environment of the enzyme must be anaerobic. Rhizobia within root nodules are in a low-oxygen environment, and leghemoglobin within the nodule scavenges any free oxygen. Although in most cases it is not known how diazotrophic endophytes protect nitrogenase from oxygen, many possibilities are available (Gallon 1992). In the case of the sugarcane endophyte, the ability of *Gluconacetobacter diazotrophicus* to fix nitrogen in the aerobic environment of the stem is attributed to "respiratory protection" whereby the extremely rapid respiration from metabolism of high levels of sucrose within the sugarcane stem leads to a microaerobic environment needed for the oxygen-sensitive nitrogenase enzyme (Flores-Encarnacion et al. 1999). Other endophytes may use physical barriers including exopolysaccharides to exclude oxygen or interior vesicles to compartmentalize the nitrogenase, or biochemical methods to scavenge interior oxygen (Gallon 1992).

9.2 Methods of Analysis

Most studies of diazotrophic endophytes begin with the isolation of microbes from within the plant that are capable of growing on nitrogen-free medium (outlined in Fig. 9.2). First, the outer surface of the plant tissue is sterilized using a bleach solution, ethanol, or mercuric chloride in order to eliminate contaminating microorganisms on the outside of the plant that may or may not be involved in any symbiosis with the plant. Adequate surface sterilization can be assessed by either placing the uncut plant tissue on medium and monitoring for bacterial growth, plating the last of the rinse solutions, or by using microscopy

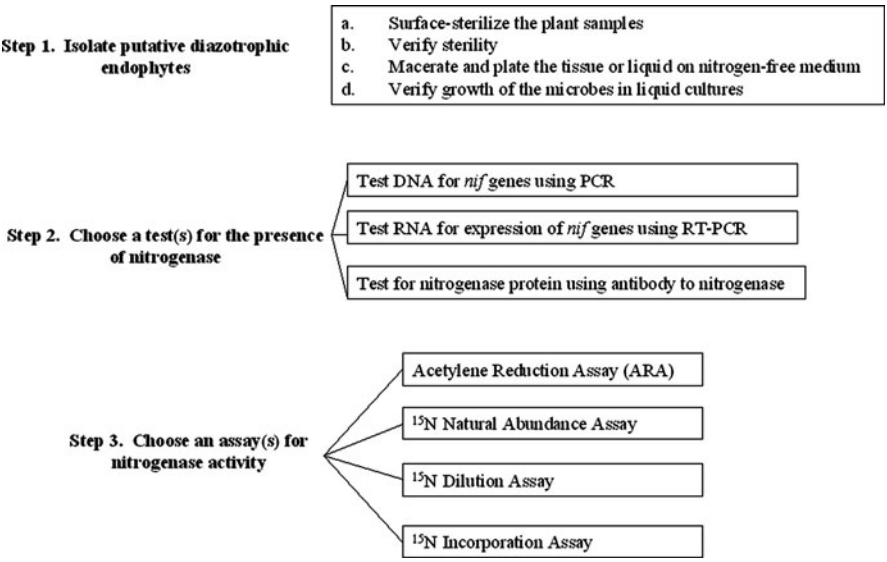

Fig. 9.2 Flowchart of the methods for analysis of diazotrophic (N-fixing) endophytes

(Dong et al. 2003). To isolate the bacteria within the plant, the tissue is macerated and then either directly placed on medium supporting the growth of most nitrogen-fixing bacteria or suspended in a solution that is then spread on the plates. Although this is the standard method for isolating endophytes, a recent paper demonstrated that hundreds of times more colonies could be counted when using the ELISA technique (Silva-Froufe et al. 2009). The standard technique assumes that the endophytes are not tightly adhering to the plant cells and that they do not aggregate, but neither assumption is probably correct. Using this fluorescent tagging method allows more accurate quantification of the number of endophytes within the plant. However, the ELISA technique requires nitrogenase antibody that can recognize a broad range of nitrogenases, so this method will need to be used mostly for quantifying specific endophyte species.

Development of appropriate methods for quantifying nitrogen fixation by microbes within plants has been challenging. Unlike the situation with rhizobia and legumes where all the nitrogen fixation is occurring in a concentrated area in root nodules, diazotrophic endophytes can fix nitrogen throughout the plant body. Furthermore, since the bacteria are within the plant, it is difficult to establish whether the fixed nitrogen is being transferred to the plant as in the rhizobium symbiosis or whether the plant is obtaining the nitrogen only after the microbial cells die and lyse open. Each of the methods described below has advantages and disadvantages, so the use of a combination of methods provides the most convincing evidence for nitrogen fixation by the endophytes.

The most common second stage of analysis following verification of growth in nitrogen-free medium is to determine if the bacterium contains the genes encoding

nitrogenase. This enzyme is required for the conversion of dinitrogen gas to ammonia, and is conserved in all nitrogen-fixing organisms including the Achaea, a separate Domain from the Bacteria. Genomic DNA is isolated from the endophyte and is subjected to PCR with primers that are general enough (degenerate) to amplify most nitrogenase sequences. Nested PCR using primers that bind within the area amplified by the first set of primers is performed to eliminate non-specific DNA bands. The resulting PCR product is then cloned and sequenced to verify that the amplified DNA does correspond to the nitrogenase gene. The nitrogenase enzyme is encoded by three genes, *nifH*, *nifD*, and *nifK*; however, most researchers test only for one of the genes, usually *nifH*. One problem with using the presence of the *nif* gene as evidence for nitrogen-fixing ability is that often the gene is not actually expressed while the endophyte is in the plant. A *nifH*-based microarray was developed to study the diazotrophs of Kallar grass (Zhang et al. 2007). The study revealed that, although there were many different species of diazotrophic endophytes present, only one species, *Azoarcus*, was the dominant species that was expressing the nitrogenase gene *in planta*. In addition to examining the endophytes for the nitrogenase genes (DNA) or expression (RNA) of those genes, is to directly assay for the presence of the nitrogenase enzyme. However, as mentioned for the ELISA study described above, antibodies against the nitrogenase enzyme are usually too specific and would work mostly for detecting the type of nitrogenase from which the antibody was raised.

A common method of testing if an endophyte has a functional nitrogenase enzyme is the acetylene reduction assay (ARA). This assay takes advantage of the fact that acetylene, like dinitrogen gas, has a triple bond that is reduced by nitrogenase. Reduction of acetylene results in the formation of ethylene gas which is easily quantified using gas chromatography. This assay is highly effective in rhizobium–legume studies since the bacteria fix nitrogen while concentrated in small root nodules. It is more difficult to do similar studies with endophytes that are unevenly distributed within the plant. Furthermore, wounded plant tissue releases ethylene, a plant stress hormone, thus interfering with the quantification of ethylene produced from acetylene reduction. To solve this dilemma, cultures of the bacteria are often tested independently of the plant.

The high percentage of endophytic strains that test negative for the ARA yet have the nitrogenase gene and are able to grow on nitrogen-free medium indicates that this assay may not be an accurate measure of diazotrophy for this class of organisms. It has been shown in studies of plant growth-promoting rhizospheric bacteria that there seems to be selection for microbes that can decrease the amount of ethylene produced by the host plant (Hardoim et al. 2008). Using the enzyme ACC deaminase, the bacteria break up the precursor for ethylene from the plant, causing the plant to not over-react to stress. But some plant-associated bacteria may also have a mechanism for degrading the ethylene directly. Work in the 1970s revealed that some soil bacteria can metabolize ethylene (de Bont and Albers 1976). Perhaps some of the putative diazotrophic endophytes that tested negative for ARA were degrading the ethylene they produced from acetylene.

The stable isotope of nitrogen, ^{15}N, is used in three different approaches for quantifying nitrogen fixation. The natural abundance assay relies on more of the lighter ^{14}N being in the atmosphere and more of the heavier ^{15}N being in soil, such that an organism that received most of its nitrogen via nitrogen fixation would have a ratio of more ^{14}N than ^{15}N. Another approach is to deliberately label the soil with ^{15}N using labeled ammonium sulfate, and then testing for a dilution of the isotopic signature with ^{14}N from the atmosphere. A possible problem with this assay is that it requires the addition of ammonium to the soil that may inhibit nitrogenase gene expression that is tightly regulated to the amount of available nitrogen. This method has the advantage of being easier to detect than natural abundance, and it is less expensive than the $^{15}N_2$ incorporation assay which is the most direct method for studying nitrogen fixation. In this assay, the plant is placed in a sealed chamber with some of the air spiked with the labeled nitrogen gas. If the plant is receiving any nitrogen through nitrogen fixation from its endophytes, the plant tissue will become labeled. Highly pure $^{15}N_2$ gas is available to avoid complication with contaminating ^{15}N-labeled ammonia gas. The best study would be to use *nifH* mutants to determine if the plant tissue was labeled only when inoculated with the wild-type strain and not with the mutant (Sevilla et al. 2001) and to verify that the label was incorporated into plant proteins (Iniguez et al. 2004).

9.3 Diazotrophic Endophytes

As described in introductory section, diazotrophic endophytes refer to nitrogen-fixing microorganisms within plants. Following is a review of the current research on these diazotrophs from different groups of crop plants.

9.3.1 Diazotrophic Endophytes from Monocot Crops: Sugarcane, Rice, and Maize

Although this chapter is focused on crop plants, the reader is strongly encouraged to read the reviews on diazotrophic endophytes of the forage grass, Kallar grass (*Leptochloa fusca*) that have been well studied (Reinhold-Hurek and Hurek 1998; Elmerich et al. 1997; Hurek et al. 2002), and other grasses (Reis et al. 2000; Dalton et al. 2004).

The best example of a monocot crop species with nitrogen-fixing endophytes is sugarcane (*Saccharum* spp.). This crop species was grown successfully for many decades without addition of fertilizer. ^{15}N experiments conducted in the early 1990s suggested that a few sugarcane varieties received large amounts of their nitrogen from BNF (Urquiga et al. 1992). Experiments by Boddey et al. demonstrated that 80% of the total nitrogen for three sugarcane varieties could be attributed to BNF

(Boddey 1995). The vegetative propagation of sugarcane by nodal sections and the traditional farming methods without fertilizer may have selected for superior diazotrophs.

The dominant diazotrophic endophyte of sugarcane is *G. diazotrophicus* (previously termed *Acetobacter diazotrophicus*). It grows best in very high levels of sucrose (100 g/L), is acid-tolerant, and exists in high numbers within plants, but it survives poorly in soil. This bacterium is capable of secreting nearly half of its fixed nitrogen in a form that the plant can utilize (Cohjo et al. 1993). A variety of different methods has been used to demonstrate that *G. diazotrophicus* fixes nitrogen and transfers that nitrogen to the host plant. Since inoculation of plants with endophytes could result in increased mass because of other factors besides fixed nitrogen, inoculation with a mutant strain that no longer fixes nitrogen makes an excellent experiment. Inoculation with *nif* mutants (non-nitrogen fixing) of this organism resulted in reduced sugarcane growth under nutrient-limited conditions compared with inoculation with the wild-type strain, suggesting that fixed nitrogen is transferred to the plant under normal symbiotic conditions (Sevilla et al. 2001). The plants inoculated with the wild-type (nitrogen-fixing) strain had more tillers, leaves, and height, and this improved growth was sustained during the 4-month field study. For example, there was an increase of 40% more fresh cane weight in the plants inoculated with the wild-type compared with the mutant. The colonization of the plants by the wild-type and mutant strains was verified and shown to be the same; therefore, the effect was specific to the ability to fix nitrogen. Immunogold labeling of nitrogenase demonstrated that the enzyme was expressed in the endophytic microbes. The ^{15}N incorporation test was also conducted. The shoots and roots of the plants inoculated with the wild-type strain were labeled with ^{15}N to a small extent, further demonstrating that nitrogen fixation was occurring within the plant. When nitrogen was not a limiting nutrient; however, the microbe still enhanced plant growth, suggesting that it helps the plant in multiple ways, probably from the production of plant hormones (IAA and gibberellins).

Although *G. diazotrophicus* was shown to fix nitrogen within the sugarcane, it seems that other endophytes must also contribute substantial nitrogen. A larger study that used the ^{15}N dilution method and seven different combinations of five endophyte species demonstrated that the best BNF growth effect occurred when all five strains were inoculated onto the plants (Oliveira et al. 2002). The endophytes were from the genera *Azospirillum*, *Burkholderia*, *Herbaspirillum*, and *Gluconacetobacter*. A doubling of the BNF contribution was seen between inoculation with the whole consortia of five strains compared to inoculation with only one strain. The dry weights were increased substantially after 400 days between the uninoculated controls and the multi-species inoculated plants. The improvements were substantial despite the fact that the sterility of the uninoculated controls was not maintained, and these control plants did get colonized during the experiment. The growth effects seen in this study demonstrate that inoculating micropropagated plants with a consortia of nitrogen-fixing endophytes has long-term yield enhancements even under normal conditions of competition with other microbes.

Burkholderia and *Herbaspirillum* species are important contributors to the growth enhancements of sugarcane. *B. vietnamiensis* colonizes sugarcane in large numbers (115,000 colony-forming units per gram of plant tissue), it reduces acetylene, the indirect assay for nitrogen fixation, and it contains the nitrogenase genes (Govindarajan et al. 2006). Growth experiments in the field demonstrated that inoculation with the strain MG43 resulted in a 20% increase in growth. In a study comparing *B. tropici* and *Herbaspirillum* using fluorescent in situ hybridization (FISH), it was demonstrated that both microbes colonized the sugarcane within 12 h after inoculation but in different sites (Oliveira et al. 2009).

As rice is the staple food in the diet for over 40% of the world's population, it is critical to develop sustainable methods to increase yields. Using traditional agriculture, 4 tons of rice per hectare can be grown with 50 kg of nitrogen per hectare coming from BNF (Stoltzfus et al. 1997). Using modern agriculture, a yield of 5–8 tons per hectare can be achieved but at the cost of 60–100 kg of nitrogen added through chemical fertilizer per hectare. In order to increase yields without chemical fertilizers, research into diazotrophic endophytes of rice began. By screening 133 rice endophytes, 13 isolates of diverse genera were identified that were positive for both the *nifD* gene and the ARA (Stoltzfus et al. 1997). In a more recent study of rice plants in Vietnam, 13 diazotrophic endophytes were identified using the same criteria but all of these were *Burkholderia* species (Govindarajan et al. 2008). The most active in the ARA was named *B. vietnamiensis* strain MGK3. By marking the strain with *gusA*, it was determined that the strain entered at lateral root junctions and the root tip, and colonized intercellular spaces of the root cortex after 15 days. Inoculation of seedlings with this strain resulted in increases of rice yield of 5–12%. When mixed with other diazotrophic endophyte species including *G. diazotrophicus*, *Azospirillum lipoferum*, and *H. seropedicae*, the combination resulted in a yield increase of 9–23%. ^{15}N dilution assays both in pots and in the field demonstrated nitrogen fixation, with up to 42% of the N derived from nitrogen fixation. Inoculation of rice with diazotrophic endophytes increased yield, root biomass, and tiller number.

As maize is also a staple crop and, like sugarcane, is now being used for biofuels, it is another crop for which sustainable agricultural practices need to be developed that also meet the growing demand. Studies in Mexico revealed that *B. unamae* was a prevalent endophyte in maize that colonized roots and stems, reduced acetylene, and had the *nif* gene cluster (Caballero-Mellado et al. 2004). In another study, *B. tropici* was identified from both maize and its ancestor, teosinte, and grew on nitrogen-free medium and was ARA-positive (Reis et al. 2004). In a more recent study, 178 endophytes were isolated from maize in Uruguay that could grow on nitrogen-free medium (Montanez et al. 2009). Only 11 of these were found to be ARA(+), and these included species from the familiar genera *Pantoea*, *Pseudomonas*, *Rhanella*, *Herbaspirillum*, *Azospirillum*, and *Rhizobium*. The maize cultivars used in this study were tested by the ^{15}N dilution assay and shown to be using BNF.

The diazotrophic maize endophyte, *Klebsiella pneumoniae*, was able to fix nitrogen not only in its original host but also in the major food crop, wheat (*Triticum aestivum*). In an excellent model paper, it was proven that fixed nitrogen

was transferred from the endophyte to the wheat cultivar, Trenton (Iniguez et al. 2004). Addition of the wild-type endophyte relieved nitrogen-deficiency symptoms and increased total nitrogen of the plant, whereas inoculation with a nitrogenase mutant or a killed control did not yield this effect. After 6 weeks without fertilizer, the control plants were stunted and chlorotic while the plants inoculated with the wild-type strain had more chlorophyll, 50% more dry weight, 285% more total N in shoots, and 654% more N in roots. ^{15}N dilution experiments verified that not only was nitrogen fixation occurring within the plant, the fixed nitrogen was utilized by the plant since the chlorophyll also had the isotopic signature. Unfortunately, these positive results were limited to one cultivar of wheat; there was no effect for two other wheat cultivars, a rice line, two barley lines, and several lines of maize.

9.3.2 *Diazotrophic Endophytes from Dicot Crops: Sweet Potato and Coffee*

Compared to the amount of research on diazotrophic endophytes of monocots, there is much less known about them in dicot crops. The nitrogen-fixing microbes, *Azospirillum* (Hill et al. 1983) and *Gluconacetobacter* (Paula et al. 1991), were isolated from sweet potato plants (*Ipomoea batatas*), the fifth most important food crop in developing countries. Nitrogen fixation was observed in sweet potato using the ^{15}N natural abundance assay (Yoneyama et al. 1998). A survey of *nif*H-containing microbes as conducted using sweet potato from farms in Uganda and Kenya that did not use chemical fertilizers (Reiter et al. 2003). This culture-independent assay revealed that there were 17 different microbial groups, as separated by RFLP analysis. About 50% of the species were *Rhizobium* sp. Sweet potato plants in Japan were also analyzed (Terakado-Tonooka et al. 2008). Using the ^{15}N natural abundance assay, it was determined that 40% of the N was from BNF, an amount of 103–135 kg N per hectare. Rather than looking for the presence of *nifH* as in the African study, they used RT-PCR to look for active expression of the nitrogenase gene in endophytes of the stems and storage roots. As with the African study, most of the sequences were of *Rhizobium* species, but there were also *H. seropedicae*, *B. vietnamiensis*, *B. unamae*, *Pelomonas saccharophila*, *Cyanobacteria*, and *Azohydromonas australica* sequences. Since there were no *nifH* sequences in the sprouts prior to transplanting, all of these microbes colonized the plants from the soil environment. By studying active *nif*H expression, the authors were able to see a seasonal effect. The highest *nifH* expression was in summer and not in autumn, correlating with the highest photosynthetic rate of the plant.

In a small study of American sweet potato endophytes, it was found that 4 out of 11 isolates produced the plant hormone, auxin, and 1 isolate was a putative nitrogen-fixing endophyte (Khan and Doty 2009). This isolate (SP-A) grew in nitrogen-free medium and had the nitrogenase gene. Although the *nifH* fragment

was 99% identical to that of *Azotobacter vinelandii*, the 16S rDNA sequence was 100% identical to *Stenotrophomonas maltophilia*. It is interesting to note that most studies do not report the sequence of both the 16S rDNA and the *nif* gene, so it is unknown how common it might be that endophytes harbor nitrogenase sequences from unrelated soil bacteria. Recent studies involving endophyte-assisted phytoremediation of pollutants have demonstrated that bacteria can conjugate *in planta*, transferring plasmids from poor colonizers to effective endophytes of the roots, and upward to endophytes of the stem (Taghavi et al. 2005; Weyens et al. 2009). It may be that plasmids containing nitrogenase genes or other symbiosis-related genes are also transferred among endophytes.

In coffee plants of Mexico, there are some diazotrophic plant-associated bacteria, namely *Gluconacetobacter* species. *G. diazotrophicus*, *G. johannae*, and *G. azotocaptans* were identified from the coffee tissues (Jimenez-Salgado et al. 1997) and in the rhizosphere (Fuentes-Ramirez et al. 2001). More studies have yet to be carried out to determine if the coffee plants are directly benefitting from these microbes, however.

More evidence of beneficial impact on a dicot species from diazotrophs has been documented for alfalfa (*Medicago* sp.). The *K. pneumoniae* strain Kp342 isolated from maize was inoculated onto the dicots, alfalfa and *Arabidopsis*, and the monocots, wheat and rice (Dong et al. 2003). This strain was a highly effective colonizer of monocots, requiring an inoculum amount of only one colony-forming unit to yield a high colonization. By contrast, the type strain for this species, a clinical isolate, required a 1,000-fold higher inoculum concentration and still colonized to a 100-fold lower level. Because of this strain difference, it is clear that the endophyte is an active participant in the colonization process. There were also differences in the colonization of monocots and dicots. The monocots were colonized to a level 100× higher than the dicots; however, the dicot species in the study were not the natural hosts for this maize endophyte.

9.3.3 Diazotrophic Endophytes from Bioenergy Crops: Poplar and Willow

In most of the previous examples of diazotrophic endophytes isolated from plants, the crop species were tropical. Only recently research has begun on diazotrophic endophytic microbes of the temperate climate, high biomass plants, poplar (*Populus* sp.) and willow (*Salix* sp.). Endophytic *Rhizobium tropici* was isolated from hybrid cottonwood (Doty et al. 2005). Although this species is known for its ability to nodulate an unusually wide diversity of legumes, its endophytic nature in non-legumes had not been previously reported.

In their native habitat, poplar and willow grow vigorously alongside rivers in rocky substrates with no organic matter (Fig. 9.3). Analysis of the stem tissues of poplar and willow in such an environment revealed large numbers of putative

Fig. 9.3 Poplar (*Populus trichocarpa*) and willow (*Salix sitchensis*) grow in nutrient-limited, rocky substrates in their native habitat in Western Washington state, USA. A variety of putative N-fixing endophytes were isolated from the stems (Doty et al. 2009)

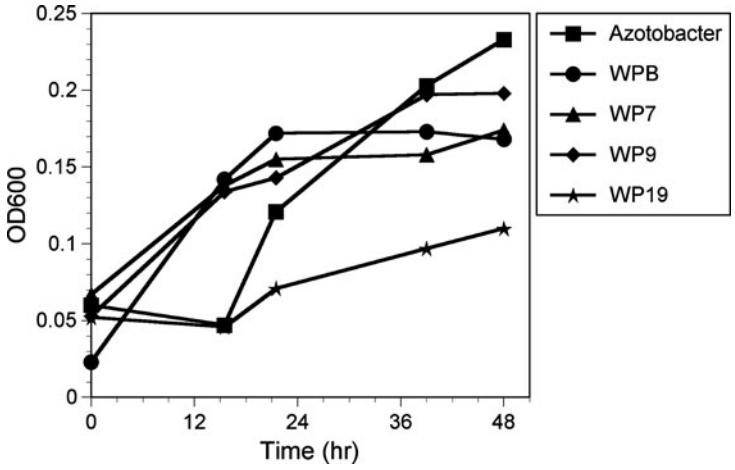

Fig. 9.4 Growth of wild poplar (WP) endophytes in nitrogen-free medium. *Azotobacter vinelandii* was included as a positive control. Although several of the endophytes had similar growth, only strain WPB was positive in the acetylene reduction assay (Doty et al. 2009)

diazotrophic endophytic bacteria (Doty et al. 2009). The bacterial strains belonged to the genera *Burkholderia*, *Rahnella*, *Herbaspirillum*, *Enterobacter*, *Acinetobacter*, *Sphingomonas*, and *Pseudomonas*. The strains grew well in nitrogen-free medium, and some examples are shown in Fig. 9.4. Of 14 isolates that grew on nitrogen-free medium that were analyzed further, only 5 were positive in the ARA, and of these, only 1 had strong activity. This isolate, *B. vietnamiensis*

strain WPB, was chosen for further study (Xin et al. 2009). The *nif*HDK cluster was cloned and sequenced, and showed a high identity with other *B. vietnamiensis* strains. The ^{15}N incorporation assay as well as the ARA demonstrated that strain WPB was fixing nitrogen aerobically. In addition, WPB produced high levels of the phytohormone, indole-3-acetic acid. When Kentucky bluegrass (Xin et al. 2009), corn, and rice (Redman and Doty, unpublished) were inoculated with WPB, the growth of the plants was increased. Inoculated bluegrass had 42% higher overall biomass, and 37% higher nitrogen content. The effectiveness of WPB for growth enhancement of poplar is currently being studied at the levels of greenhouse growth and in the field at sites with different levels of nitrogen using both wild-type and *nif* H mutant strains.

Given the natural habitat of poplar and willow alongside rivers that flood regularly, an advantage of diazotrophic endophytes over diazotrophs requiring root nodulation seems clear (Doty et al. 2009). While any investment into the development of root nodules would be lost after each flooding event that brings in additional silt and rocks, investments into symbioses with endophytes within the stems would be unaffected. In the areas from which the poplar and willow endophytes were isolated, alder trees with their root nodules of nitrogen-fixing *Frankia* were also present, but much further from the banks of the river, outside the flood zone. It remains to be seen if other colonizing plant species subjected to regular flooding are more likely to have symbioses with diazotrophic endophytes rather than with nitrogen-fixing bacteria requiring root nodules.

9.4 Conclusions

The use of nitrogen-fixing endophytes to enhance the growth of agricultural crops and bioenergy crops could become an effective strategy for more environmentally sustainable production. With the growing need for increased food and bioenergy biomass but with a greater understanding of the implications of conventional-intensive agriculture, the time is right for a greater emphasis on biological mechanisms for improved plant growth. Adding beneficial microbes to only the rhizosphere of agricultural crops has not been very successful (Sturz and Nowak 2000). Endophytes may have an advantage over this approach since there would be less competition as there is when adding soil bacteria to the established rhizosphere communities. In some of the cases described in this review, it is clear that diazotrophic endophytes can provide a substantial amount of fixed nitrogen to the plants. The strain and cultivar specificities seen in some of the examples point to the necessity of further research to understand the plant–microbe communications necessary for effective nitrogen fixation in non-legumes.

Some caution must be exercised before widespread application of this technology. In many of the studies, *Burkholderia* isolates were found to perform best in the nitrogen fixation assays and strongly increased plant growth. However, some isolates of *Burkholderia* species are associated with human lung diseases

(Parke 2005). Species in the Burkholderia cepacia complex (Bcc) have enormous potential as biocontrol agents, as degraders of harmful environmental pollutants, and as plant growth promoters. They are naturally abundant in soil, water, and plants. Some isolates are registered for use as microbial pesticides. But on the other side, some Bcc strains are opportunistic human pathogens, and some can cause fatal lung infection in the patients with cystic fibrosis (CF). Persons with this genetic disorder have an abnormally thick mucus in the lungs that can be colonized by some bacteria. At this time, it is not clear what marks certain strains as being pathogenic. In a study of several isolates of *B. cenocepacia* from clinical and environmental settings, it was determined that the environmental samples were not invasive according to an in vitro assay (Pirone et al. 2008). However, all the isolates were able to persist in lungs of infected mice, and all had the virulence-associated genes (*opc*I, *cci*L, and *ami*L). *B. vietnamiensis* was one of the species with strong plant growth-promoting activity. This species is present in 5.9% of the patients with CF, but there are no reports of it being associated with Cepacia syndrome, decreased survival, or transmissibility (Chiarini et al. 2006). In 2003, the US Environmental Protection Agency ruled that Bcc strains can be used only for bioremediation (Chiarini et al. 2006). However, since Bcc strains have high identity in the 16S rDNA genes, but only 30–60% DNA hybridization, it seems that an all-encompassing ban on Bcc strains is not appropriate given the wide diversity in this genus. It has been suggested that a revised ruling be made such that non-pathogenic Bcc strains can be used in agriculture (Chiarini et al. 2006).

Considering the enormous potential impact of the use of nitrogen-fixing endophytes on global crop production, funding for research in this area is limited. Many of the papers described in this review cited only partial support, institutional support, or individual fellowships as the funding sources for the research. There needs to be a greater public awareness of this environmentally-friendly, biologically based method for increasing plant growth. The study of diazotrophic endophytes impacts both basic and applied science. The discovery of diazotrophic endophytes in poplar and willow helps explain the biology of these and maybe other colonizing tree species. It may be that many non-leguminous plants in nutrient-poor environments have symbiotic relationships with nitrogen-fixing endophytes. It is essential that sustainable agricultural practices are developed that use resources more efficiently, maintain environmental health, and yet increase the food supply. With more study of the impacts of diazotrophic endophytes on plants, we can better understand the role of endophytes in natural systems as well as utilize that information for a new revolution in agriculture that is better for the environment.

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Chapter 10
Endophytic Actinomycetes: Biocontrol Agents and Growth Promoters

Masafumi Shimizu

10.1 Introduction

There has been a large body of literature describing potential uses of plant-associated bacteria (including actinomycetes) as agents to stimulate plant growth and/or management of soil and plant health, as reviewed by various workers (Hallmann et al. 1997; Winding et al. 2004; Compant et al. 2005; El-Tarabily and Sivasithamparam 2006). Most of these bacteria and actinomycetes (sometimes described as actinobacteria) were isolated from soil, the rhizosphere, and the phyllosphere. Their antipathogen potentials have been demonstrated in the laboratory but their direct association with plants has not definitively been demonstrated. It was Smith (1957) who successfully isolated *Micromonospora* sp. from tissue sections of an apparently healthy tomato and found that this actinomycete showed a strong inhibitory effect to *Fusarium oxysporum* f. sp. *lycopersici*. He reisolated the same strain from 4 of 20 tomato plants previously inoculated with this strain, suggesting its endophytic residence in tomato. Endophytic microbes colonizing inside plant tissues usually get nutrition and protection from the host plant. In return, they confer profoundly enhanced fitness to the host plants by producing certain functional metabolites (Tan and Zou 2001). These metabolites may promote growth of the host plants and give adverse effects to other pathogenic microbes growing within the same plants. Endophytic actinomycetes probably produce antimicrobial metabolites within their host plants, because actinomycetes, for example *Streptomyces* species, are well known for their ability to produce biologically active secondary metabolites, particularly antibiotics (Loria et al. 1997). In parallel with pharmaceutical interest of endophytic actinomycetes as a source of novel antibiotics (Strobel 2003), few microbiologists have considered the use of

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endophytic actinomycete biocontrol agents over the last 2 decades. Kunoh and colleagues (Kunoh 2002; Shimizu et al. 2001a) assumed that if useful endophytic actinomycetes isolated from field-grown plants can successfully colonize tissue-cultured seedlings, the seedlings could become resistant to various plant pathogens. Because tissue-culture flasks are usually axenic, such a novel technique should allow this actinomycete to colonize its host plant without competition and/or antagonism by any other microbes in soil (Kunoh 2002). Most of endophytic actinomycetes latently reside in the internal tissues of nonsymptomatic plants without causing any adverse effects to the host (Hasegawa et al. 2006). Endophytic actinomycetes have been demonstrated to improve and promote growth of host plants, as well as to reduce disease symptoms and/or various environmental stresses. Management of beneficial potentials of endophytic actinomycetes to favor plant growth could be realized by a better understanding of the physiological and molecular interactions between these microbes and plants.

This chapter examines the current states and approaches to help realize the potential of endophytic actinomycetes in plant-disease control and plant growth promotion.

10.2 Isolation of Endophytic Actinomycetes

This section reviews brief information on isolation of endophytic actinomycetes with respect to various sources and procedures involved.

10.2.1 Isolation Sources

In the nineteenth century some actinomycetes were proved to be closely associated with living plants, giving beneficial and/or adverse effects to their host. As reviewed by Okazaki (2003), Brunckhorst isolated the genus *Frankia* from nonlegume root nodules in 1886, suggesting its role of nitrogen fixation in roots, similar to *Rhizobium* in legume plants. Later, Thaxter first reported pathogenic actinomycetes, *Streptomyces scabies*, a causal pathogen of potato scab in 1890. The scab organism sometimes occurs in soils where potatoes have never been cultivated, indicating that this species is both soil-inhabiting and endophytic (Loria et al. 1997). Hasegawa et al. (1978) isolated a new genus of actinomycetes, *Actinosynnema*, from a grass blade, the first actinomycete of plant origin in Japan. Matsukuma et al. (1994), Okazaki et al. (1995) and Matsumoto et al. (1998) reported that a variety of actinomycetes inhabit a wide range of plants as symbionts, parasites or saprophytes, and most of them belonging to the genera, *Streptomyces* and *Microbispora*. They isolated these species because of their interest in sources of novel antibiotics for medicinal purposes. As evident from these earlier papers,

a variety of endophytic actinomycetes inhabit various organs and tissues of many terrestrial and aquatic vascular plants. Commonly, multiple strains of endophytic actinomycetes have been isolated from a single plant (Table 10.1).

Table 10.1 Endophytic actinomycete genera within past 10 years

Plant species	Actinomycete taxa[a]	References
Crop plants		
Triticum aestivum	*Streptomyces*, *Microbispora*, *Micromonospora*, *Nocardioides*	Coombs and Franco (2003a)
Cucumis sativus	*Streptomyces*	Shimizu et al. (2009)
Lupinus termis	*Actinoplanes*	El-Tarabily (2003)
Zea mays	*Microbispora*, *Streptomyces*, *Streptosporangium*	de Araújo et al. (2000)
Lycopersicon esculentum	*Streptomyces*, *Streptoverticillium*, *Nocardia*	Cao et al. (2004b)
Oryza sativa	*Streptomyces*, *Nocardioides*	Tian et al. (2007)
Brassica campestris	*Microbispora*, *Streptomyces*, *Micromonospora*	Lee et al. (2008)
Musa acuminata	*Streptomyces*, *Actinomadura*, *Streptovarticillium*, *Streptosporangium*, *Nocardia*	Cao et al. (2004a)
Woody plants		
Taxus spp.	*Streptomyces*, *Micromonospora*, *Nocardioforme*, *Actinoplanes*, *Actinomadura*, *Kitasatospora*	Caruso et al. (2000)
Acacia auriculiformis	*Actinoallomurus*	Thamchaipenet et al. (2010)
Rhododendron sp.	*Streptomyces*	Shimizu et al. (2000)
Kalmia latifolia	*Streptomyces*	Nishimura et al. (2002)
Aquilaria crassna	*Streptomyces*, *Nonomuraea*, *Actinomadura*, *Pseudonocardia*, *Nocardia*	Nimnoi et al. (2010)
Medicinal plants		
Sambucus adnata	*Glycomyces*	Gu et al. (2007)
Alpinia galanga	*Streptomyces*, *Nocardia*, *Microbispora*, *Micromonospora*	Taechowisan et al. (2008)
Kennedia nigricans	*Streptomyces*	Castillo et al. (2002)
Maytenus austroyunnanensis	*Saccharopolyspora*, *Actinomadura*	Qin et al. (2008, 2009)
Thottea grandiflora	*Streptomyces*	Ghadin et al. (2008)
Azadirachta indica	*Streptomyces*, *Streptosporangium*, *Microbispora*, *Streptoverticillium*, *Saccharomonospora*, *Nocardia*	Verma et al. (2009)
Others		
Monstera sp.	*Streptomyces*	Ezra et al. (2004)
Paphiopedilum appletonianum	*Streptomyces*	Tsavkelova et al. (2007)

[a]Genera are listed in order of reported abundance

10.2.2 Isolation Procedures

Colonization of actinomycetes in plant tissues is largely influenced by the environmental circumstances surrounding the host plants such as the type and pH of soil, the content of inorganics and organics in soil, rainfall, sunshine, air and soil temperatures. In addition, endophytic actinomycetes may occur in low numbers and sometimes in localized positions within plants, so that it is almost impossible to find their specific affiliation to their host plant.

For isolation of endophytes, attention needs to be paid to avoid contamination with undesirable epiphytic microbes. It is recommended to first sterilize the entire surface of the samples, followed by cutting their organs and tissues into pieces with a sterilized knife, if necessary. Sodium hypochlorite (NaOCl) is the most commonly used disinfectant. Plant samples usually are sterilized by sequential immersion in 70–99% ethanol for 1–5 min, and 1–5% NaOCl for 3–20 min, followed by repeated rinsing in sterile water to remove residual NaOCl. Hydrogen peroxide and mercuric chloride are also effective disinfectants (Bandara et al. 2006; Ghodhbane-Gtari et al. 2010; Gurney and Mantle 1993; Merzaeva and Shirokikh 2010). Sardi et al. (1992) used vapor of propylene oxide instead of the liquid disinfectants. The surface treatment with ethanol alone is not sufficiently effective to epiphytic bacteria (Zin et al. 2010). Double or triple surface-sterilization with a combination of ethanol and other disinfectants is also recommended to eliminate epiphytic microbes. The efficacy of surface sterilization can be enhanced by common use of a disinfectant with surfactants such as Tween 20 and Tween 80 which increase affinity of the disinfectant to hydrophobic waxy plant surfaces.

Segments of the sterilized plant are placed onto an appropriate agar medium, followed by incubation at an appropriate temperature (normally 25–30°C). Another troublesome matter is often caused by other unwanted endophytic fungi and/or bacteria harboring within the plant segments. To suppress growth of such undesirable endophytes, several selective media were devised: media of humic acid-vitamin agar (Hayakawa and Nonomura 1987), starch casein agar (Küster and Williams 1964), water yeast-extract (Crawford et al. 1993), and S medium (Baker 1990). Supplementation with antimicrobial compounds such as nalidixic acid and trimethoprim (Hayakawa et al. 1996), nystatin (Williams and Davies 1965), or cycloheximide (Caruso et al. 2000) improves selectivity of actinomycetes. Because actinomycetes grow slowly relative to other bacteria and fungi, a few weeks to several months are generally required before visualization of their colonies on the surface of specimens. Rapidly growing microbes that appear within the first 2 weeks are bacterial and/or fungal contaminants.

Individual mycelial colonies are picked-up with a sterilized toothpick and streaked to newly prepared agar plates. Repeating this step results in the recovery of single isolates. The dilution plating method is also sometimes employed. Aliquots of the mycelial suspensions serially diluted with sterile water or buffer solution are spread onto newly prepared agar medium plates. Following incubation, single colonies are individually transferred onto another set of newly prepared agar

media. Mycelia and spores are taken from the medium surface and stored in 20% glycerol solution or 10% glycerol solution supplemented with 10% dimethyl-sulfoxide solution at −80°C.

The final culture is often contaminated with nonactinomycetous microbes, mostly bacteria. The microfilter technique developed by Hirsch and Christensen (1983) is effective to eliminate such contaminants. A trituration technique is another method used to isolate endophytic actinomycetes (El-Tarabily 2003), although complete surface sterilization is required for this technique and validation of sterility should hold another key for success of isolation (Hallmann et al. 1997). Coombs and Franco (2003a) imprinted the surface-sterilized samples onto agar medium and confirmed no growth of any epiphytic contaminants after several days of incubation. El-Tarabily (2003) focused attention to the final buffer solution that was used for rinsing the disinfectant-sterilized specimens. He incubated the agar plates on which the solution had been spread, and then confirmed no growth of any organisms on the plates. His careful protocol assured that the isolated actinomycete was endophytic. Furthermore, Cao et al. (2004b) submerged the *Streptomyces* isolate in the disinfectant solutions that had been originally used for surface sterilization of plant segments to confirm no growth of this isolate after incubation on medium plates. This result assured that the target isolate was not epiphytic but endophytic. They considered that the obtained isolate was protected from the surface-sterilizer by plant tissues and thus was not killed during the surface sterilization with the disinfectant. This simple validation test was accepted and used by other researchers (Qin et al. 2009; Tan et al. 2006; Tian et al. 2004; Verma et al. 2009).

10.3 Diversity and Organ-Specificity of Endophytic Actinomycetes

Information on diversity of endophytic actinomycetes and their organ-specificity is significant not only to help in the screening of beneficial strains but also to understand their ecological roles. As described above, to date a number of endophytic actinomycetes were found by various culture-dependent methods. *Streptomyces* is the largest and most dominant genus, comprising nearly 50% of the total population of soil-inhabiting actinomycetes, which are probably saprophytes (Thakur et al. 2007). *Streptomyces* species are most frequently isolated from plant tissues as well as *Microbispora* species (Okazaki 2003; Rosenblueth and Martinez-Romero 2006; Takahashi and Omura 2003). Inderiati and Muliani (2008) reported that the majority of endophytic actinomycetes recovered from tobacco plants were classified as species of *Streptomyces*. Likewise, Sardi et al. (1992) reported that 482 of 499 isolates from a variety of plants were *Streptomyces* spp. Moreover, Verma et al. (2009) showed that *Streptomyces* spp. occupied about 50% of 55 separate strains obtained from Indian lilac. Interestingly, the majority of endophytic *Streptomyces* species frequently isolated from various countries are

limited to species that include *S. aureus*, *S. galilaeus*, *S. caviscabies*, *S. setonii*, *S. cyaneus* and *S. thermocarboxydus* (Castillo et al. 2007; Coombs and Franco 2003a; Inderiati and Franco 2008; Tan et al. 2006; Tian et al. 2007, Verma et al. 2009), suggesting that these species could have a higher compatibility to a wide range of plants than other species. The genus *Microbispora* appears to be predominantly comprised of endophytes. Among a total of 53 actinomycete isolates that de Araújo et al. (2000) obtained from maize, 33 were *Microbispora* spp. and only 6 were *Streptomyces* spp. According to Lee et al. (2008), *Microbispora* spp. (67%) were the most common isolates of Chinese cabbage, followed by *Streptomyces* spp. (12%) and *Micromonospora* spp. (11%). Takahashi and Omura (2003) isolated 33 strains of *Microbispora*, 32 *Streptomyces*, and 10 other rare actinomycetes from fallen leaves of nine genera of higher plants. Thus, the genera *Streptomyces* and *Microbispora* seem to be both soil-inhabiting and endophytic, although only a limited number of species may have this two-way habitation. Kizuka et al. (1998) noted that *Microbispora* spp. were isolated more frequently from plant leaves than soil, suggesting that they could have mutualistic associations with plants.

The majority of endophytic actinomycetes have been isolated from roots rather than other organs (Verma et al. 2009; Zin et al. 2010). El-Tarabily and colleagues (2003, 2009) estimated the culturable population of endophytic actinomycetes in cucumber and lupin roots as approximately 10^5 cfu/g fresh root weight. Diversity of actinomycetes is also relatively broader in roots. Tian et al. (2007) analyzed the 16S rRNA genes of 45 and 33 clones of endophytic actinomycete isolates obtained from roots and stems, respectively, and found that the clones of root origin were affiliated with nine genera of known actinobacteria and other uncultured (unidentified) actinobacteria, whereas those of stem origin comprised only four genera of the former and other uncultured actinobacteria. This result suggests that a variety of known and unidentified actinobacteria reside inside roots than stem region.

The relatively larger population and broader diversity of endophytic actinomycetes in roots indicate that soil-inhabitant actinomycetes like endophytic bacteria can readily move to soil-contacting roots (Zinniel et al. 2002). In fact, Conn and Franco (2004) demonstrated that the density of endophytic actinobacteria in wheat roots detected by terminal restriction fragment length polymorphism (T-RFLP) was closely related to that of soil microflora: a larger diversity was detected in roots of wheat grown in soil having a large population of actinobacteria. Several other factors such as edaphic factors, cultivars, and physiological status of plants may also influence the population and diversity of endophytic actinomycetes. According to Tian et al. (2004), rice plants grown in alkaline soil harbor diverse endophytic *Streptomyces* spp. Cao et al. (2004a) reported that more diverse species of *Streptomyces* were isolated from leaves of banana trees wilted due to infection of *F. oxysporum* f. sp. *cubensis* than those of uninfected trees. However, the same species of the genus were isolated from roots of both infected and uninfected trees. They considered that in healthy leaves the defense response might be sufficient to block endophytic habitation of *Streptomyces* spp., while in leaves of infected trees the suppressed defense response was insufficient to interfere with entry of opportunistic actinomycetes on phylloplane, resulting in their successful endophytic colonization.

10.4 Mode of Penetration and in Planta Localization of Endophytic Actinomycetes

How do endophytic actinomycetes enter into host plants? And how do they colonize inside plant organs and/or tissues? These are very attractive and important research themes for plant–microbe interactions. Although Rosenblueth and Martinez-Romero (2006) emphasized the importance of in planta evidence of colonization for true endophytes, detailed reports on localization of endophytic actinomycetes are still limited (Hasegawa et al. 2006). In general, individual bacterial cells are unable to penetrate intact epidermal cells because they do not possess penetration structures such as penetration pegs of fungi (Huang 1986). Bacteria commonly enter plant tissues via stomata, wounds, lenticels, projecting areas of lateral roots, and broken trichomes by forming clump of their cells. Most of actinomycetes, however, form branching hyphae that grow on plant surfaces and enter their host plants through natural openings and mechanical and insect wounds. The infection mode of phytopathogenic *Streptomyces* spp. has been well-studied at the ultrastructural level (Loria et al. 1997). *Streptomyces scabies* produces extensive hyphal networks on the surface of potato tubers. The substrate hyphae penetrate the tuber through immature lenticels and mechanical wounds which often occur when tubers are rapidly enlarging. Following penetration, the pathogen grows in the intercellular spaces between or through a few layers of cells which then die, and the pathogen feeds on these cells as a saprophyte (Loria et al. 1997). Hyphae of *S. ipomoeae*, a causal pathogen of soil rot of sweet potato, grow on the root surface and then enter the root through tissues at cell–cell conjunction sites (Clark and Matthews 1987). Both pathogenic *Streptomyces* species often form short branches that emerge from the main hyphae and directly penetrate host cell walls. Yoshida and Yamaguchi (2004) also observed root tumors of melon by scanning electron microscope (SEM) and found that hyphae of a pathogenic *Streptomyces* sp. assembled on the surfaces of the epidermis and the endodermal layers exposed when branch roots were erupted through the root cortex. Proliferation of this pathogen was observed only in the intercellular spaces of the first and second outer layers of tumor tissue. However, details of their entry into the host plant remains unclear.

Sardi et al. (1992) showed hyphae of *Streptomyces* sp. in cortical tissues of tomato by SEM but were unable to resolve the detail of the interaction with the host cells. Taechowisan and Lumyong (2003) showed SEM images of aerial hyphae of unidentified actinomycetes which had grown through the root epidermis of *Zingiber officinale*. Similarly, Okazaki (2003) showed the presence of hyphae and sporophores of *Microbispora* sp. on the surface and inside epidermal cells of a host plant. El-Tarabily et al. (2009) also showed distribution of sporangia of *Actinoplanes campanulatus* within cells of the cucumber root cortex, but again, they did not provide any information on the mode of its entry into the host plant. Earlier, Coombs and Franco (2003b) traced infection process of endophytic *Streptomyces* sp. strain EN27 by tagging it with green fluorescent protein (GFP). They coated germinating seeds of wheat with GFP-tagged *Streptomyces* sp. EN27

and harvested at every 24 h for detection of its localization in the seed tissues using a laser scanning confocal microscope. They detected the strain only in the embryo and around the break in the seed husk where the embryo emerged from the seed during the 24 h incubation. After 3 days, GFP-expressing microcolonies of the strain were seen more frequently in the embryo tissue of the seed, the emerging radicle of the embryo, and in the endosperm than at 24 h, indicating continuation of its active growth in the seed. Although they failed to demonstrate the initial penetration stage of the strain, they speculated that initially the strain may enter the embryo through the breaks of the seed husk and then expand to the endosperm as the seed hydrates from the embryo end. Their observations showed that the endophytic actinomycetes were able to associate with its host at a very early stage in the development of the plant. Subsequently, Franco et al. (2007) provided further information of long-term microscopic observation of wheat seedlings inoculated with GFP-tagged EN27 for up to 4 weeks. They observed that the strain colonized the seminal roots, especially at the root junctions, and the cracks surrounding lateral roots, and sporulated inside the epidermal cells during 3–4 weeks postinoculation. This observation indicates that the strain invaded plant roots through the crevices at lateral root emergence and then multiplied within the cells. Shimizu et al. (2009) inoculated *Streptomyces* sp. MBCu-56 to cucumber leaves and monitored the behavior of its mycelia. The inoculum developed mycelia actively on the leaf surfaces and formed dense mycelial masses especially at the epidermal cell junctions. Some substrate mycelia penetrated the cuticle layer and expanded into the underlying epidermal cells (Fig. 10.1). However, their observation was not enough to resolve inter- and intracellular growth in the epidermis. In more detail, Minamiyama et al. (2003) demonstrated by SEM that when the endophytic *Streptomyces galbus* R-5 (renamed as MBR-5) was spread on the surface of the tissue-culture medium in which rhododendron seedlings were growing, its hyphae extended on leaf surfaces and entered leaf tissues via stomata, and the internal hyphae grew out of stomata and multiplied in substomatal cavities. Interestingly, such hyphae formed short, perpendicular branches similar to those observed in *S. ipomoeae* (Clark and Matthews 1987) and *S. scabies* (Loria et al. 2003). Suzuki et al (2005) examined by transmission electron microscope (TEM) the infection mode of *S. galbus* MBR-5 in leaves of tissue-cultured rhododendron seedlings and confirmed that hyphae entered the leaves through stomatal openings but not through intact cuticle layers (Fig. 10.2). Hyphae were observed individually or in colonies in intercellular spaces between epidermal and mesophyll cells but not inside both cells (Fig. 10.2). Such an infection mode is similar to that of a number of bacterial pathogens (Huang 1986). More interestingly, hyphae colonizing leaf surfaces, penetrating through stomatal openings, and colonizing within intercellular spaces were embedded in an electron-dense, amorphous, mucilage-like material (Fig. 10.2). These observations were consistent with the SEM observation by other workers (Loria et al. 2003; Minamiyama et al. 2003; Sardi et al. 1992; Shimizu et al. 2009). Minamiyama et al. (2003) hypothesized that such materials were probably required for massive colonization of hyphal cells in the stomatal

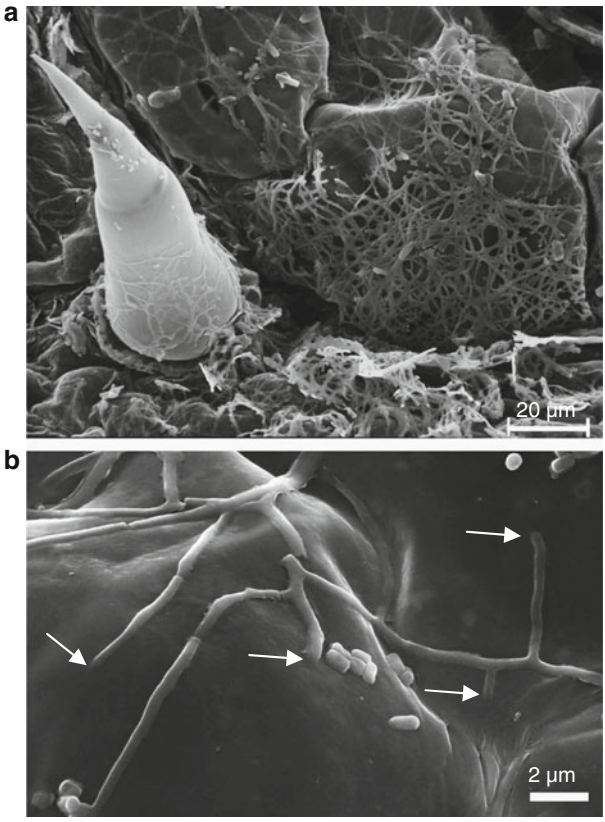

Fig. 10.1 Scanning electron micrographs of the surface of cucumber leaves 8 days after inoculation with *Streptomyces* sp. strain MBCu-56. (**a**) Hyphal networks on the leaf surface. (**b**) Substrate mycelia growing on and beneath the surface of the cuticle layer. *Arrows* indicate the sites of direct penetration of cuticle layer by the hyphae. From Shimizu et al. (2009). Reproduced by permission from Journal of General Plant Pathology vol. 75, © 2009 Springer Japan

openings and adherence of hyphal cells to the surface of the host cells. Likewise, Loria et al. (2003) speculated that a matrix deposited at the interface between pathogenic *Streptomyces* and host plant cells probably contains hydrophobin-like proteins similar to fungal hydrophobins that function in morphogenetic development and in the attachment of hyphae with hydrophobic surfaces (Tucker and Talbot 2001). In fact, production of hydrophobin-like proteins by several *Streptomyces* species was reported by Claessen et al. (2002). Minamiyama et al. (2003) and Suzuki et al. (2005) demonstrated that on the leaf surface of rhododendron, the wax that covered the undulating cuticle layer was degraded beneath the growing hyphae of *S. galbus* MBR-5. This observation was supported by the presence of indigo blue crystals that formed as hydrolytic products of hyphal nonspecific esterase along hyphae on fresh leaves (Suzuki et al. 2005). In addition, MBR-5 was proved to

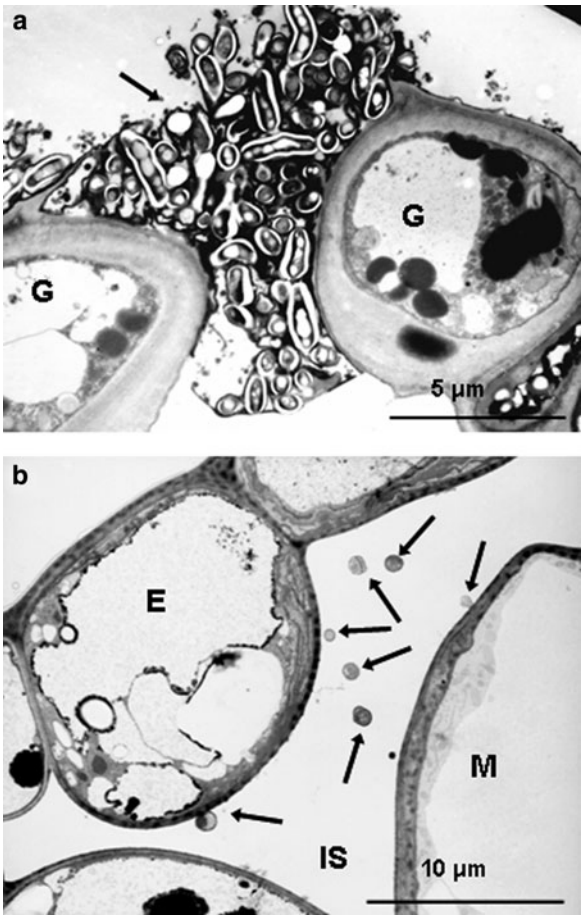

Fig. 10.2 Stomatal penetration and intercellular colonization by *Streptomyces galbus* strain MBR-5. TEM images of rhododendron leaves 60 days after inoculation with MBR-5. (**a**) Mycelia penetrating stomata. A mycelial colony is embedded in an electron-dense material (*G*, guard cell). (**b**) Hyphal cells (*arrows*) in an intercellular space beneath the epidermis (*E*, epidermal cell; *IS*, intercellular space; *M*, mesophyll cell). From Suzuki et al. (2005). Reproduced by permission from Actinomycetologica vol. 19, © 2005 The Society for Actinomycetes Japan

produce other hydrolytic enzymes such as cellulase, xylanase, and pectinase. This strain was able to grow on cellulose, xylan, and pectin as single carbon sources (Minamiyama et al. 2003). Hydrolytic enzymes in the extracellular matrix from fungal spores or hyphae are considered to contribute to adhesion and preparation of the infection court (Mendgen et al. 1996). Considering these reports, it is plausible that endophytic actinomycetes adhere firmly to the host plant surfaces and acquire the nutrition from host surfaces by using extracellular polymers containing adhesive compounds and hydrolytic enzymes, similar to that of fungi.

10.5 Endophytic Actinomycetes as Biological Control Agents

Biological control has gained momentum to mitigate the effects of chemicals widely used for over a century. Actinomycetes group have strong potential for use as biological control agent.

10.5.1 Biocontrol of Soil-Borne Diseases

Soil-borne diseases are very problematic in crop management strategies. The frequent use of highly toxic chemicals for eradication of the soil pathogens often causes environmental hazards. It has been generally considered that biocontrol of such diseases is more desirable than chemical control unless the agent exerts too much pressure on the native microflora. To date, a number of bacteria and fungi with biocontrol activity against soil-borne pathogens have been isolated from soil and the rhizosphere (Chet et al. 1990; Handelsman and Stabb 1996). For example, Thomashow and Weller (1988) showed that *Pseudomonas fluorescens* 2-79 is suppressive to take-all, a major root disease of wheat caused by *Gaeumannomyces graminis* var. *tritici*. Elad et al. (1980) reported a biocontrol isolate of *Trichoderma harzianum* that significantly reduced diseases caused by *Sclerotium rolfsii* and *Rhizoctonia solani* in field experiments with beans, cotton, and tomatoes. Many soil actinomycetes have also been reported as candidates of biocontrol agents for soil-borne pathogens (El-Tarabily et al. 1997; Rothrock and Gottlieb 1984; Tahtamouni et al. 2006).

Endophytic strains of *Microbispora rosea* subsp. *rosea* and *Streptomyces olivochromogenes* were found to be effective in suppressing clubroot of Chinese cabbage caused by *Plasmodiophora brassicae* (Lee et al. 2008). These strains were originally isolated from surface-sterilized roots of Chinese cabbage collected from various regions in Korea. Pretreatment of germinated seeds of cabbage with each of the strains reduced the severity of clubroot by 33–58%. However, the mechanism of their control effects remains unsolved.

Meguro et al. (2004) and Shimizu (2007) conducted noteworthy experiments employing endophytic streptomycete strains (MBR-5 and AOK-30 from rhododendron and mountain laurel, respectively) to tissue-cultured seedlings of rhododendron and mountain laurel in flasks. They found that the seedlings preinoculated with these strains in flasks became resistant to damping-off caused by *Phytophthora cinnamomi* and *Rhizoctonia* sp. after transplanted to plug-tray soil. They isolated the same strains from the inoculated seedlings to assure that the resistance was associated with endophytic colonization of the inoculated strains. This technique has been applied in commercial seedling production in Japan (Hasegawa et al. 2006).

El-Tarabily (2003) reported that an endophytic strain of *Actinoplanes missouriensis* isolated from lupin roots was effective in suppressing root rot of lupin caused by *Plectosporium tabacinum*. In the laboratory he observed degradation of the pathogen hyphae by chitinase, a product of this isolate, and in further glasshouse

experiments he confirmed colonization of the isolate inside lupin roots 8 weeks after inoculation, resulting in significant reduction of the severity of root rot of the host plant. His subsequent experiments aroused our interest in the control mechanism of this isolate. Their results indicated that another endophytic isolate, *A. italicus*, and a separate mutant strain of *A. missouriensis*, both having no potential to produce chitinase, did not lyse the pathogen hyphae and did not affect the severity of root rot in glasshouse tests. Nevertheless, both isolates colonized the lupin roots as similarly as the original isolate of *A. missouriensis*. They suggested that the protective mechanism of the *A. missouriensis* isolate might be attributable to its production of chitinase enzymes and lysis of *P. tabacinum* hyphae with these enzymes. In a separate paper (El-Tarabily et al. 2009), the potential use of endophytic actinomycetes for controlling *Pythium aphanidermatum* in cucumber was described. They evaluated the biocontrol potential of three antagonistic isolates of *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis*, all of which produced high levels of cell-wall degrading enzymes (β-1,3, β-1,4 and β-1,6-glucanases). Irrespective of their individual and combinatorial application to cucumber roots, they significantly reduced the incidence of damping-off and crown- and root-rot with the latter mode of application being more effective than the former. Besides the cell-wall degrading enzymes, *A. campanulatus* and *S. spiralis* produced antifungal volatiles and *M. chalcea* showed a hyperparasitic activity. Hence, the authors assumed that biocontrol effects enhanced by the combinatorial application of three isolates could be attributed to synergistic effects of multiple mechanisms.

Coombs et al. (2004) examined biocontrol efficacy of endophytic actinomycetes against *G. graminis* var. *tritici* of wheat. The six strains which showed significant biocontrol effects in naturally infested soil tests had varied degrees of antifungal activities, from undetectable to strong, in laboratory tests. No consistent correlation between in vitro antibiosis and in planta control activity was observed suggesting that antibiosis might be only one of the contributory factors for biocontrol ability but not enough to account for the control effect in field soil. Similar results on the interaction of endophytic actinomycetes with tomato were obtained by Inderiati and Franco (2008). They noted that 13 of 15 strains having varying levels of antagonistic activity showed the suppressive effects on damping-off caused by *R. solani* significantly. By contrast, one isolate was inactive in vitro but had relatively high disease suppression in vivo, while another isolate of *Streptomyces thermocarboxydus* showed strong in vitro antagonism but resulted in the lowest disease suppression in vivo. They considered that although antibiosis might be the most important mechanism, involvement of other mechanisms cannot be denied because of inconsistent correlation between in vitro antagonism and in vivo biocontrol activity in several strains.

Cao et al. (2004b) reported biocontrol activity of an endophytic *Streptomyces* sp. against damping-off disease of tomato seedlings caused by *R. solani*. A typical antagonist among isolated strains was evaluated for its suppressive effect on damping-off disease of cucumber and tomato seedlings. Unexpectedly, this strain promoted growth of the host plants as well as exhibited disease-suppressive effects on only tomato seedlings, suggesting that this strain could have host specificity to tomato but not to cucumber.

The strain S96 of *Streptomyces* sp. isolated from surface-sterilized banana roots by Cao et al. (2005) concerns the possible association of siderophore production with biocontrol activity. The treatment of roots with this strain was effective to protect the banana plantlet from infection of *F. oxysporum* f. sp. *cubense* (Foc), the causal pathogen of Panama disease. The antagonism of this strain against Foc detected on banana tissue extract medium became undetectable if the medium was supplemented with an excess of $FeCl_3$. They assumed that siderophore production by the strain could hold a key for its biocontrol potential. However, further work is desired to give more evidences by experiments using mutant strains lacking siderophore activity.

10.5.2 Biocontrol of Foliar Diseases

A search of the available literature indicated that an effective biocontrol strain of endophytic actinomycete for control of foliar disease was first reported by Shimizu et al. (2001a). Ten actinomycete strains were isolated from field-grown rhododendron plants. Among them, the strain MBR-5 identified as *Streptomyces galbus* showed significant antagonistic activity against major rhododendron pathogens, *Phytophthora cinnamomi* and *Pestalotiopsis sydowiana*. Advanced inoculation of tissue-cultured seedlings of rhododendron with MBR-5 protected them from challenge-inoculation of leaves with *P. sydowiana*. Later, Meguro et al. (2004) demonstrated the biocontrol effect of *S. padanus* strain AOK-30 against *P. sydowiana* in tissue-cultured seedlings of mountain laurel. In fact, MBR-5 was found to produce two antibiotics, actinomycin X_2 and fungichromin, in liquid medium (Shimizu et al. 2004). Although actinomycin(s) was detected in tissue-cultured rhododendron seedlings previously treated with MBR-5, the external supply of analogues of actinomycin X_2 and fungichromin to the seedlings did not enhance their disease resistance (Shimizu et al. 2001b). These results led us further to establish whether the two nonantagonistic *Streptomyces* strains could induce disease resistance in rhododendron seedlings (Shimizu et al. 2006). Interestingly, colonization of both strains induced disease resistance in the seedlings similar to that of MBR-5, suggesting that induction of disease resistance could be attributable to colonization of an endophytic streptomycete in the seedlings rather than in planta antibiosis. This hypothesis was verified by another molecular study using an *Arabidopsis*–MBR-5 system: MBR-5 inoculation enhanced disease resistance of *Arabidopsis thaliana* toward *Colletotrichum higginsianum*, which was characterized by an accumulation of phytoalexin and increased *PDF1.2* gene expression in this host plant (Shimizu et al. 2005) as also evidenced by the molecular study by Conn et al. (2008), who investigated expression variation of the key genes in systemic acquired resistance (SAR) or jasmonate/ethylene (JA/ET) pathways in *A. thaliana* inoculated with endophytic actinomycetes (*Streptomyces* sp. strains EN27 and EN28, *Micromonospora* sp. strain EN43, and *Nocardioides albus* EN46). All these endophytes "primed" both SAR and JA/ET pathways that linked with systemic

resistance via up-regulation of pathogenesis-related proteins and other antimicrobial proteins which were effective against several pathogens.

Streptomyces sp. strain MBCu-56 showed a similar biocontrol effect on cucumber anthracnose disease caused by *Colletotrichum orbiculare* (Shimizu et al. 2009). Only 6 of 178 strains of endophytic actinomycetes isolated from healthy cucumber and pumpkin plants had in vitro antagonistic activity against *C. orbiculare*. Spraying of MBcu-56 strain at $10^{7–9}$ cfu/ml onto the cucumber leaves suppressed the incidence of the disease by 72–93%. Light microscopy revealed that hyphae of MBCu-56 massively colonized around germinating and appressorium-forming conidia of *C. orbiculare* on leaf surfaces. No intercellular hyphae of *C. orbiculare* were seen in epidermal cells of these leaves, suggesting that superficial colonization of MBCu-56 could block wall penetration from *C. orbiculare* appressoria (unpublished data). Although this interaction between both organisms is interesting, the suppressive mechanism resulting in reduced pathogen growth remains elusive.

The earlier reports regardless of soil- and air-borne diseases revealed the noteworthy findings that the biological effect obtained in the laboratory cannot always explain results and their causes in field experiments. El-Tarabily and Sivasithamparam (2006) studied the mechanism of action of actinomycetes that have focused mainly on in vitro screens, especially in relation to antibiosis. Within plant tissues, various factors such as pH, nutritional balance and native endophytes may affect the production of antimicrobial substances by the introduced endophytic actinomycetes, or their antibiotics and enzymes may be inactivated by host metabolism. In some cases, systemic resistance in host plants could be involved in a biocontrol mechanism so that the in vitro antagonism might not be directly linked to the biocontrol effects. Indeed, more detailed studies are needed to understand the significant roles of endophytic actinomycetes and host plant responses in biocontrol effects.

10.6 Endophytic Actinomycetes as Plant Growth Promoters

As well as soil actinomycetes (El-Tarabily and Sivasithamparam 2006), plant-associated bacteria and fungi have been known to show the activity of plant growth promotion and disease control (Compant et al. 2005; Hallmann et al. 1997; Joseph et al. 2007). Although plant growth-promoting rhizobacteria (PGPR) is one of the well-studied plant-promoting microbes, more and more genera are yet to be researched for their successful application in field comprising biotic and abiotic stress conditions. Cattelan et al. (1999) noted the possible mechanism for this phenomenon: (a) the ability to produce or change the concentration of the plant hormones indoleacetic acid (IAA), gibberellic acid, cytokinins, and ethylene; (b) asymbiotic N_2 fixation; (c) antagonism against phytopathogenic microorganisms by production of siderophore, β-1,3 glucanase, chitinase, antibiotics, and cyanide; and (d) solubilization of mineral phosphates and other nutrients.

Few reports are available on the association of "endophytic actinomycetes" with plant growth promotion. Several recent studies demonstrated that endophytic

actinomycetes produced plant growth-promoting compounds such as IAA and siderophores in vitro (de Oliveira et al. 2010; Ghodhbane-Gtari et al. 2010; Nimnoi et al. 2010). For example, Nimnoi et al. (2010) examined the productivity of IAA and siderophores by endophytic actinomycetes isolated from eaglewood. All of the ten isolates produced IAA and eight of them produced siderophores in culture broth. Earlier, Igarashi et al. (2002) purified pteridic acids A and B from the culture broth of an endophytic *Streptomyces hygroscopicus*. These metabolites accelerated formation of adventitious roots in hypocotyls of kidney beans at 1 nM as effectively as IAA. El-Tarabily et al. (2009) reported that some strains of endophytic actinomycetes produced IAA and IPYA (indole-3-pyruvic acid) that enhanced growth of cucumber plants significantly. Prior to this, Meguro et al. (2006) reported an endophytic strain of *Streptomyces* sp. MBR-52 that accelerated emergence and elongation of plant adventitious roots.

10.7 Conclusion

A number of endophytic actinomycetes inhabit tissues of a wide variety of native and cultured crop plants. Because in planta microfloras are so diverse and complicated associations of endophytic actinomycetes with host plants and/or other endophytes still remain poorly understood. Nevertheless, some of them are undoubtedly beneficial to the host plant life: the endophytic presence of some actinomycetes may play important role in plant development and health because of their role in nutrient assimilation and in secondary metabolite production.

In natural environments plants encounter various environmental stresses such as fluctuation of climate, chemical and physical conditions of soil, rhizosphere microflora, and pest attacks. The defense and tolerance potentials of plants to endure such stresses are significant and essential for maintenance of plant life. Interestingly, Hasegawa et al. (2004, 2005) discovered that tissue-cultured seedlings of mountain laurel harboring endophytic *Streptomyces padanus* became highly tolerant to drought. In those tolerant seedlings callose deposition and lignification of cell walls as well as osmotic pressure of cells were accelerated, suggesting that all these events could be closely associated with enhanced drought-tolerance. Their reports showed another unexpected beneficial use of endophytic actinomycetes in crop management, although further research is required to apply them in horticultural and cropping systems.

As Coombs et al. (2004) noted, when applied in field situations, antagonistic endophytic actinomycetes do not always show biocontrol effects as expected due to their ability to colonize the internal tissues of the target host plant. A variety of techniques have been used for the application of endophytic microbes at the experimental level, ranging from several variations of seed treatments, soil drenches, stem injections, and foliar sprays of microbial suspensions (Hallmann et al. 1997). Apparently, more research on the bioformulation and delivery of the endophytic actinomycetes is needed for employing them in practical agronomical production.

Research on endophytic actinomycetes will contribute to the development of novel technologies and methodologies in the agricultural, medical, and pharmaceutical fields in future days to come.

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Chapter 11
Bacteria Associated with Orchid Roots

Elena Tsavkelova

11.1 Introduction

Orchids are monocotyledonous plants and form the largest family of angiosperms, Orchidaceae Juss., with over than 24,000 described species, and the list is constantly growing by the number of newly discovered plants and selected horticultural hybrids (Dressler 1993; Fay and Chase 2009). Orchids, particularly the tropical species, are considered as the most desirable ornamentals; they have the highest value in commercial horticultural production. However, the survival of these plants is nowadays severely threatened due to habitat loss, deforestation, diseases and pests, weed encroachment, anthropogenic impacts on tropical rainforests, illegal poaching, and commercial overexploitation and cultivation (Franco et al. 2007; Roberts and Dixon 2008; Swarts and Dixon 2009). Many orchids that have been classified as extinct or threatened species in the wild are currently maintained in the natural reserves and greenhouses. However, the extreme specializations of orchids including pollination strategy, epyphytism, and symbiotic seed germination present additional obstacles in orchid propagation both in natural and artificial environment. The interactions between orchids and mycorrhizal fungi have been studied for a long time, although little is known about orchid "satellite" bacteria and their role in plant growth and development. This chapter discusses current knowledge of diversity, specificity, and functional activity of microbial communities associated with the roots of greenhouse and wild grown orchids

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11.2 Orchids

Orchids are ubiquitous; they adapted themselves to different climate conditions from equatorial rainforests and temperate zones to the boreal territories, although the maximum diversity occurs in the humid tropical and subtropical regions. In general, they are composed of adventitious roots, rhizome, stem, leaves, floral ramets, and flowers (Kramer 1975; Moreira and Isaias 2008; Vakhrameeva et al. 2008). Broadly distributed orchid ecotypes are terrestrial that grow in the ground and epiphytic plants, which live in tree canopies or on the rocks (lithophytes); two-thirds of orchid species are epiphytes (Atwood 1986; Gravendeel et al. 2004; Roberts and Dixon 2008). The growth of orchids is either sympodial (from a rhizome emitting shoots in several directions) or monopodial (from one shoot), however the environmental variety of the orchids contributes to morphological, anatomical, and physiological alterations in their vegetative organization (Pabst and Dungs 1975; Dressler 1993). The majority of the world's orchids are photosynthetic. There is also a small specific group of myco-heterotrophic orchids with low (mixotrophy) or even lost capacity of photosynthesis; the mycorrhizal fungus represents an energy source that may supplement or totally replace plant's photosynthetic activity (Rasmussen and Whigham 2002; Dearnaley 2007).

Among structural variations, the formation of a highly absorbent multilayered velamen covering the roots is one of the distinguishing features of epiphytic orchids. The velamen is filled with air and formed by dead mature air-filled cells with adjacent perforations and wall thickening strands (Noel 1974); the number of cell layers does not change under modified environmental conditions, although the rate of maturation, size of cells, and thickness of the velamen can be markedly altered (Pridgeon 1987; Dressler 1993; Dycus and Knudson 1957; Moreira and Isaias 2008). When the root is wet, the velamen is saturated passively with water, aided by perforations that are elliptical slits or holes between secondary bulges of the plant cell walls (Noel 1974; Pridgeon 1986a). The principle role of the velamen is mechanical protection, retaining of mineral salt solutions, water conservation, and the prevention of excess loss of water from the cortex (Dycus and Knudson 1957; Sanford and Adanlawo 1973; Noel 1974). Besides, the velamen contributes to reducing transpiration, reflecting solar radiation, and exchanging oxygen and carbon dioxide between the root and atmosphere (Dycus and Knudson 1957; Cockburn et al. 1985). The cortical cells of the aerial roots possess chloroplasts, thus being capable of photosynthesis, although its contribution is insignificant for the green mature plant (Dycus and Knudson 1957; Erickson 1957; Arditti 1962; Katiyar et al. 1986; Gehrig et al. 1998). If aerial roots penetrate a substratum or attach themselves to a solid object, they serve as climbing or adhesive roots; they may then function like terrestrial roots, acting as organs of absorption (Dycus and Knudson 1957).

Among other intriguing adaptive strategies of the orchids are high levels of pollinator specialization, minuscule seeds, and germination depending on the symbiotic relations with the mycorrhizal fungus (Arditti and Ghani 2000; Roberts

and Dixon 2008; Dearnaley 2007; Fay and Chase 2009). A great diversity of orchid pollination systems for sexual reproduction includes a variety of deception strategies, where the pollinator receives no reward; approximately one-third of orchid species have evolved mechanisms of food deception, food-deceptive floral mimicry, brood-site imitation, shelter imitation, pseudoantagonism, rendezvous attraction, and sexual deception (Jersáková et al. 2006; Roberts and Dixon 2008; Waterman and Bidartondo 2008). Besides, pollinator imitation (most often an insect, but in rare instances a bird) among orchids appears to be extremely common as well (Ackerman 1989; Tremblay et al. 2005).

Orchids produce the smallest seeds by size (0.05–6 mm) or weight (0.31–24 μg) of any seed-bearing plants (Arditti and Ghani 2000; Roberts and Dixon 2008); to compensate for this, the seeds are found in thousands and millions per one seed capsule. However, the number of germinated seeds under natural conditions is negligible and barely exceeding 5% (Cherevchenko and Kushnir 1986). Orchids are entirely dependent on mycorrhiza for successful germination and seedling establishment; tiny orchid seeds have no endosperm and get their energetic and nutritional supply from symbiotic fungus (Burgeff 1959; Benzing and Friedman 1981; Clements 1988; Smresiu and Currah 1989; Smith and Read 1997; Waterman and Bidartondo 2008). The dependence on the fungal partner changes during the orchid ontogenesis (Vakhrameeva et al. 2008; Roberts and Dixon 2008). Adult photosynthesizing plants may dispense with the fungus, whereas the orchids lacking chlorophyll need these symbiotic relations throughout their lives. However, mycotrophy has been shown to be not directly dependent on the prevalence of phototrophic activity (Rasmussen and Whigham 2002). Existance of specificity between orchids and fungi still remains a controversial issue (Hadley 1970; Clements 1988; Brundrett 2004; Bonnardeaux et al. 2007). Although there are fungi that can infect different species of some orchid genera (Hadley 1970; Warcup 1971; Katiyar et al. 1986; Dearnaley 2007), many orchids have been found to have highly specific mycorrhizal association (Burgeff 1959; Clements 1988; Shefferson et al. 2005; Bonnardeaux et al. 2007). Molecular identification of orchid mycorrhizal fungi has revealed high fungal specificity in orchids that get organic nutrients from fungi as adults (Waterman and Bidartondo 2008). In any case, interactions between the partners depend very much on the external conditions and nutrient access, and that is why the subtle balance in the orchid–fungus symbiosis can be easily broken and shifted to parasitism and killing of the orchid seedling by the fungus (Clements 1988; Beyrle et al. 1995; Roberts and Dixon 2008).

Like no other plants, orchids associate with a wide range of endophytic fungi from morels (Ascomycetes) to mushrooms (Basidiomycetes) and even species of truffles (Roberts and Dixon 2008). Most orchid mycorrhizal fungi belong to the genus of rhizoctonia-forming fungi, a diverse group of pathogens, endophytes, saprophytes, and symbiotic fungi from three basidiomycete families (Sebacinaceae, Ceratobasidaceae, and Tulasnellaceae) (Burgeff 1959; Warcup 1981; Currah et al. 1987; Roberts 1999; Rasmussen and Whigham 2002; Bonnardeaux et al. 2007). Upon infection of the orchid seedling, fungal hyphae penetrate the cell walls and form characteristic coil-like pelotons within the cortical cells (Clements 1988).

Growth of the fungus is restricted to cortex due to production of phenolic and antifungal compounds (Beyrle et al. 1995; Shimura et al. 2007; Waterman and Bidartondo 2008). Pelotons are digested and through this process, the orchid obtains the essential nutrients and carbon that it needs to grow (Burgeff 1959; Clements 1988; Beyrle et al. 1995; Waterman and Bidartondo 2008). Besides, orchid-associated fungi are known to produce plant growth regulators, such as auxin and gibberellic acids (Hayes 1969; Barroso et al. 1986; Wu et al. 2002; Tsavkelova et al. 2003b, 2008) that may stimulate plant growth and development. The ability of orchids to go into intimate relations with their fungal symbionts facilitates and enlarges the adaptive capacities of each partner to different environmental conditions (Rasmussen et al. 1991; McCormick et al. 2006).

After elucidating the role of mycorrhiza in orchid seed germination by Noël Bernard (1874–1911), the next greatest advantage was the discovery of asymbiotic seed germination, axenic propagation on a defined medium in vitro that was developed by Lewis Knudson (1884–1958), followed by a modern mass rapid clonal propagation (Arditti 1984). Nevertheless, there is no such method of artificial orchid propagation which would be universal and applicable for all known species of the vast Orchidaceae family. Among the obstacles the botanical gardens are facing with, there are complexities of orchid pollination process, fruit set and germinating seed production, and selection of the specific mycorrhizal fungus for symbiotic and appropriate media for asymbiotic germination (Yamazaki and Miyoshi 2006). In the artificial conditions, the interactions between the host plant and its mycobiont are often unpredictable, and it is hard to control the establishment of the subtle and beneficial balance between the partners (Clements 1988; Peterson and Currah 1990; Beyrle et al. 1995; Dijk and Eck 1995). Asymbiotic germination despite its great impact on the orchid industry by making possible a large scale of hybridization (Arditti 1992), requires a usage of complex agar media supplemented with various nutrient substances, vitamins, hormones and uncertain compounds, such as potato extract, beech sap, fish semen, banana pulp, coconut water, tomato, pineapple, or orange juice (Hadley and Harvais 1968; Fonnesbech 1972; Cherevchenko and Kushnir 1986; Arditti 1992). The same ingredients are added to the media for orchid tissue culture (Kusumoto 1980) which is widely used for mass micropropagation of *Phalaenopsis* and other commercial orchids (Gu et al. 1987a, b; Chen et al. 2005). Nevertheless, it is known that many orchids are scarcely ever germinating in the aseptic conditions, and the widespread application of tissue culture is limited by cost of production and restricted number of orchid species with acceptable micropropagation protocols (Govil and Gupta 1997).

Orchid conservation and artificial seed germination presents severe difficulties, but the sustainable cultivation of the adult plants under greenhouse conditions is also an uneasy and labor-intensive process. When cultivated ex situ, the orchids undergo different biotic and abiotic stresses that are aggravated by lack of natural consortive relations with associative microorganisms (Kolomeitseva et al. 2002). Thus, management and development of effective conservation strategies for orchid propagation require a wide range of integrated scientific approaches

(Roberts and Dixon 2008) to overcome the continuous anthropogenic pressing on existence and survival of these vulnerable plants.

11.2.1 Orchid-Associated Bacteria

One of the possible beneficial methods for conservation and better adaptation of rare orchids in the artificial conditions could become in vitro bacterization, seed inoculation with the resident PGPR (plant growth promoting rhizobacteria) cultures. Associative rhizobacteria are recognized to have a great and often favorable impact on plant growth and development due to nitrogen fixation, production of plant growth regulators, improvement of water uptake and mineral nutrition, and biosynthesis of fungicidal and/or bactericidal substance reducing the number of phytopathogens (Tien et al. 1979; Cacciari et al. 1989; Hill et al. 1994; Noel et al. 1996; Dileep et al. 1998; Patten and Glick 2002; Yasmin et al. 2004; Ayyadurai et al. 2006; Mehnaz and Lazarovits 2006; Spaepen et al. 2007; Ahmad et al. 2008; Gulati et al. 2009; Deepa et al. 2010). However, there is a lack of information about composition and functional activity of the orchid PGPR; only few research groups are known for their studies on this subject (Wilkinson et al. 1989, 1994a, b; Tsavkelova et al. 2003c, 2005, 2007a, b), whereas the knowledge on interactions between the orchid and its "satellite" bacteria might add to understanding of orchid biology and result in more effective cultivation, propagation, and conservation of these plants, particularly under artificial greenhouse conditions.

11.2.2 Cyanobacteria

Cyanobacteria are an ancient group of prokaryotic microorganisms, varying in their morphology from unicellular to differentiated filamentous and branched types, and capable of oxygenic photosynthesis and nitrogen fixation (Fay 1992; Adams 2000; Sergeeva et al. 2002). Cyanobacteria possess several cell types and carry out different physiological and biochemical reactions, shifting from ordinary photolithoautotroph metabolism to facultative light-dependent growth on a carbon source other than CO_2, such as sugars; quite a few of them are capable of heterotrophic mode of C nutrition (Rippka et al. 1979; Lambert and Stevens 1986; Rai et al. 2000). Complex life cycle and both structural and functional plasticity of cyanobacteria contribute to a great stability of their microbial communities and allow them to colonize different habitats as well as adapt to strong variation of the environmental conditions (Pankratova 2000; Badger et al. 2006). They are found in cold and hot, alkaline and acidic, marine and freshwater, saline and terrestrial environments (Badger et al. 2006). Cyanobacteria form dense microscale communities of so-called microbial mats in which a wide range of diverse microbes and their metabolism may be present (Stal 2000; Badger et al. 2006).

Slimy cyanobacterial coatings protect their cells against drying out, mechanical damage, and diverse abiotic factors (Pankratova 2000).

In nature, cyanobacteria exist not only as free-living but also as symbiotic organisms (Adams 2000). They have strong associations with sponges and protists, algae and fungi (to form lichens); hosts are also found within bryophytes, a water-fern *Azolla*, cycads, and *Gunnera* (angiosperm) (Grobbellaar et al. 1987; Bergman et al. 1993; Korzhenevskaya et al. 1999; Rai et al. 2000; Costa et al. 2001; Sergeeva et al. 2002; Adams and Duggan 2008; Lindblad 2009). The most prominent role of cyanobacteria to the plants is nitrogen fixation, although in lichens and, in the case of non-photosynthetic hosts, cyanobionts act both as diazotrophic and autotrophic component. Despite that, in most cases the host benefits from the provision of metabolites that contain both nitrogen and carbon (Rai et al. 2000; Badger et al. 2006; Adams and Duggan 2008; Lindblad 2009), the involvement of other substances in cyanobacterial–plant interactions, such as plant growth stimulators, has also been reported (Sergeeva et al. 2002). In one's turn, host plants provide cyanobacteria with a stable environment and enriched pool of nutrients under microaerobic conditions (Bergman et al. 1993; Rai et al. 2000).

Despite some cyanolichens, freshwater and seawater algae, unicellular cyanobacteria such as *Gloeothece*, *Gleocapsa*, and *Synechocystis* are rarely found in host plants; filamentous and heterocyst-forming species of *Nostoc*, *Anabaena*, *Scytonema*, *Calothrix*, *Fischerella* are more frequent symbiotic partners (Bergman et al. 1997; Rai et al. 2000; Rasmussen and Nilsson 2002; Adams and Duggan 2008). The most common cyanobacteria with a range of taxonomically different hosts from algae and fungi, liver- and hornworts, ferns and cycades to some angiosperms belong to the genus *Nostoc* (Vagnoli et al. 1992; Korzhenevskaya et al. 1999; Rai et al. 2000; Rasmussen and Nilsson 2002; Lindblad 2009). The cyanobionts are located in a variety of host organs and tissues (bladder, thalli and cephalodia in fungi; cavities in gametophytes of hornworts and liverworts or fronds of the *Azolla*; coralloid roots in cycads; stem glands in *Gunnera* (Adams 2000; Rai et al. 2000; Rasmussen and Nilsson 2002; Adams and Duggan 2008). *Nostoc* strains infect and colonize different tissues and organs of hosts, and are usually inter- or intracellularly located in the host cells (Rasmussen and Nilsson 2002). Combination of CO_2 assimilation and N_2 fixation allows the partners of cyanobacterial symbioses to flourish in a nutrient-poor environment. Cyanobacterial–plant symbioses occur throughout the world and might be the dominant vegetation in some regions; lichens and bryophytes are rarely found inhabiting rich substrates, where the competitive relations are strong and disadvantageous (Rai et al. 2000; Costa et al. 2001). Moreover, it is believed that only nutrient-poor habitats contribute to the development and functional stability of cyanobacterial associations with the plants (Rai et al. 2000).

The intimate relations of cyanobacteria with their hosts creates a unique symbiotic consortium, which guarantee an ecological stability and nutrient supply that enable both partners to enlarge the adaptive amplitude of the system (Rai et al. 2000; Pankratova 2000; Badger et al. 2006). Nitrogen-fixing cyanobacteria are known to occur epiphytically in phyllosphere and/or rhizosphere of plants growing

in aquatic and high humidity environments (Freiberg 1999; Rai and Bergman 2002). Besides the hosts, such as a water-fern *Azolla*, cycads, and *Gunerra* that are residents of the origins of warm moist habitats, *Nostoc* and *Anabaena* strains has been reported to successfully colonize and in certain cases penetrate the roots of rice, corn, sugar beet, marine mangroves, and wheat seedlings (Gantar et al. 1991; Spiller et al. 1993; Svircev et al. 1997; Rai et al. 2000; Rai and Bergman 2002). Nitrogen-fixing cyanobacteria cocultivatated with wheat and rice seedlings in liquid hydroponic and sand cultures are known to form associations and promote plant growth and yield in nitrogen-free media (Spiller et al. 1993; Toledo et al. 1995; Svircev et al. 1997; Rai and Bergman 2002). Another artificial microbial consortium consisted of different species of *Rhizobium* spp. introduced into the extracellular slime of *Nostoc paludosum* that has been shown to penetrate into the nodules, resulted in an increase of the nitrogen-fixing ability and productivity of legumes (Pankratova et al. 2008).

The presence of cyanobacteria in the orchid's velamen has been previously noted (Gladkova 1982), although no information on taxonomy, localization, and possible role of orchid-associated cyanobacteria has been provided. Nevertheless, epiphytic orchids, producing numerous air roots covered with the spongy velamen represent a perfect econiche for associative cyanobacteria. We have studied various tropical orchids cultivated under greenhouse conditions (Tsavkelova et al. 2001; 2003c, d). Among the examined specious, there were epiphytic *Acampe papillosa* (Lindl.) Lindl., *Dendrobium moschatum* (Buch. – Ham.) Swartz., *D. phalaenopsis* Pfitz., *Phalaenopsis amabilis* (Lindl.) Blume., and one terrestrial species of *Calnthe vestita* Lindl. var. *rubro-oculata*. Natural habitat of *A. papillosa* covers the territories of Bhutan, Burma, India, Laos, Nepal, Thailand, and Vietnam; in addition to the earlier mentioned countries, *D. moschatum* also occurs in South China. Under conditions of selected greenhouse, these epiphytes are cultivated as pot plants, so they are able to form both aerial and substrate roots. *P. amabilis* and *D. phalaenopsis* occur in Indonesia, New Guinea, Philippines, and Queensland, Australia. In the greenhouse, these two orchids are cultivated on the bars of bark or inert (plastic) materials, which are used to support a plant. Under constant warm and moist conditions, with relative humidity of about 90%, these orchids form numerous long and interlaced aerial roots, whose surface is fully covered by 1–3 mm thick sheath-like folds of dark green biomass of phototrophs. *A. papillosa* and *D. moschatum* also produce aerial roots with a silvery-gray velamen, but cultivated under less humid conditions (about 80%), they lack a folder of associative photosynthetic microorganisms. The phototrophic microorganisms are disseminated along the roots of *D. moschatum* in a threads-looking shape, and the larger roots of *A. papillosa* contain fibrous greenish films of phototrophs on their surface.

Diverse cyanobacteria have been revealed in the sheaths of aerial roots of *P. amabilis* and *D. phalaenopsis* (Tsavkelova et al. 2003d). This phototrophic covering consists of various morphotypes of cyanobacteria (Figs. 11.1–11.3). Figures 11.1 and 11.2 show *P. amabilis* root fragments with filamentous cyanobacteria represented by chains of ellipsoid and diskshaped flattened cells.

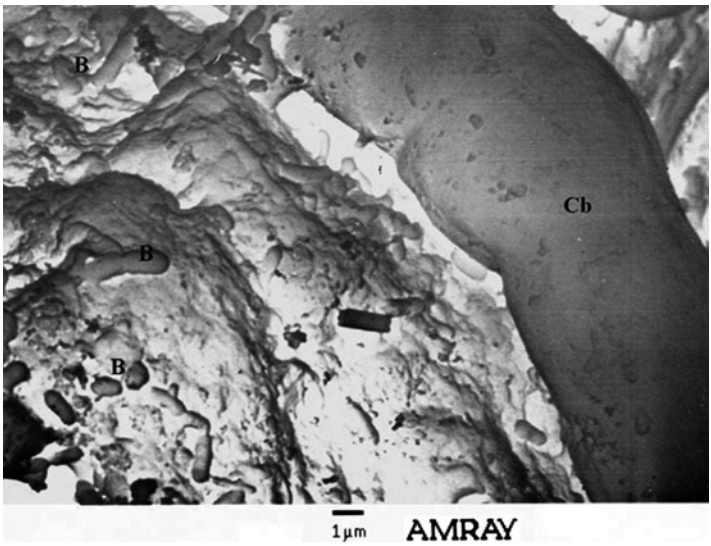

Fig. 11.1 Fragment of the *Phalaenopsis amabilis* aerial root surface. Scanning electron microscopy (SEM). *B* bacteria, *Cb* cyanobacteria (originally published in Tsavkelova et al. 2003c)

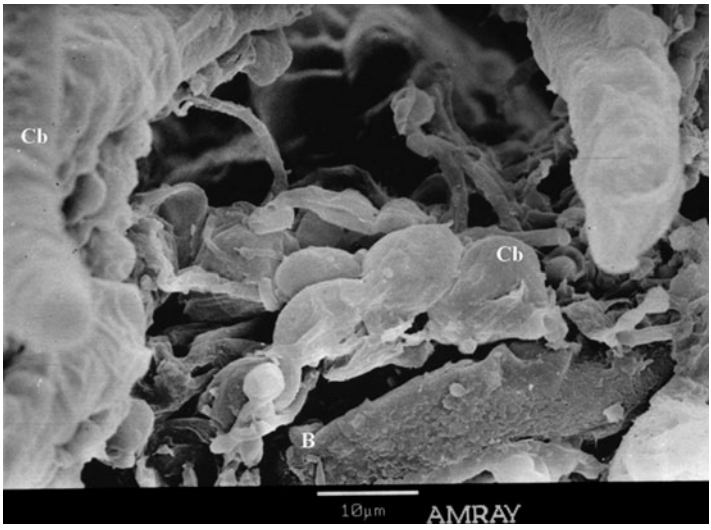

Fig. 11.2 Fragment of the *Phalaenopsis amabilis* aerial root surface. SEM. *B* bacteria, *Cb* ellipsoid and diskshaped filamentous cyanobacteria (originally published in Tsavkelova et al. 2003c)

The surface of aerial roots of *D. phalaenopsis* exhibited similar morphological organization of the associative phototrophic community (Fig. 11.3). In addition to filamentous cyanobacteria, short chains, individual microbes and agglomerated

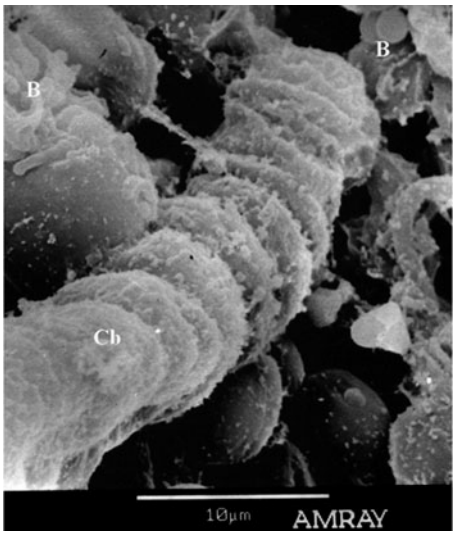

Fig. 11.3 Fragment of the *Dendrobium phalaenopsis* aerial root surface. SEM. *B* bacteria, *Cb* cyanobacteria (originally published in Tsavkelova et al. 2003c)

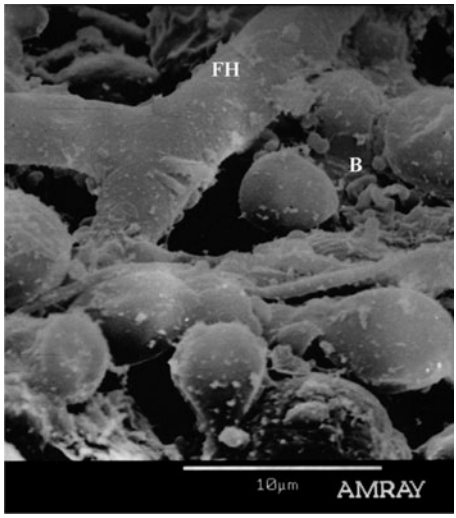

Fig. 11.4 Fragment of the *Dendrobium phalaenopsis* aerial root surface. SEM. *B* bacteria, *FH* fungal hyphae (originally published in Tsavkelova et al. 2003c)

bacterial cells, as well as fungal hyphae are abundant on the surface of aerial roots of all investigated orchids (Figs. 11.1–11.7).

The velamen represents a suitable econiche for colonization by autotrophic and diazotrophic microorganisms. Along with phototrophic cyanobacteria, other microbial cells penetrate the internal layers of the velamen. By the example of *D. phalaenopsis* and *A. papillosa*, it has been shown to lack filamentous-looking cyanobacteria, while numerous phototrophic cells grouped in spherical clusters were found inside the velamen (Fig. 11.8). Morphophysiological changes usually occur when cyanobacterias are involved in symbiotic relationships with fungi

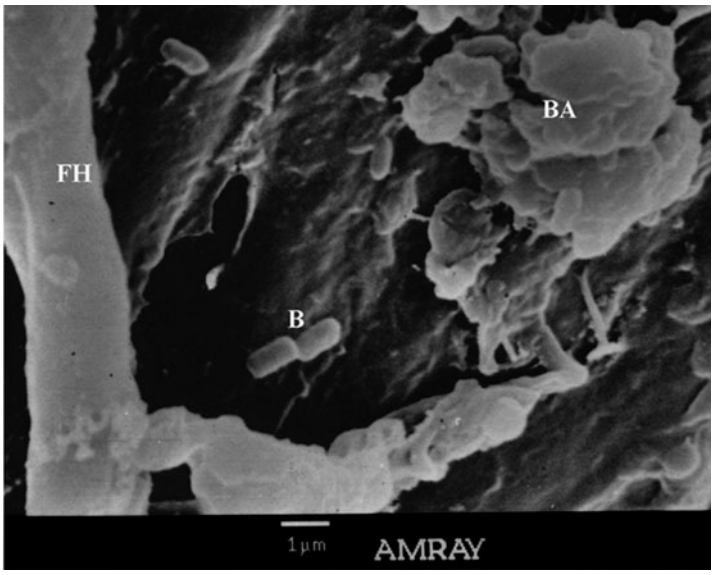

Fig. 11.5 Fragment of the *Acampe papillosa* aerial root surface. SEM. *B* bacteria, *BA* bacterial agglomerates embedded in extracellular matrix, *FH* fungal hyphae

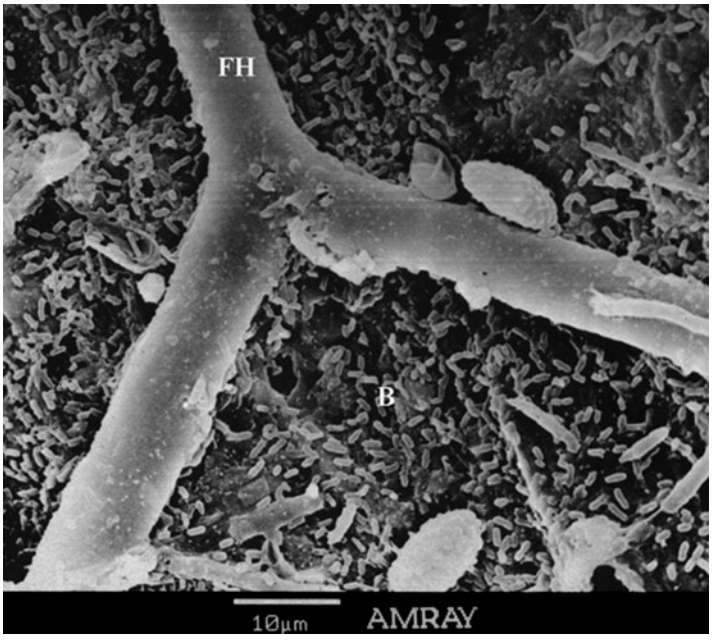

Fig. 11.6 Fragment of the *Acampe papillosa* aerial root surface. SEM. *B* bacteria, *FH* fungal hyphae (originally published in Tsavkelova et al. 2003c)

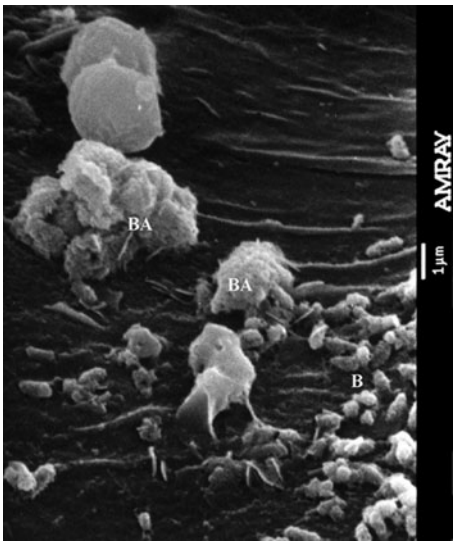

Fig. 11.7 Fragment of the *D. moschatum* aerial root surface. SEM. *B* bacteria, *BA* bacterial agglomerates (originally published in Tsavkelova et al. 2001)

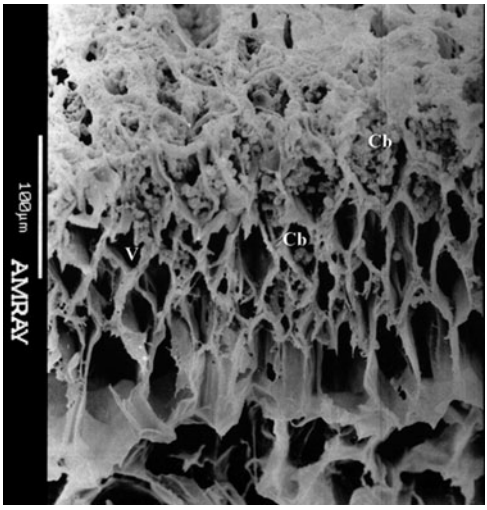

Fig. 11.8 Fragment of a cross section of *Acampe papillosa* aerial root. SEM. *V* the velamen, *Cb* cyanobacteria (originally published in Tsavkelova et al. 2003c)

(in lichens) or plants during their compartmentalization within the host tissues (Korzhenevskaya et al. 1999; Rai et al. 2000; Gorelova and Korzhenevskaia 2002; Meeks and Elhai 2002; Adams and Duggan 2008; Lindblad 2009). Within the symbiotic colony, the vegetative cells show altered morphology, being enlarged and irregular in shape, and even heterocysts become difficult to recognize due to loss of some of their morphological characteristics that distinguish them from vegetative cells (Meeks and Elhai 2002; Adams and Duggan 2008). Slimy mucous covering formed by cyanobacteria is also favorable for inhabitation by other microbes. Polysaccharides are common constituents of cyanobacterial cell

envelopes (Drews and Weckesser 1982; Stewart et al. 1983; Plude et al. 1991), and cyanobacterial slime containing carbohydrates and various organic compounds serves as a nutrient and energy source to heterotrophic microorganisms. In turn, "satellite" bacteria are able to remove toxins produced by cyanobacteria and utilize oxygen, which inhibits nitrogen fixation.

In subtropical and tropical forests, nutrients are constantly cycling through the ecosystem, and most of the available nutrients (over 75%) are not in the soil but in the organic matter, and use of nitrogen-fixing species for hedgerows and mulching is a common technique for soil improvement (Elevitch et al. 1998). Since insufficient amount of minerals is common in the tropics, nitrogen may be the limiting factor for growth and development of epyphitic orchids. Involvement of various cyanobacteria in formation of the sheath-shaped covering suggests that nitrogen fixation is the primary function of orchid-associated cyanobacteria. All filamentous cyanobacteria of the genera *Anabaena*, *Nostoc*, *Scytonema*, and *Calothrix*, isolated from the investigated plants under nitrogen-limiting conditions formed specialized nitrogen-fixing cells, heterocysts (Figs. 11.9–11.11). Potential nitrogen-fixing activity of the

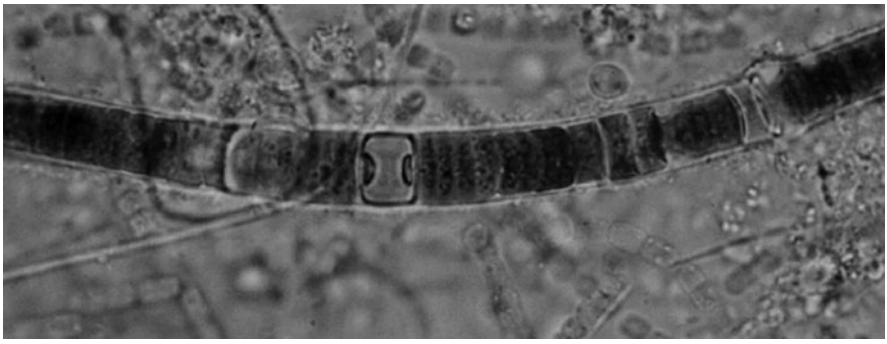

Fig. 11.9 *Scytonema* sp. (enrichment culture in nitrogen-free medium; isolated from aerial roots of *P. amabilis*). Light microscopy ×3,200 (originally published in Tsavkelova et al. 2003d)

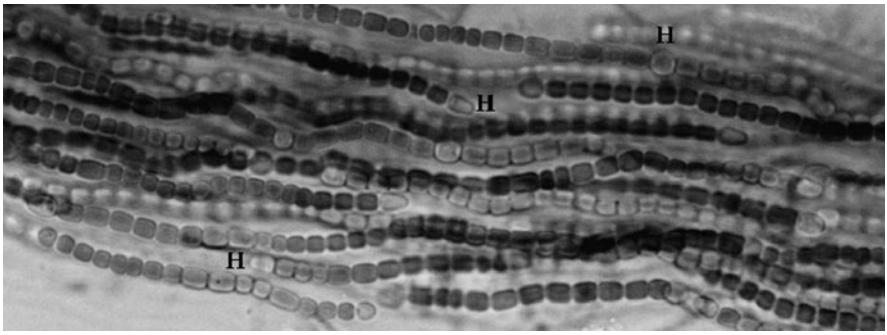

Fig. 11.10 *Nostoc* sp. (enrichment culture in nitrogen-free medium; isolated from aerial roots of *P. amabilis*). *H* heterocyst. Light microscopy ×3,550 (originally published in Tsavkelova et al. 2003d)

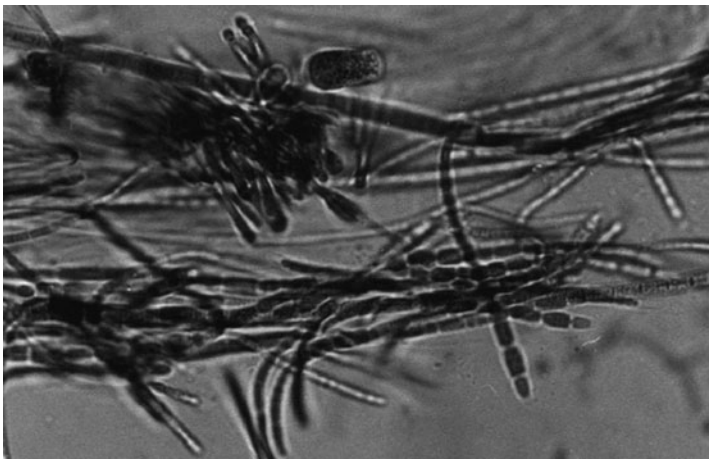

Fig. 11.11 *Calothrix* sp. (enrichment culture in nitrogen -free medium; isolated from aerial roots of *P. amabilis*). Light microscopy ×1,400 (originally published in Tsavkelova et al. 2003d)

Table 11.1 Associative filamentous cyanobacteria of greenhouse orchids (Tsavkelova et al. 2001, 2003d)

Orchid	Cultivation medium	
	Nitrogen-free BG_0-11	Complete BG-11
Substrate roots		
D. moschatum	*Nostoc*	*Nostoc*, LPP, *Fischerella*
A. papillosa	*Nostoc*	*Nostoc*, *Oscillatoria*, LPP
C. vestita	*Nostoc*	*Nostoc*, *Oscillatoria*
Aerial roots		
D. moschatum	*Nostoc*	*Nostoc*
A. papillosa	*Nostoc*, *Anabaena*	*Nostoc*, *Anabaena*, *Calothrix*
D. phalaenopsis	*Nostoc*, LPP, *Scytonema*	*Nostoc*, LPP, *Scytonema*
P. amabilis	*Nostoc*, *Scytonema*, *Calothrix*, LPP	*Nostoc*, *Scytonema*, *Calothrix*, LPP, *Spirulina*, *Oscillatoria*

microorganisms, forming the most developed sheaths on the *P. amabilis* aerial roots has been shown to comprise about 800 nmol C_2H_4/h per gram biomass (as measured by acetylene reduction) (Tsavkelova et al. 2003c). High activity of microbial nitrogen fixation confirms the great importance of cyanobacteria to the orchid, since a great part of the fixed nitrogen is usually provided to the host (Fay 1992; Rai et al. 2000; Adams and Duggan 2008; Lindblad 2009).

Experiments on isolation and cultivation of associative cyanobacteria revealed that abundance of phototrophs and composition of cyanobacterial community depend on climate and ecological conditions (aerial or substrate roots) (Tsavkelova et al. 2003d), although the strains of the genus *Nostoc* were invariably isolated from the roots of all investigated plants (*C. vestita*, *A. papillosa*, *D. moschatum*, *D. phalaenopsis*, and *P. amabilis*) (Table 11.1). *Nostoc* spp. isolated from

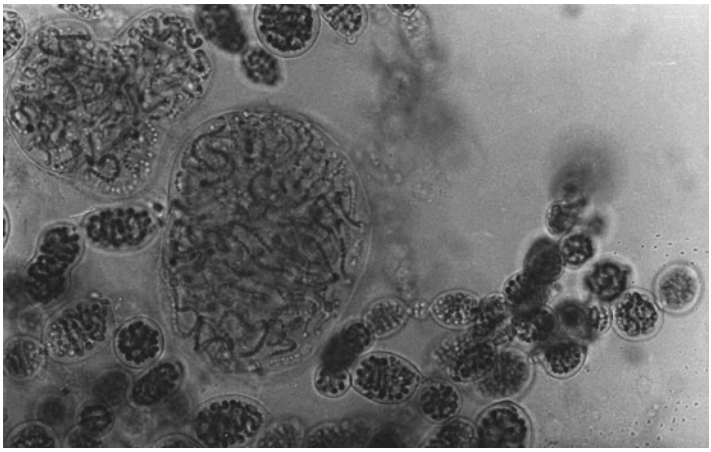

Fig. 11.12 Clusters of *Nostoc* sp. isolated from aerial roots of *A. papillosa* in nitrogen-free medium. Light microscopy ×1,750 (originally published in Tsavkelova et al. 2003d)

A. papillosa and *P. amabilis* formed globe-shaped colonies of various sizes at initial stages of growth both in BG_o-11 and BG_N-11 media (Fig. 11.12). The spherical clusters of trichomes are usually fixed due to a jellylike mucous coat produced by cyanobacteria. *Nostoc* and *Anabaena* cyanobionts are known to lack a visible peptidoglycan layer and occasionally an outer membrane when they enter in artificial associations with higher plants (Gorelova and Korzhenevskaia 2002). The morphology of such symbiotic cyanobacteria changes markedly; the filamentous forms appears unicellular because of increased cell size, thinner cell walls, and altered cell shape that enable the cyanobiont to arrange in more rounded shape (Galun and Bubrick 1984; Korzhenevskaya et al. 1999; Rai and Bergman 2002; Adams and Duggan 2008).

In the nitrogen-free BG_o-11 medium, *Anabaena* spp. was also detected on the aerial roots of epiphytic *Acampe papillosa*. In the complete BG-11 medium, cyanobacteria of the genera *Nostoc*, *Oscillatoria* and of the LPP group (*Lyngbia*, *Phormidium*, and *Plectonema*), and cyanobacteria of the genera *Nostoc*, *Anabaena,* and *Calothrix* were isolated from the substrate and aerial roots of this plant, respectively (Table 11.1). Less favorable conditions for cyanobacterial growth presents the submerged roots of terrestrial *Calanthe vestita*. Nevertheless, the strains of *Oscillatoria* and *Nostoc* were also detected, with *Oscillatoria* as the dominant cyanobacterial culture (Tsavkelova et al. 2001). *Nostoc*. spp. was the only diazotrophic specious, found in BG_o-11 medium on the substrate roots of another epiphytic orchid, *D. moschatum*, whereas the usage of complete BG_N-11 medium revealed a broader spectrum of associative cyanobacteria (Table 11.1); they were *Nostoc* spp., *Fischerella* spp., and LPP strains (Tsavkelova et al. 2001, 2003d). Cyanobacteria of *D. moschatum*'s aerial roots were represented mainly by *Nostoc* strains. The specificity of the substratum (pine bark) used for cultivation of these epiphytic orchid provides enough moisture and an access of air and light both to the

substrate roots and its associative cyanobacteria that explains the presence and diversity of phototrophic microorganisms. The composition of the cyanobacterial community differs between the plants even if they are cultivated in the same greenhouse; as shown by the examples of *D. moschatum*/*A. papillosa* and *P. amabilis*/*D. phalaenopsis* (Tsavkelova et al. 2001, 2003d). *Nostoc*, *Scytonema*, and LPP group have been isolated from the roots of *D. phalaenopsis* (Table 11.1). These cyanobacteria were revealed on *P. amabilis* aerial roots as well. However, besides the earlier mentioned microorganisms, *Calothrix*, *Spirulina,* and *Oscillatoria* cultures were also detected. Thus, the aerial roots of orchids cultivated under the conditions of constant high humidity and temperatures perform the most favorable econiche for cyanobacteria; the spectrum of photosynthetic microbial community of *Phalaenopsis amabilis* and *Dendrobium phalaenopsis* is much broader than that of orchids grown in a drier greenhouse environment (Tsavkelova et al. 2001, 2003d).

Other beneficial effect of the cyanobacterial community is that it provides nutrients for associative microorganisms of rhizoplane, such as bacteria and fungi. In case, when the epiphytes are cultivated without substratum, being attached to the bars of bark or any inert material, the cyanobacterial sheath may play an important role in providing nutrients to orchid mycorrhiza. Although the dependence of adult orchids on their symbiotic fungi reduces, many mature terrestrial and epiphytic plants retain their symbionts and remain mycotrophic (Rasmussen and Whigham 2002; Vakhrameeva et al. 2008; Roberts and Dixon 2008). The substrate roots of greenhouse orchids *Calnthe vestita*, *Acampe papillosa*, and *Dendrobium phalaenopsis* have been shown to contain mycorrhizal hyphae and their coiled aggregates (pelotones) in cortical cells (Figs. 11.13–11.15). Pelotones of mycorrhizal fungi

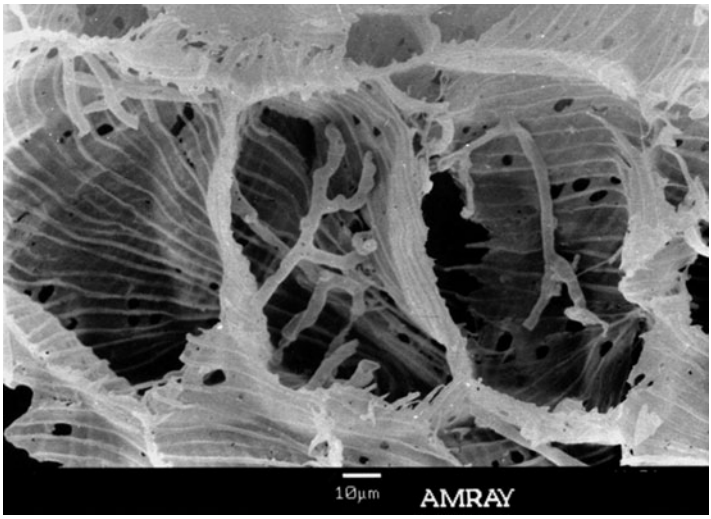

Fig. 11.13 Mycorrhizal fungus hyphae penetrating the substrate roots of *D. moschatum*. SEM (originally published in Tsavkelova et al. 2003a)

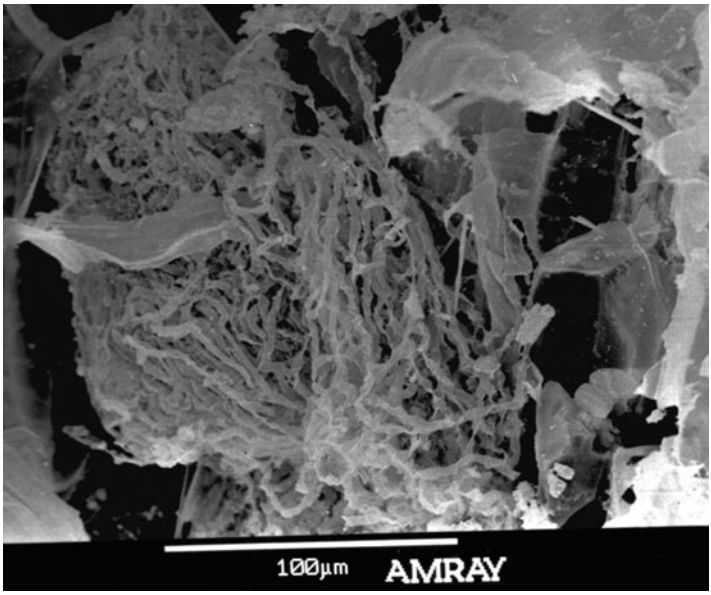

Fig. 11.14 Fungal hyphae in parenchyma cells of substrate roots of terrestrial *Calanthe vestita* var. *rubro-oculata*. SEM

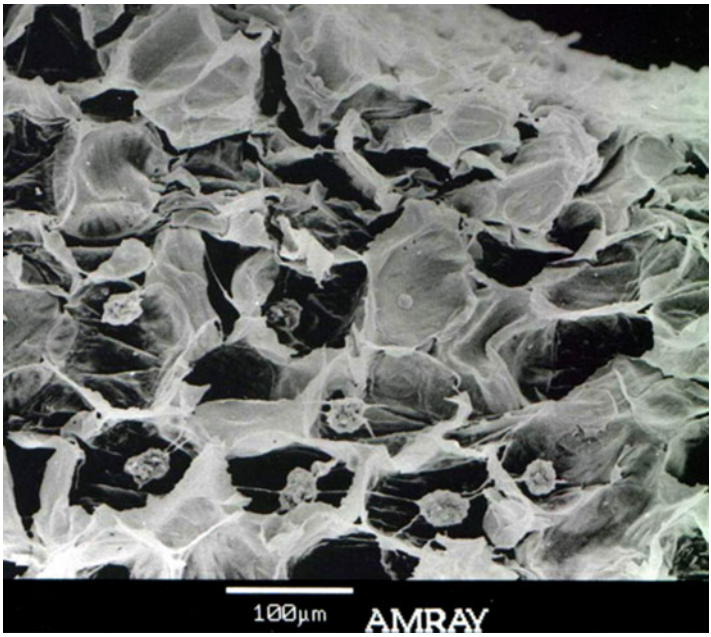

Fig. 11.15 Pelotones of mycorrhizal fungus in parenchyma cells of substrate roots of *Acampe papillosa*. SEM (originally published in Tsavkelova et al. 2003a)

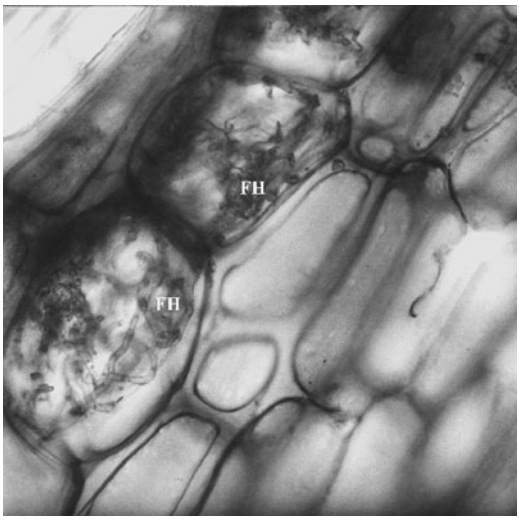

Fig. 11.16 Fungal hyphae (FH) within aerial root of epiphytic *Phalaenopsis amabilis*. Light microscopy ×3,150 (originally published in Tsavkelova et al. 2003d)

are usually digested by the host cells shortly after their formation (Peterson and Farquhar 1994). The aerial roots which are not in touch of bark or other substratum are believed not to have any mycorrhizal associations (Katiyar et al. 1986; Zalukaeva 1990). However, we revealed that the cyanobacterial coating of aerial roots contributes to fungal growth and development that enable the fungus to penetrate the root and to form symbiotic associations with the host plant (Tsavkelova et al. 2003d). Fungal hyphae and half-digested pelotones have been detected within the aerial roots of *Phalaenopsis amabilis* (Fig. 11.16). The aerial roots covered with a dense cyanobacterial sheath become incapable of photosynthesis, while the sheath itself may serve as a particular econiche, colonized by mycorrhizae-forming fungi.

Isolated strains of associative cyanobacteria were tested for their ability to produce a principle plant growth stimulator, indole-3-acetic acid (IAA, auxin) (Tsavkelova and Cherdyntseva 2005). Investigated cyanobacteria (several strains of *Nostoc* sp., *Anabaena* sp., and *Calothrix* sp.) did not produce high amounts of IAA, according to the method of colorimetric Salkowski assay. Without supplementation of IAA precursor, tryptophan, the level of produced auxin scarcely exceeded 1.5 μg/ml. Addition of tryptophan to the cultivation media enhanced auxin production; the IAA amounts ranged between 2.6 and 12.8 μg/ml in the complete BG-11 medium. In BG_o-11 nitrogen-limited medium, the strains of *Anabaena* sp. and *Calothrix* sp. were more active and produced about 20.0 μg IAA/ml; whereas the strains of *Nostoc* sp. did not show any significant difference in auxin production. Tryptophan-dependent biosynthesis of IAA has been previously reported in cyanobacteria both free-living and symbiotic (Sergeeva et al. 2002). Associative cyanobacteria may use the ability of producing auxins in their strategy to interact with the host plant, particularly when the cyanobacterial partners are localized within the plant tissues (Sergeeva et al. 2002). Nevertheless, the most

evident functional characteristic and primary role of the orchid cyanobacterial community is rather nitrogen fixation and nutrient supply to both the host plant and the "satellite" microorganisms, including mycorrhizae-forming fungi. Thus, a well-structured cyanobacterial community may contribute to formation and stable functioning of the microbial consortium of orchid rhizoplane that in one's turn is an important constituent of complex orchid–microbial interactions.

11.2.3 Rhizobacteria

The plant–microbial interactions have been studied for a long time, however little is known about diversity and functional activity of orchid-associated bacteria. Beneficial influence of rhizobacteria on growth and development of orchids still remains unexplored, although this information could provide a better insight into relations between the host plant and its associative microorganisms and thus, help in orchid cultivation and conservation. About 30 years ago, an Australian group of researches reported on isolation of endophytic bacteria from the substrate roots of wild grown terrestrial *Pterostylis vittata* orchid (Wilkinson et al. 1989, 1994a, b). Associative bacteria were classified as belonging to the genera of *Kurthia*, *Pseudomonas*, *Bacillus*, *Xanthomonas*, and *Arthrobacter*.

In order to study microbial diversity, localization and functional activity of orchid-associated bacteria, several greenhouse and wild grown terrestrial and epiphytic orchids have been investigated (Tsavkelova et al. 2001, 2004, 2007a). The scanning electron microscopy analysis of orchid roots showed that the surface and inner root tissues, particularly the velamen, were abundantly occupied by various bacteria (Figs. 11.1–11.8). Together with individual bacterial cells of different morphology, microbial clusters submerged within intercellular matrix were revealed on the aerial roots of epiphytic *D. moschatum* and *A. papillosa* (Figs. 11.5–11.7). Interestingly, the aerial roots of epiphytic orchids were more densely occupied by bacteria than the substrate roots. Quantitative assessment of the *A. papillosa* bacterial complex showed that the amount of bacteria isolated from the aerial roots was about 30 times higher than that of the substrate roots of the same plant (Tsavkelova et al. 2004). Evidently, the rhizoplane of epiphytic orchids become a zone of maximal microbial activity. Multilayered structure of the velamen may protect the associative bacteria of various biotic and abiotic factors, and the root exudates provide a better nutrient support for microbial community. Beneficial interactions between a host orchid and rhizobacteria are of special importance, particularly when epiphytes are cultivated without any substratum, attached to a piece of bark or on the blocks. Further investigation of this phenomenon revealed that microbial abundance in orchid rhizoplane differs significantly between the epiphytic and terrestrial plants of the same location. Associative bacteria of wild grown Vietnamese orchids populated the roots of epyphitic *Pholidota articulata* 55 times more densely than the substrate roots of terrestrial *Paphiopedilum appletonianum* (Tsavkelova et al. 2007a).

Besides, the diversity of rhizobacteria also differs between epiphytic and terrestrial orchids (Table 11.2). Regardless the conditions of plant growing, the variety of associative microorganisms of epiphytes is much wider than that of terrestrial plants (Tsavkelova et al. 2001, 2004, 2007a). Bacteria detected on the roots of terrestrial *Calanthe vestita* were *Arthrobacter*, *Bacillus*, *Pseudomonas*, and *Mycobacterium* (Table 11.3). At the same time, the strains of *Rhodococcus*,

Table 11.2 Associative bacteria of selected wild grown orchids (Tsavkelova et al. 2007a)

Plant	Bacteria of rhizoplane	Endophytic bacteria
Terrestrial orchid		
Paphiopedilum appletonianum	*Bacillus* *Burkholderia* *Erwinia* *Nocardia* *Pseudomonas* *Streptomyces*	*Bacillus* *Erwinia* *Pseudomonas* *Streptomyces*
Epyphitic orchid		
Pholidota articulata	*Agrobacterium* *Bacillus* *Burkholderia* *Chryseobacterium* *Erwinia* *Flavobacterium* *Pantoea* *Paracoccus* *Stenotrophomonas* *Pseudomonas*	*Bacillus* *Flavobacterium* *Pseudomonas*

Table 11.3 Associative bacteria of selected epiphytic greenhouse orchids (Tsavkelova et al. 2001, 2004, 2007b)

Plant/root type	Bacteria of rhizoplane	Plant/root type	Bacteria of rhizoplane	Endophytic bacteria
Dendrobium moschatum		*Acampe papillosa*		
Aerial roots	*Bacillus* *Flavobacterium* *Microbacterium* *Nocardia* *Pseudomonas* *Rhodococcus* *Sphingomonas* *Xanthomonas*	Aerial roots	*Bacillus* *Flavobacterium* *Micrococcus* *Pseudomonas* *Rhodococcus* *Streptomyces* *Xanthomonas*	*Bacillus* *Flavobacterium* *Pseudomonas* *Rhodococcus* *Xanthomonas*
Substrate roots	*Acinetobacter* *Bacillus* *Mycobacterium* *Pseudomonas* *Rhizobium* *Rhodococcus*	Substrate roots	*Acinetobacter* *Bacillus* *Cellulomonas* *Gluconobacter* *Mycobacterium* *Pseudomonas* *Rhodococcus* *Streptomyces*	*Alcaligenes* *Bacillus* *Gluconobacter* *Pseudomonas*

Micrococcus, *Flavobacterium,* and *Xanthomonas*, which inhabit the roots of epiphytic *Acampe papillosa* and *D. moschatum* were not found on the roots of *C. vestita* cultivated in the same greenhouse as previously mentioned plants. As for the wild grown orchids, the most commonly isolated bacteria of the terrestrial *Paphiopedilum appletonianum* were Gram-positive strains of *Streptomyces* and *Bacillus*, while the dominants of epiphytic *Pholidota articulata* roots were Gram-negative *Pseudomonas* and *Flavobacterium* (Table 11.2, Tsavkelova et al. 2007a). The microbial community is not identical within one plant. *Acinetobacter*, *Rhizobium*, *Bacillus*, *Pseudomonas*, *Mycobacterium*, and *Rhodococcus* were isolated from the substrate roots of epiphytic *D. moschatum*, whereas the aerial roots of this plant were populated by *Bacillus*, *Microbacterium*, *Sphingomonas Flavobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, and *Xanthomonas* (Table 11.3). Bacteria classified into genera of *Acinetobacter*, *Bacillus*, *Cellulomonas*, *Gluconobacter*, *Mycobacterium*, *Rhodococcus*, *Pseudomonas,* and *Streptomyces* were isolated from the substrate roots of another epiphyte, *A. papillosa*, and bacteria of the genera *Bacillus*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, *Rhodococcus*, *Streptomyces,* and *Xanthomonas* were isolated from its aerial roots (Table 11.3, Tsavkelova et al. 2004).

Surface sterilized roots of the greenhouse *A. papillosa* also revealed the presence of a wide range of endophytic bacteria. The inner layers of *Acampe* aerial roots were colonized by *Bacillus*, *Flavobacterium*, *Pseudomonas*, *Rhodococcus,* and *Xanthomonas* strains, while *Alcaligene*, *Gluconobacter*, *Bacillus,* and *Pseudomonas* were detected within its substrate roots. Among endophytic bacteria of *Paphiopedilum*, the majority belonged to bacilli, while the strains of *Pseudomonas* comprised the primary microbial population of the *Ph. articulate* (Table 11.2). Species of bacilli, *Burkholderia*, enterobacteria, *Flavobacterium*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas*, and *Xanthomonas* are well known plant-associated bacteria and frequent inhabitants of rhizosphere and rhizoplane of numerous plants (Garbeva et al. 2001; Halda-Alija 2003; Loiret et al. 2004; Park et al. 2005; Unno et al. 2005; Young et al. 2005; Gnanamanickam 2007; Ferreira et al. 2008; Deepa et al. 2010). Wilkinson et al. (1989, 1994a) had previously reported the prevalence of endophytic *Pseudomonas* and *Bacillus* strains, and frequent occurrence of *Arthrobacter*, *Xanthomonas*, and *Kurthia* spp. strains as associative bacteria of several wild grown Australian terrestrial orchids. The authors suggest that the orchids are characterized by limited specificity in choosing their bacterial partners. However, despite that the common species of *Pseudomonas*, *Bacillus*, *Xanthomonas,* and *Burkholderia* are typical for the orchids of the same origin, the plants host diverse microbial populations (Tables 11.2 and 11.3). The composition of bacterial community differs even within one particular plant depending on the root type; it may result from the different morphology and physiological characteristics of orchid roots as well as various environmental conditions in which the aerial and substrate roots function.

Associative microorganisms attach to the root surface without formation of specialized visible structures such as nodules, as it is in plant–*Rhizobium* symbiosis (Döbereiner and Day 1974; Kameneva and Muronets 1999). Bacterial cells embedded

in the adhesive films of extracellular exopolysaccharide matrix have been found on the root surface of greenhouse orchids (Figs. 11.5 and 11.7), and were more noticeable on the aerial roots of epiphytes (Tsavkelova et al. 2001, 2003c). Except for the strains of *Nocardia* and *Streptomyces*, all isolated orchid-associated bacteria produced colonies with a developed matrix. Microbial matrix is essential for providing a beneficial environment for agglomerated bacteria; it accumulates nutrients and ions; in addition to a structure-forming and anchoring function, it protects bacterial cells from dehydration, radiation, and other negative abiotic factors (Pavlova et al. 1990; Safronova and Botvinko 1998; Skorupska et al. 2006; Khan et al. 2009). Production of a variety of active polysaccharides is considered to allow rhizobial strains to adapt to changing environmental conditions and interact efficiently with legumes (Skorupska et al. 2006). Microbial glycocalyx is a highly hydrated polyanionic matrix (>90% water) surrounding the bacterial cells and can be composed of hundreds to thousands of monomeric units (Sutherland 2001; Erlandsen et al. 2004). Typically, bacterial matrix consists of polysaccharides and glycosyl phosphate-units or, less frequently, glycoproteins and peptides (Safronova and Botvinko 1998; Sutherland 2001; Skorupska et al. 2006). The majority of studied orchid-associate bacteria produced the polysaccharide matrix of polyanionic nature (Tsavkelova et al. 2004); carbohydrate-rich matrix was shown for the strains of the genera *Pseudomonas*, *Rhizobium*, *Mycobacterium*, and *Rhodococcus*, although some *Pseudomonas* strains possess equal proportions of carbohydrate/protein complex.

Other characteristic of associated bacteria colonizing the aerial roots of epiphytic orchids is that they form brightly colored, pigmented colonies. The pigments usually offer UV radiation (UVR) protection, and such UVR tolerance is known to be a common phenotype among phyllosphere bacteria (Sundin and Jacobs 1999). Since the aerial roots of epiphytic orchids are exposed to sunlight, only selected bacteria with sufficient UVR protection can survive in this environment. The solar radiation has a great impact on microbial ecology and formation of the whole consortium of orchid-associated microorganisms. Thus, orchid-associated bacteria actively colonize host plant's roots and form microbial communities; they attach to the root surface by means of extracellular matrix and they penetrate inside the root. The composition, abundance, and diversity of microbial associations depend on orchid species (terrestrial versus epiphytic) as well as on the root type (aerial roots versus the substrate ones).

11.2.3.1 Auxin Production by Orchid-Associated Rhizobacteria

Plant hormones, such as auxins, gibberellins, cytokinins, abscisic acid, and ethylene are essential in coordination of different aspects of plant physiology. Auxins are considered to be the most active plant growth stimulators; cell division, elongation and enlargement, tissue differentiation, root initiation and growth, branching, tropistic responses to light and gravity, blossom, and fruit set formation are just some of the effects of IAA, a naturally occurring auxin (Davies 1995; Woodward

and Bartel 2005). Although IAA producers have been found among free-living microorganisms, IAA biosynthesis is mostly widespread among soil and plant-associated bacteria (White 1987; Patten and Glick 1996, 2002; Kameneva and Muronets 1999; Spaepen et al. 2007; Ahmad et al. 2008). Auxin production has been reported for the strains of the genera *Acetobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Aminobacter*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Methylobacterium*, *Methylovorus*, *Paracoccus*, *Pseudomonas*, *Rhizobium*, *Rhodococcus*, *Xanthomonas*, and many others (Tien et al. 1979; Cacciari et al. 1989; Costacurta and Vanderleyden 1995; Ivanova et al. 2001; Halda-Alija 2003; Khalid et al. 2004; Unno et al. 2005; Mehnaz and Lazarovits 2006; Spaepen et al. 2007; Ali et al. 2009; Gulati et al. 2009; Deepa et al. 2010). The ability of auxin production is determinant for both plant growth promoting rhizobacteria (PGPR) and plant pathogens (Patten and Glick 1996; Patten and Glick 2002; Spaepen et al. 2007). Stimulation of root growth is one of the major characteristics by which the positive effects of PGPR are measured (Patten and Glick 2002; Spaepen et al. 2007). However, IAA overproduction by pathogenic strains of *Agrobacterium*, *Pseudomonas*, *Erwinia,* and *Stenotrophomonas* causes imbalance in plant endogenous auxin pool that can lead to abnormal tissue proliferation (Patten and Glick 1996; Glickmann et al. 1998; Liu and Nester 2006; Spaepen et al. 2007).

Till now, only insufficient and fragmentary data have been reported on the ability of orchid-associated bacteria to produce auxin (Wilkinson et al. 1989, 1994a). The influence of rhizobacteria on orchid growth and development under natural and artificial conditions of cultivation remains unclear. Wilkinson and coworkers (1989, 1994a) reported on the orchid endophytic strains of *Pseudomonas* spp., *Bacillus* spp., and *Xanthomonas* spp., which were capable of IAA production. In our studies, we have also shown that associative bacteria of greenhouse orchids were active in producing IAA (Tsavkelova et al. 2005). Without supplementation of tryptophan which is believed to be the main precursor of IAA, some bacterial strains produced up to 10–30 μg/ml when they were cultivated in nutrient broth medium. The amounts of produced IAA were much lower in mineral Czapek medium (about 0.3–6.6 μg IAA/ml), indicating that there is a lack of substances needed for auxin biosynthesis. Strains belonging to one particular genus differed significantly (3–58 μg/ml) in amounts of produced auxin (data for selected strains are performed in Table 11.4); this corresponds to the previously published data on variations in IAA production within the strains of the genus *Pseudomonas* (Mordukhova et al. 1991; Olyunina and Shabaev 1996). The most active orchid-associated IAA producers were also found among *Pseudomonas* strains, although *Rhodococcus*, *Rhizobium*, *Microbacterium*, *Sphingomonas*, *Bacillus*, *Mycobacterium*, and *Xanthomonas* produced high amounts of auxin as well (Table 11.4).

The amino acid tryptophan (Trp) is known to enhance considerably microbial IAA yield in vitro (Ivanova et al. 2001; Halda-Alija 2003; Khalid et al. 2004; Spaepen et al. 2007). It is also believed that root exudates of higher plants supply the rhizosphere with Trp, which may undergo further conversion into IAA by plant-associated bacteria (Jaeger et al. 1999; Asghar et al. 2002; Kravchenko et al. 2004;

Table 11.4 Microbial auxin production by selected orchid-associated bacteria as dependent on tryptophan concentration in Czapek medium (originally published in Tsavkelova et al. 2005)

Orchid	Bacterial culture	Tryptophan (μg/ml)	IAA (μg/ml)	Maximal increase of IAA biosynthesis, times
D. moschatum	*Sphingomonas* sp. 18	0	1.8[a]	28
		50	17.4	
		100	38.4	
		200	50.2	
	Microbacterium sp. 23	0	2.5	21
		50	3.1	
		100	14.6	
		200	53.1	
	Mycobacterium sp. 1	0	3.1	30
		50	34.0	
		100	86.1	
		200	92.9	
	Bacillus sp. 3	0	6.6	6
		50	13.2	
		100	28.4	
		200	37.6	
	Rhizobium sp. 5	0	1.5	34
		50	2.8	
		100	16.3	
		200	60.4	
A. papillosa	*Sphingomonas* sp. 42	0	1.1	63
		50	17.5	
		100	43.4	
		200	69.4	
	Rhodococcus sp. 37	0	0.4	124
		50	6.2	
		100	22.7	
		200	49.6	
	Cellulomonas sp. 23	0	1.0	54
		50	19.9	
		100	35.6	
		200	53.9	
	Pseudomonas sp. 24	0	0.3	103
		50	14.2	
		100	21.2	
		200	31.0	
	Bacillus sp. 12	0	1.9	4
		50	1.9	
		100	3.7	
		200	8.4	
	Micrococcus sp.	0	1.0	39
		50	13.4	
		100	25.3	
		200	39.2	

[a]Standard deviation values are ±(0.07–0.15)

Kamilova et al. 2006). Orchid rhizobacteria were also shown to converse Trp effectively into auxin (Table 11.4). Usually, IAA production by PGPR strains ranges in response to tryptophan addition from 5–10 to 100–200 μg IAA/mL and higher, depending on exogenous tryptophan concentration (Tien et al. 1979; Halda-Alija 2003; Khalid et al. 2004; Park et al. 2005; Ahmad et al. 2008). Trp significantly enhanced IAA amounts produced by orchid-associated microbial strains both in mineral and complex media. Auxin accumulation in the culture medium reached its maximum during the stationary phase of bacterial growth (Tsavkelova et al. 2005, 2007a) that has also been shown for diverse plant-associated *Pseudomonas*, *Azospirillum*, *Arthrobacter* and *Agrobacterium* strains (Tien et al. 1979; Cacciari et al. 1989; Olyunina and Shabaev 1996; Ivanova et al. 2001; Leveau and Lindow 2005). At 200 μg/ml concentration, Trp showed the most effective response; the strains isolated from roots of *D. moschatum* (*Sphingomonas* sp., *Microbacterium* sp., *Mycobacterium* sp., *Bacillus* sp., and *Rhizobium* sp.) produced 50.2, 53.1, 92.9, 37.6, and 60.4 μg IAA/ml, respectively, and bacteria isolated from *Acampe papillosa* (*Sphingomonas* sp., *Rhodococcus* sp., *Cellulomonas* sp., *Pseudomonas* sp., and *Micrococcus* sp.) produced 69.4, 49.6, 53.9, 31.0, and 39.2 μg IAA/ml, respectively. Thus, the supplementation of growth media with 200 μg/ml of exogenous Trp increased the level of produced IAA by 30–170-fold in comparison to "no Trp" cultivation media, confirming that Trp is efficiently consumed by investigated strains as an IAA precursor.

Thin layer chromatography showed the identity of microbial auxin to the authentic IAA (Tsavkelova et al. 2005). Other indole compounds related to diverse intermediates of IAA biosynthetic pathways have been also detected. Microbial production of IAA is realized via several routes (Costacurta and Vanderleyden 1995; Patten and Glick 1996; Spaepen et al. 2007), although auxin formation through indole-3-pyruvic acid (IPA), which is then converted into indole-3-acetaldehyde (IAAld) and IAA, is the primary route of auxin biosynthesis among PGPR (Koga et al. 1994; Costacurta and Vanderleyden 1995; Brandl and Lindow 1996; Ona et al. 2005; Spaepen et al. 2007). For the most active IAA-producing bacteria associated with greenhouse orchids (*Sphingomonas* sp., *Mycobacterium* sp., *Rhizobium* sp., and *Microbacterium* sp.), the auxin biosynthetic routes have been studied by using HPLC analysis (Tsavkelova et al. 2007b). IAAld, the direct precursor of IAA, has been identified in all investigated strains, whereas IPA and/or indole-lactic acid (ILA) have been detected only in *Sphingomonas* sp., *Rhizobium* sp., and *Microbacterium* sp. strains, thus indicating the activity of IPA pathway. Another intermediate, indole-3-acetamide (IAM), has been found in *Mycobacterium* sp., confirming the presence of the IAM biosynthetic pathway (Fig. 11.17). This pathway is believed to be the major IAA-forming route in phytopathogenic bacteria of *Agrobacterium*, *Pseudomonas,* and *Erwinia* genera (Weiler and Schröder 1987; Costacurta and Vanderleyden 1995; Glickmann et al. 1998; Manulis et al. 1998; Patten and Glick 1996, 2002; Spaepen et al. 2007). At the same time, this substance has also been reported in *Azospirillum brasilense* and certain strains of streptomycetes and methylobacteria (Magie et al. 1963; Manulis et al. 1994, 1998; Bar and Okon 1993; Ivanova et al. 2001). The genes controlling

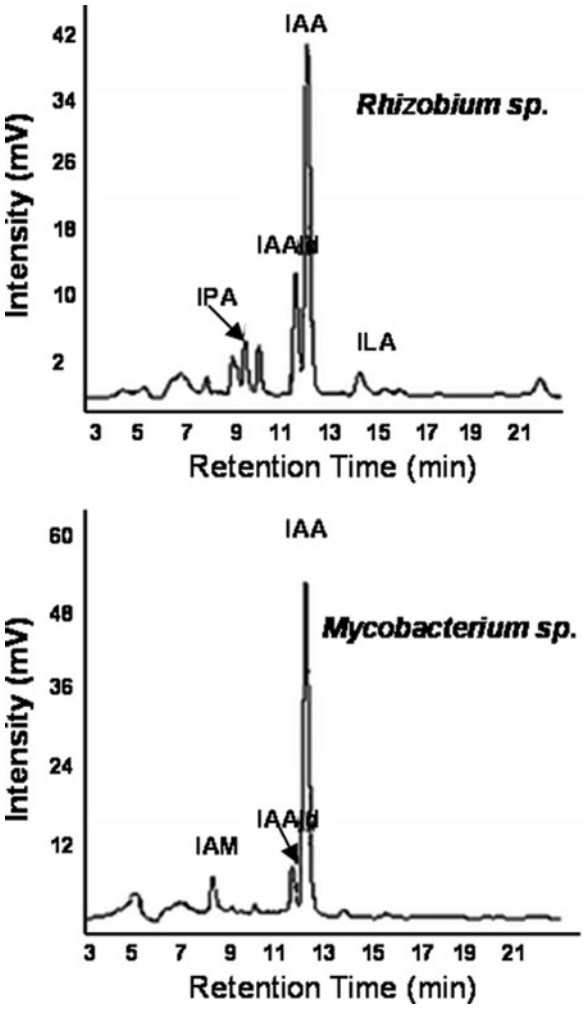

Fig. 11.17 HPLC analysis of indolic compounds in the supernatants of orchid-associated *Rhizobium* sp. and *Mycobacterium* sp. growing in mineral Czapek medium. *IAA* indole-3-acetic acid, *IAAld* indole-3-acetaldehyde, *IPA* indole-3-pyruvic acid, *ILA* indole-3-lactic acid (originally published in Tsavkelova et al. 2007b)

IAA synthesis through IAM were also detected in symbiotic *Rhizobium* sp. and *Bradyrhizobium* sp., although the activity of the corresponding enzymes was negligible (Sekine et al. 1989; Costacurta and Vanderleyden 1995). The absence of indole-3-acetonitrile (IAN) suggests that the IAN pathway is not operative in the studied orchid-associated bacteria (Tsavkelova et al. 2007b).

The strains of *Bradyrhizobium*, *Pseudomonas*, *Alcaligenes,* and *Agrobacterium* genera are also recognized as effective IAA degraders (Libbert and Risch 1969; Egebo et al. 1991; Jensen et al. 1995; Leveau and Lindow 2005). It is believed that the IAA degraders are abolishing the harmful auxin effects on plants, when IAA is overproduced by plant pathogens (Jensen et al. 1995; Leveau and Lindow 2005). Rhizobacteria can combine IAA production and its degradation, although IAA consumption depends on the medium composition and growth stage of microbial

culture (Libbert and Risch 1969; Leveau and Lindow 2005). In order to verify whether the selected strains of *Sphingomonas* sp., *Mycobacterium* sp., *Rhizobium* sp., and *Microbacterium* sp. were able to degrade exogenous IAA, they were cultivated in both complete nutrient and minimal media with glucose supplementation decreased to (1) 10 μg/ml, (2) 1.5 μg/ml, and (3) 0 μg/ml (Tsavkelova et al. 2007b). However, not a single strain showed any significant reduction in IAA content; bacterial growth was not supported by exogenous IAA as the only source of carbon and energy. The investigated strains did not utilize IAA as a nutrient substrate. Moreover, some bacteria augmented the auxin content in the cultivation media by additional excretion of newly produced IAA. It demonstrates that exogenous IAA might act as an autoinducer of microbial IAA biosynthesis. Previously, Vande Broek et al. (1999) reported that exogenous IAA induced transcription of the *Azospirillum brasilense ipdC* gene, encoding indole-3-pyruvate decarboxylase, involved in IPA-biosynthetic pathway of IAA.

In order to determine an influence of auxin on IAA-producing microbes, orchid-associated bacteria were treated with different amounts of exogenous IAA (Tsavkelova et al. 2007b). Auxin is believed to have the prominent effects on root formation in 10–100 μg/ml range; these concentrations have been tested and shown to be the most effective in stimulation of the microbial growth. Thus, 10 μg IAA/ml revealed the maximal beneficial influence on growth and development of *Microbacterium* sp. and *Rhizobium* sp. strains, and the growth of *Sphingomonas* sp. and *Mycobacterium* sp. cultures was significantly stimulated by exogenous IAA in concentration of 100 μg/ml (Fig. 11.18). The best results have been observed for

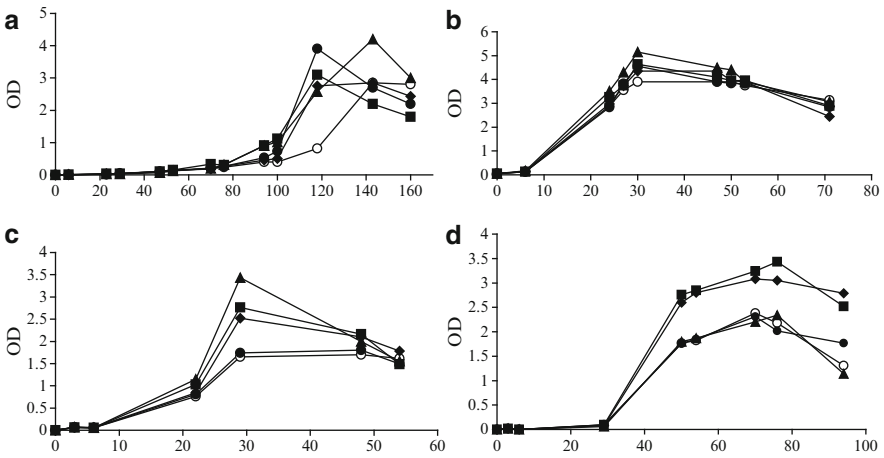

Fig. 11.18 IAA effects on bacterial biomass accumulation in mineral Czapek medium: *a* as *Mycobacterium* sp., *b* as *Sphingomonas* sp., *c* as *Rhizobium* sp., *d* as *Microbacterium* sp. Symbols are: *open circle* bacterial growth in the absence of IAA; *filled circle* in the presence of 0.1 g IAA/ml; *filled diamond* in the presence of 1.0 g IAA/ml; *filled square* in the presence of 10.0 g IAA/ml; *filled triangle* in the presence of 100.0 g IAA/ml. Data shown are mean of three independent experiments. Variations within the experiments are less than 10% (originally published in Tsavkelova et al. 2007b)

Rhizobium sp., which increased its biomass production two times in comparison to auxin-untreated culture. It has been also noted that IAA showed the most stimulating effects in exponential phase of bacterial growth, shortening the initial lag- and stationary phases. Previously (Berliner 1981), it has been demonstrated that exogenous IAA decreased and eliminated the lag phase of *Cosmarium botrytis*. It seems likely that IAA promotes development of "auxin-sensitive" plant-associated bacteria, so they could gain an advantage in inhabiting the rhizosphere and rhizoplane. Besides, the fact that IAA stimulates the microbial growth without being used as a nutrient source of carbon and energy suggests that it may act as a hormone substance.

Resident PGPR strains are widely applied in cultivation of agricultural, industrial, and ornamental plants to stimulate seed germination, root formation, and improve plant growth, development and productivity (Tien et al. 1979; Noel et al. 1996; Dileep et al. 1998; Eneba et al. 1998; Belimov et al. 1999; Khalid et al. 2004; Yasmin et al. 2004; Kamilova et al. 2006; Mehnaz and Lazarovits 2006; Ferreira et al. 2008; Ahmad et al. 2008; Deepa et al. 2010). The tiny orchid seeds require different combinations of diverse plant growth stimulators and/or the presence of mycorrhizal fungi for initiation of germination (Burgeff 1959; Clements 1988). The supplementation of the nutrient medium with plant and fungal extracts sometimes replaces the need for the orchid's symbiotic partner (Fonnesbech 1972; Hadley and Harvais 1968). However, this in vitro germination technique still remains a labor-intensive and not always a successful process. The first attempt on the orchid seed bacterization was made by Lewis Knudson in 1922 (Knudson, 1922). He reported on successful inoculation of the seeds of *Epidendron* and *Laelia-Cattleya* epiphytic orchids with a root-nodule bacterium, *Bacillus radicicola* (*Rhizobium leguminosarum*), originally isolated from alfalfa; more recent studies showed that orchid seed germination is also promoted by co-inoculation with a mycorrhizal fungus and orchid-associated bacteria (Wilkinson et al. 1989, 1994a). The authors suggest that the ability to produce auxin is essential for selected strains to bring the favorable impact into orchid germination; *Pseudomonas putida*, *Bacillus cereus*, *B. sphaericus*, and *Xanthomonas maltophilia* jointly with the symbiotic fungus, were able to promote germination of *Pterostylis vittata* seeds (Wilkinson et al. 1989, 1994a). Auxins, in comparison to other known plant growth stimulators, are considered of the most importance for orchid seed germination (Hadley and Harvais 1968).

Bacteria isolated from greenhouse orchids were also shown to be active in stimulating plant development by promoting root formation and increase the number of roots (Tsavkelova et al. 2005, 2007a). Treatment of the kidney bean cuttings with bacterial filtrates of several PGPR strains significantly simulated the adventitious root growth, and no inhibiting or suppressing effects have been observed (Table 11.5 and Fig. 11.19). The strains that showed the most beneficial effect on the kidney beans were taken for the orchid symbiotic seed germination assays. Originally isolated from the roots of epiphytic *D. moschatum*, *Rhizobium* sp., *Sphingomonas* sp., and *Mycobacterium* sp. were among the most active and stable IAA producers. They were studied for the ability to promote germination and further development of the orchid seeds under the artificial conditions of the

Table 11.5 Effects of selected bacterial culture supernatants on root formation of kidney bean cuttings (originally published in Tsavkelova et al. 2005, 2007a)

Bacteria associated with greenhouse orchids (Tsavkelova et al. 2005)			Bacteria associated with wild grown orchids (Tsavkelova et al. 2007a)		
Test variant	Height of root formation (mm)	Root's number	Test variant	Height of root formation (mm)	Root's number
Rhizobium sp. K2	63 ± 3.4	98 ± 4.0	*Burkholderia* sp. 1	48 ± 4.6	56 ± 6.2
Rhizobium sp. 5	57 ± 3.2	64 ± 4.0	*Erwinia* sp. 7	46 ± 5.8	28 ± 5.2
Sphingomonas sp. 42	70 ± 3.8	68 ± 2.0	*Bacillus* sp. 8	42 ± 2.5	38 ± 5.0
Sphingomonas sp. 18	67 ± 3.4	82 ± 2.0	*Bacillus* sp. 3	51 ± 3.8	56 ± 4.5
M. luteus	66 ± 3.1	62 ± 3.0	*Bacillus* sp. 21	50 ± 4.2	54 ± 6.2
Microbacterium sp. 37	46 ± 2.5	44 ± 3.0	*Chryseobacterium* sp. 15	41 ± 3.5	40 ± 5.0
Rhodococcus sp. 23	62 ± 3.5	20 ± 4.0	*Pseudomonas* sp. 10	53 ± 2.8	48 ± 6.2
Cellulomonas sp. 23	58 ± 3.4	54 ± 3.0	*Stenotrophomonas* sp. 13	53 ± 4.2	24 ± 2.8
Bacillus sp. 3	60 ± 2.9	63 ± 2.0	*Erwinia* sp. 17	21 ± 2.5	18 ± 3.2
Nocardia sp. K5	37 ± 1.8	64 ± 4.0	*Pseudomonas* sp. 20	45 ± 3.0	52 ± 8.0
Mycobacterium sp. 1	47 ± 3.4	78 ± 3.0	*Flavobacterium* sp. 11	51 ± 5.0	33 ± 4.0
Pseudomonas sp. 24	57 ± 3.2	69 ± 4.0	*Flavobacterium* sp. 22	42 ± 3.5	34 ± 3.6
Bacillus sp. 12	35 ± 2.2	46 ± 2.0	*Agrobacterium* sp. 16	33 ± 4.7	28 ± 4.7
Streptomyces sp. 32	46 ± 2.5	69 ± 3.0			
Streptomyces sp. 40	42 ± 2.6	65 ± 4.0			
IAA (50 μg/ml)	57 ± 2.7	77 ± 4.0	IAA (50 μg/ml)	57 ± 2.7	65 ± 5.5
Tap water	5 ± 0.45[a]	5 ± 2.0	Tap water	6 ± 1.5[a]	14 ± 5.0
Czapek medium	5 ± 0.45[b]	5 ± 2.0	Czapek media	4 ± 1.6[b]	11 ± 4.2

Values are the means of three replicates ± SD

[a]Control treatment

[b]Not significantly different from the control ($p \leq 0.05$); other values are significantly different ($p \leq 0.05$) from the control

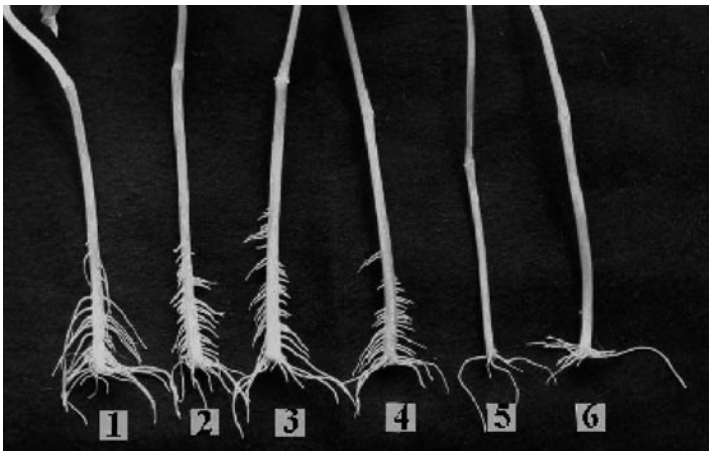

Fig. 11.19 Bioassay with the kidney bean cuttings; adventitious root formation under treatment of: (1) 50 μg/ml of IAA (auxin); (2) supernatant of *Rhizobium* sp.; (3) supernatant of *Sphingomonas* sp.; (4) supernatant of *Mycobacterium* sp.; (5) tap water; and (6) mineral Czapek medium. Data from five replicates of several independent experiments

greenhouse (Tsavkelova et al. 2007b). It turned out that no germination happened under treatment with *Rhizobium* sp. producing abundant extracellular matrix, which covered the seeds with thick (up to 3 mm) layer mucus. Two other bacteria perfectly initiated germination of *D. moschatum* seeds that resulted in seed swelling, protocorm formation, and its subsequent growth by accelerating both cell enlargement and cell division, favored in shoot bud induction and rhizome enlargement. *Sphingomonas* sp. was the most effective in symbiotic orchid propagation; more than 10% of the seeds germinated and the juvenile plants formed the chlorophyll-containing leaves. By contrast, no germination occurred in the control samples (without bacterial inoculation). Another strain, *Bacillus* sp., originally isolated from *Dendrobium leonis* (Lindl.) Rchb., has been also shown to significantly promote symbiotic germination and development of diverse epiphytic and terrestrial orchids of *Dendrobium*, *Paphiopedilum*, *Ponthieva*, and *Dactylorhiza* genera under greenhouse conditions (Kolomeitseva et al. 2002). These data on in vitro orchid germination, which takes place in the absence of mycorrhizal fungus or any of additional exogenous plant growth regulators, confirm a successful application of PGPR in orchid germination and propagation. It also shows the significant role of orchid-associated bacteria to promote the development of the endosperm-lacking orchid seeds under non-mycorrhiza-assisted conditions. Thus, treatment of the orchid seeds with IAA-producing microbial strains is helpful and advantageous for orchid propagation in vitro under artificial conditions. Associative bacteria may use their ability to produce and release auxin as a strategy to influence and trigger diverse philological processes in orchids, thus successfully affecting plant growth and development.

11.3 Conclusion

The orchid rhizoplane is well populated by different phototrophic and heterotrophic microorganisms. The spongy layers of the velamen provide a favorable econiche for various "satellite" bacteria. Microbial diversity depends on the environmental conditions in which plant is grown (wild grown environment versus the greenhouses) and on the plant ecotype (terrestrial and epiphytic orchids). Even within one epiphytic plant, the microbial consortium of its aerial and substrate roots differs considerably; the bacterial population of the aerial rhizoplane is much more intense than that of the substrate roots. Nevertheless, the common species of PGPR, such as *Bacillus*, *Burkholderia*, *Flavobacterium*, *Pantoea*, *Pseudomonas*, and *Stenotrophomonas* are among the most popular strains isolated from both greenhouse and wild grown orchids. The specific characteristic of epiphytic plants is that their aerial roots harbor nitrogen-fixing cyanobacteria, which form a sheath-like covering under conditions of constant high humidity and warm temperatures. The ability of nitrogen fixation by associative cyanobacteria is essential to support and improve plant growth under nitrogen-limited conditions. Cyanobacterial community also provides nutrients for other members of microbial community of rhizoplane, such as bacteria and mycorrhizal fungi. Another characteristic of orchid PGPR is that they are active in auxin production, although the strains vary in amounts of produced IAA. Regardless the functional pathways of auxin production, all investigated bacteria performed tryptophan-dependent IAA biosynthesis. Since the resident PGPR strains promote orchid seed germination, symbiotic bacterization might be considered as an additional method for orchid in vitro propagation.

Orchids are known to produce a number of antimicrobials, phytoalexins, and related substances that inhibit a number of fungi and bacteria (Takagi et al. 1983; Stoessl and Arditti 1984; Yamaki et al. 1993). It is believed that a continuous production of reasonable amount of degradable phytoalexin(s) is optimal in that it would keep the fungal infection functional without damaging the fungus, and the host plant would not be parasitized either (Fisch et al. 1973). In one turns, orchid endophytic fungi have also been reported to possess strong antibacterial activity and produce compounds that repress bacterial growth (Vaz et al. 2009), whereas plant-associated rhizobacteria display wide range of antifungal activity and antibiosis towards different pathogenic fungi (Ayyadurai et al. 2006; Recep et al. 2009). It may therefore be concluded that the complex interactions between host orchid and its associative microorganisms are directed to selection of the most beneficial partners that is resulted in the formation of a stable community; its functional activity guarantees the nutrient supply and better adaptation of the members of such consortium to the environmental conditions.

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Chapter 12
Diversity and Beneficial Interactions Among *Methylobacterium* and Plants

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12.1 Introduction

Environmental factors controlling biological methanol oxidation are not well understood, as this process is mediated by microbial communities consisting of multiple species besides abiotic and biotic factors which set limits for the activity and growth of each species (Kolb 2009). Terrestrial ecosystems are the main source of atmospheric methanol. Methanol contributes to the atmospheric pool of reactive volatile organic compounds (Galbally and Kirstine 2002) and triggers the formation of tropospheric ozone (Wennberg et al. 1998). Globally, 25×10^{12} mol/year of methanol is released from dead plant materials and 3×10^{12} mol/year from growing plants. However, only a minor fraction, i.e., 4.9×10^{12} mol/year enters the atmosphere. The gap between production and emission may be due to biological methanol oxidation in terrestrial ecosystems. Conversely, the primary source of atmospheric methanol is the emission from plants (Galbally and Kirstine 2002), i.e., 67% of global annual production. Methanol from plants is released by chemical and enzymatic demethylation of methoxy groups wherein plant cell wall-associated pectin polymers are demethylated during plant growth (Fall and Benson 1996) also during the degradation of pectin (Donnelly and Dagley 1980; Schink and Zeikus 1980; Schink et al. 1981) and lignin (Warneke et al. 1999). Pectin methylesterases (PMEs, EC 3.1.1.11) are important cell wall enzymes involved in modeling the pectin matrix. Pectin is a major compound of plant cell walls and accounts for about one-third of all the macromolecules in dicotyledonous species. PMEs catalyze the C-6 demethylation of homoglacturonan (HG) within plant cell walls. In this process, methanol (MeOH) is released and negatively charged carboxyl groups are created (Körner et al. 2009).

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In soils, methanol concentrations could be spatially and heterogeneously distributed. In proximity to plant material, hot spots of methanol might exist that are not detectable in mixtures with bulk soil due to dilution effect (Conrad and Claus 2005). In spite of our knowledge of the mechanism of methanol production and consumption in soils, there still are no biogeochemical data on soil internal production and consumption rates. Hence, global soil emission rates in relation to the aboveground emission from plants are not available till date. Global methanol emission from terrestrial ecosystems is similar to fluxes of some other atmospheric chemistry relevant monocarbon (C_1) compounds (4.9×10^{12} mol/year). Wetlands usually are the sources and aerated soils the sinks. A significant part of the emitted methane is degraded in the atmosphere (Denman et al. 2007). The natural production of halomethanes in terrestrial ecosystems is associated with demethylation of plant polymers. These compounds cause depletion of ozone in the upper troposphere and stratosphere (Hines et al. 1998; Keppler et al. 2000; Redeker et al. 2000; Warwick et al. 2006; Rhew and Abel 2007). There are no validated global estimates because global rates are insufficiently assessed to date. Currently available data on single compounds, i.e., methylbromide (Hines et al. 1998) suggest that fluxes of halomethanes are much lower than those of methane and methanol ($<0.01 \times 10^{12}$ mol/year) (Kolb 2009).

It is well known that methanol is a key substrate for many methylotrophic species, which is probably also true for those in soils. This point of view is supported by the fact that most of the soil-derived aerobic methylotrophic isolates (83%) utilize methanol. It is not yet clear as it how many of the species of methylotrophs are capable of utilizing methanol in situ. The growth-positive concentrations tested were in the millimolar range, whereas in situ concentrations are probably much lower (<10 mmol/L, Conrad and Claus 2005). Nonetheless, very low Km values for methanol dehydrogenase of single species, e.g., *Hyphomicrobium denitrificans* (0.3 mmol/L), suggest that at least some known soil isolates can thrive under micro- and nanomolar concentrations (Kolb 2009).

Soil-derived methanol oxidizers are primarily facultative methylotrophs. Only 31% of soil species are restricted to methanol and/or other C1 compounds. Bacteria of the genus *Methylobacterium* are facultative methylotrophs that are capable of growth on methanol and methylamine as well as C_2, C_3, and C_4 compounds. They belong to the Alphaproteobacteria and are sometimes referred to as pink-pigmented facultative methylotrophs. *Methylobacterium* strains are widespread in the environment and have been isolated from soils, dust, and lake sediments. They have also been found in association with plants, specifically with leaf surfaces, and they have been hypothesized to potentially dominate the phyllosphere bacterial population. Some *Methylobacterium* strains possess nitrogen-fixing and nodulation capabilities, which they use in symbiosis with *Crotalaria* and *Lotononis* plant species. Recently published data suggest that the degrees of plant–*Methylobacterium* association vary from very strong, as exemplified by symbiosis, to semi-tight, as exemplified by endophytic association, to loose, and epiphytic association on plant surfaces. In the case of symbiosis, the benefit for the plant is evident, in contrast to the looser forms of association between methylotrophs and plants. Recent studies have been carried

out on plant–*Methylobacterium* interactions including early growth promotion, induce systemic resistance, photosynthetic activity, heavy metal resistance, etc. Presented chapter will focus on the current status of methanol-oxidizing microbes, diversity and their beneficial interactions with associated plants. This will also address some of these arising issues as well as the established facts of methylotrophic microorganisms, diversity of methylotrophs and their interaction with plants and what these processes imply at both the plant and ecosystem level.

12.2 Methanol-Oxidizing Microorganisms

Methanol-oxidizing microorganisms utilize methanol as a carbon and energy source (Lidstrom 2006). Most known species are facultative methylotrophic and strictly aerobic, and belong to the domains Bacteria and Eukaryota. Little is known about eukaryotic methanol oxidizers in soils. Some methanol-utilizing yeasts have been isolated, i.e., *Pichia pastoris*, *Hansensula polymorpha*, *Candida* spp., and *Trichsporon* spp. (Lee and Komagata 1980; Kaszaki et al. 2006; Ito et al. 2007). However, their relevance for the aerobic conversion of C_1 compounds in soils is largely unknown and needs further research. In addition to aerobic methanol oxidizers, some strict anaerobes also utilize methanol, e.g., *Moorella mulderi* (Balk et al. 2003) utilizes methanol as a substrate with thiosulfate as an electron acceptor. Similarly, *Thermotoga lettingae* (Balk et al. 2002) oxidizes methanol as an electron donor with thiosulfate, ferric iron, or elemental sulfur as electron acceptors. Anaerobic, methanogenic Archaea may also utilize methanol (Ferry 1999) and might be important methanol consumers in wetlands (Conrad and Claus 2005; Zhang et al. 2008).

Aerobic methanol-oxidizing Archaea have yet not been discovered. Thus, in aerated soils (e.g., forest and grassland soils) and at oxic–anoxic interfaces of wetland soils, aerobic methylotrophic bacteria are the key organisms in methanol turnover. The first aerobic methanol-utilizing bacterium *Bacillus methylicus* was described in the year by Loew (1892), and many novel species have been isolated since then (Table 12.1). Methylotrophic bacteria are divided into methane-utilizing, i.e., methanotrophs and other methylotrophic species. Since the nineteenth century, 154 species with 58 genera within the Proteobacteria, Verrucomicrobia, Cytophagales, Bacteroidetes, Firmicutes, and Actinobacteria have been described. Within this group, 46 methane-utilizing species within 18 genera belonging to the Gamma- and Alphaproteobacteria, and Verrucomicrobia are known till date. Methanotrophs are principally obligate methylotrophic, only one genus (*Methylocella*) comprises three species that can also utilize multi-carbon compounds (Dedysh et al. 2005a, b). In culture, methanotrophs possess the physiological potential to grow on methanol, and labeling of bacteria in soil samples with ^{13}C from ^{13}C-methanol revealed methanotrophic genotypes (Radajewski et al. 2002). This observation revealed that methanotrophs may consume methanol from soil, if it is present in millimolar concentrations. However, in situ relevance of these observations needs

Table 12.1 List of methylotrophic bacteria (Kolb 2009)

Genus	Species	Type strain	Source
Methanol-utilizing methylotrophic bacteria			
Gammaproteobacteria			
Methylomicrobium	*album*	VKM-BG8	Soil
	alcaliphilum	20Z	Soda lake
	agile	NCIMB11124	Sewage
	japanense	NI	Marine
	kenyense	AMO1	Soda lake
	buryatense	VKM B-2245	Soda lake
	pelagicum	NCIMB2265	Marine
Methylobacter	*luteus*	VKM-53B	Sewage
	marinus	ACM4717	Marine
	psychrophilus	VKM B-2103	?
	tundripaludum	SV96	Soil
	whittenburyi	NCIMB11128	?
Methylomonas	*aurantiaca*	UQM3406	Soil
	fodinarum	UQM3268	Sediment
	methanica	VKM B-2110	Soil
	scandinavica	strain SR5	Ground water
Methylococcus	*capsulatus*	UNIQEM1	Soil, water
	chroococcus	Strain9	?
	mobilis	LMD77.28	?
	themophilus	IMV-2Yu	Soil
Methylohalobius	*crimeensis*	10Ki, 4Kr	Sediment
Methylosarcina	*fibrata*	AML-C10	Soil
	lacus	LW14	Lake
	quisquiliarum	AML-D4	Soil
Methylosphaera	*hansonii*	ACAM549	Marine
Methylosoma	*difficile*	LC2	Sediment
Methylothermus	*thermalis*	MYHT	Hot spring
Clonothrix	*fusca" B*		Drinking water
Crenothrix	*polyspora" B*		Drinking water
Photobacterium	*indicum*	ATCC19614	Marine sediment
Methylophaga	*alcalica*	M39	Soda lake
	aminisulfidivorans	MP	Marine
	marina	ATCC35842	Marine
	thalassica	ATCC33146	Marine
	sulfidovorans	LMD95.210	Marine
Methylohalomonas	*lacus*	HMT1	Lake
Methylonatrum	*kenyense*		Soda lake
Klebsiella	*oxytoca*	MMA/F	River
Enterobacter	*arachidis* sp. nov.		Rhizosphere soil
Betaproteobacteria			
Methylophilus	*leisingerii*	DM11	?
	methylotrophus	AS1	Activated sludge
	quaylei	MT	Air
Methylobacillus	*pratensis*	F31	Grass

(*continued*)

Table 12.1 (continued)

Genus	Species	Type strain	Source
	flagellatus	KT	?
	glycogenes	TK0113	Soil
Methylovorus	*mays*	C	Soil
	glucosotrophus	6B1	Waste water
Methyloversatilis	*universalis*	FAM5	Lake
Methylibium	*aquaticum*	MCC1728	Fresh water pond
	petroleiphilum	PM1	compost biofilter
Alphaproteobacteria			
Methylocystis	*echninoides*	IMET10491	Soil
	heyeri	H2	Soil
	hirsuta	CSC1	Ground water
	parvus	ACM3309	Soil
	rosea	SV97	Soil
Methylosinus	*sporium*	ACM3306	Soil
	trichsporium	OB3b	Soil
Methylocapsa	*acidiphila*	B2	Soil
Methylocella	*palustris*	K	Soil
	silvestris	BL2	Soil
	tundrae	T4, Ch1, Y1	Soil
Paracoccus	*alkenifer*	A901/1	Biofilter
	alcaliphilus	TK1015	Soil
	versutus	VKM B-2163	Soil
	methylutens	DM12	Ground water
	denitrificans	NCCB22021	Soil
	kondratievae	GB	Soil
	pantotrophus	Soil	
Sagittula	*stellata*	E-37	Salt marsh
Xanthobacter	*autotrophicus*	ATCC35674	Soil
	flavus	301	Soil
	agilis	SA35	Water
	viscosus	7d	Activated sludge
Afipia	*felis*	RD1	Soil, sediment
Rhodobacter	*capsulatus*	Kb1	Marine
	sphaeroides	ATH2.4.1	Marine
Xanthomonas	*campestris*	ATCC33913	*Brassica campestris*
Bradyrhizobium	*japonicum*	USDA110	Soil
Mesorhizobium	*loti*	??	Soil
Azorhizobium	*caulinodans*	ORS571	Soil
EnsiferF	*meliloti*	RCR2011	Soil
	fredii	PRC205	Soil
Rhodoblastus	*acidophilus*	M402	Hot spring
	sphagnicola	RS	Soil
Ancylobacter	*natronum*	VKM B-2242	Soda lake
Hyphomicrobium	*chloromethanicum*	CM2	Soil
	denitrificans	TK0415	Soil
	coagulans	B-2176	Soil

(*continued*)

Table 12.1 (continued)

Genus	Species	Type strain	Source
	sulfonivorans	BAA-113	Soil
	facile	IFAM H-526	Soil
	vulgare	TK0401	Soil
	aestuarii	ATCC27497	River
	zavarzinii	DSM1566	Soil
	hollandicum	ATCC27500	Sewage
	methylovorum	TK0412	Soil
Angulomicrobium	*tetraedrale*	1037	Fresh water
Marinosulfonomonas	*methylovora*	PSCH4	Marine water
Methylorhabdus	*multivorans*	DM13	Ground water
Methylobacterium	*persinicum*	002-165	Water
	komagatae	002-079	Water
	brachiatum	B0021	Water
	tardum	RB677	Water
	gregans	002-074	Water
	isbiliense	AR24	water
	populi	BJ001	Poplar tissue
	variabile	GR3	Water
	aquaticum	GR16	Water
	aminovorans	TH-15	Soil
	adhaesivum	AR27	Water
	platani	PMB02	*Platanus orientalis*
	salsuginis	MR	Marine
	hispanicum	GP34	Drinking water
	jeotgali	S2R03-9	Sea food
	mesophilicum	A47, TK0034	?
	nodulans	ORS2060	Root nodule, soil
	oryzae	CBMB20	Oryza sativa
	podarium	VKM B-2144	Human skin
	lusitanum	RXM	Sewage
	suomiense	F20	Soil
	thiocyanatum	ALL/SCN-P	Soil
	extorquens	TK1	Soil
	aerolatum	5413S-11	Air
	iners	5317S-33	Air
	radiotolerans	VKM B-2142	Oryza sativa
	organophilum	ATCC27886	?
	rhodesianum	NCIB12249	Fermentor
	rhodinum	ATCC14821	Soil
	zatmanii	ATCC43883	Fermentor
	beijerinckiamobilis	DSM2326	Soil
	fujisawaense	CBMB37	Soil
	goesingense	CBMB5	Soil
	phyllosphaerae	CBMB27	Rice phyllosphere
Methylosulfonomonas	*methylovora*	M2	Soil
Hansschlegelia	*plantiphila*	S1	Plant buds

(*continued*)

Table 12.1 (continued)

Genus	Species	Type strain	Source
Methylopila	*helvetica*	DM9	Ground water
	capsulata	37AA	?
Albibacter	*methylovorans*	DM10	Ground water
Acidomonas	*methanolica*	ATCC43581	Waste water
Sphingomonas	*melonis*	ET35	River
Firmicutes			
Bacillus	*methanolicus*	PB1	Soil
Actinobacteria			
Arthrobacter	*sulfinovorans*	ALL	Soil
	methylotrophus	TGA	Soil
Mycobacterium	*flavescens*	ATCC14474	Guinea pig
	gastri	ATCC15754	Human
	neoaurum	ATCC25795	Soil
	parafortuitum	ATCC19686	Soil
	peregrinum	ATCC14467	Soil
	phlei	ATCC11758	Grass
	smegmatis	ATCC19420	Soil
	vaccae	ATCC15483	Soil
Amycolatopsis	*methanolica*	239	Soil
Brevibacterium	*casei*	MSQ5	River
Bacteroidetes			
Flavobacterium	sp.	MMA/2,MSA/1	River
Verrucomicrobia			
Methylokorus	*infernorum*	V4	Soil
Methyloacida	*kamchatkensis*	Kam1	Hot spring
Acidimethylosilex	*fumarolicum*	SoilV	Soil
Non-methanol-utilizing methylotrophic Bacteria			
Gammaproteobacteria			
Methylocaldum	*gracile*	VKM-14L	?
	szegediense	OR2	Hot spring
	tepidum	LK6	Soil
Pseudomonas	*mendocina*	TSQ4	River
Beggiatoa	*alba*	LSUB18LD	Marine
Alphaproteobacteria			
Aminobacter	*lissarensis*	CC495	Soil
	ciceronei	IMB-1	Soil
Methylobacterium	*dichloromethanicum*	DM4	Soil
	chloromethanicum	CM4	Soil
Methyloarcula	*marina*	h1	Estuary
	terricola	h37	Soil
Leisingera	*methylohalidivorans*	MB2	Marine
Labrys	*methylaminiphilus*	JLW10	Sediment
Xanthobacter	*aminoxidans*	14a	Activated sludge
Paracoccus	*aminovorans*	VKM B-2140	Soil
	solventivorans	VKM B-2190	Soil
	aminophilus	VKM B-2141	Soil
	kocurii	VKM B-2139	Activated sludge

(*continued*)

Table 12.1 (continued)

Genus	Species	Type strain	Source
Betaproteobacteria			
Methylotenera	*mobilis*	JLW8	Lake
Burkholderia	*graminis*	H C4D1M	Soil
Xenovorans	H	LB400	Soil
Actinobacteria			
Nocardioides	sp.	SAC-4	Wood
Rhodococcus	*erythropolis*	DSQ4	River
Mycobacterium	*fluoranthenivorans*	DSQ3	River

? unknown source

to be proven. Non-methanotrophic methanol oxidizers have a primarily facultative methylotrophic lifestyle (91%, 100 species) and only 9% of species are restricted to C_1 compounds, i.e., methanol and sulfur- or nitrogen-containing C_1 compounds (species of *Methylobacillus*, *Methylophilus*, *Methylovorus*, and *Methylophaga*).

In all Gram-negative methylotrophic bacteria that have been studied, methanol oxidation is catalyzed by the pyrroloquinoline quinine (PQQ)-linked enzyme methanol dehydrogenase (MDH). This enzyme catalyzes the oxidation of methanol to formaldehyde and is distinct from the alcohol dehydrogenase of Gram-positive methylotrophic bacteria (de Vries et al., 1992) and methylotrophic yeasts (Williamson and Paquin 1987). Recently, a novel MDH was found in strains belonging to Burkholderiales (Kalyuzhnaya et al. 2008). An alternative MDH coupled to nicotinic acid amide (NAD) was discovered in *Bacillus methanolicus* (Arfman et al. 1997), and in Actinobacteria, a further methanol-oxidizing enzyme catalyzes methanol oxidation (Bystrykh et al. 1993). The gene *mxaF* encodes the PQQ-dependent MDH in many *Proteobacteria* (Lidstrom 2006). The evidence of the homologous gene mxaF0 in root-nodule inducing Alphaproteobacteria (e.g., *Bradyrhizobium japonicum*) might indicate that some root-nodule inducing species (1) are capable of utilizing methanol or (2) possessing at least structurally similar, but functionally dissimilar, enzymes. Some soil-derived *Burkholderia* species contain the *mxaF*-homologue *xoxF* (Chain et al. 2006). Its gene product might be involved in the oxidation of methanethiol (CH_3SH) during growth on dimethylsulfide (Schäfer 2007). However, its definite function is still unresolved, and conclusive experiments addressing the role of mxaF0 and *xoxF* in these two proteobacterial groups are missing, and, hence, it is unclear whether these organisms may utilize methanol.

The need to reinvestigate already isolated heterotrophic species may be emphasized by the fact that, recently, aerobic methanol utilization was proven when already described heterotrophic species such as *Beijerinckia mobilis* and *Mycobacterium* spp. were tested with methanol as a substrate. It is obvious that the utilization of methanol is widespread among heterotrophic aerobic bacteria and novel species, e.g., *Hansschlegelia plantiphila* are still being isolated (Ivanova et al. 2007). Recently, some novel methanol-utilizing bacteria *Enterobacter arachidis* sp. nov., *Methylobacterium phyllosphaerae* sp. nov., were isolated from the rhizosphere and phyllosphere (Madhaiyan et al. 2009a, b; Anandham et al. 2008;

Poonguzhali et al. 2008b). Thus, it is reasonable to expect a large unveiled diversity in the environment. Some methylotrophic bacteria do not utilize methanol (24 species in 15 genera). These organisms are obligate (e.g., *Methylotenera mobilis*) or facultative methylotrophs and may utilize dimethylsulfide, dimethylsulfoxide, halomethanes, mono-, di-, and trimethylamine, tetramethylammonium, or formamide. However, the same C_1 compounds are also used by some methanol-utilizing species (Kolb 2009, Table 12.1).

12.3 Diversity of Soil Methanol-Utilizing Bacteria

Methylotrophic communities in soils were assessed by the detection of biomarkers (McDonald et al. 2008). Most studies addressed methanotrophs and analyzed genes of the particulate or soluble methane monooxygenases, i.e., *pmoA* and *mmoX* (McDonald et al. 2008). Few studies targeted gene markers that allow for the detection of methylotrophs that do not utilize methane. Genes of the proteobacterial MDH (*mxaF*) (McDonald and Murrell 1997; Radajewski et al. 2002), tetrahydromethanopterin pathway-linked enzymes catalyze the oxidation of formaldehyde to formate in various methylotrophs (*fae*, *mtdB*, *mch*, and *fhcD*) (Kalyuzhnaya et al. 2004; Kalyuzhnaya and Chistoserdova 2005; Nercessian et al. 2005), and a gene of the chloromethane methyl transferase (*cmuA*) (Miller et al. 2004; Borodina et al. 2005) that oxidizes chloromethane have been used. A direct link between substrate assimilation and diversity provides stable isotope probing of membrane phospholipids fatty acids or nucleic acids. This approach has been applied to detect species that were actively involved in the consumption of isotope-labeled methanol and methane in soils (Bull et al. 2000; Lueders et al. 2004; Dumont et al. 2006; Chen et al. 2008; Maxfield et al. 2008). Biomarker studies assessing methanol-utilizing communities in soils confirmed the phyla-level diversity retrieved by cultivation. The known soil-retrieved methanol-utilizing species belongs to the class Proteobacteria, Verrucomicrobia, Firmicutes, and Actinobacteria (Fig. 12.1). The permanent establishment of a species in a community is based on its

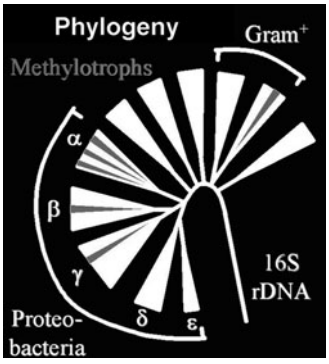

Fig. 12.1 Phylogeny of methylotrophic bacteria based on 16S rDNA analysis

fundamental ecological niche (Hutchinson 1957) that is determined by abiotic and biotic environmental factors. Environmental factors that are spatially and/or temporarily structured in soils may create habitats that favor adaptation and hence niche differentiation of soil prokaryotes. In the following sections, potential key factors of methanol-utilizing methylotrophic bacteria will be identified.

Soil-derived methanol oxidizers are primarily facultative methylotrophs. Only 31% of soil species are restricted to methanol and/or other C_1 compounds. A choice of the following soluble substrates was usually positive for growth: mono-, di-, and soluble polysaccharides (e.g., inulin, dextrin), sugar acids and polyols, primary alcohols (ethanol, butanol, and isopropanol), glycerol, amino acids, mono-, di, tricarbonic acids, aromatic, and various other nitrogen- and sulfur-containing carbon compounds (Kutschera 2007). The marine species *Sagittula stellata* is the only documented cellulolytic methanol-oxidizing methylotroph (Gonzalez et al. 1997). Because cellulose is, like pectin and lignin, an essential compound in plant cell walls, it is conceivable that cellulolytic methanol-utilizing methylotrophs may exist in soils. Facultative methylotrophic isolates have been tested for various alternative substrates, but with different spectra of carbon sources and in millimolar concentrations. In soils, however, concentrations of dissolved carbon compounds may be lower, i.e., micro- and nanomolar. Nonetheless, it has been demonstrated that species of different genera utilize very different spectra of substrates. For instance, most *Hyphomicrobium*, *Methylocella* species, and *Rhodoblastus sphagnicola* do not utilize saccharides or derived acids and polyols, whereas *Xanthobacter*, *Paracoccus*, and *Mycobacterium* species, *Beijerinckia mobilis*, *Methylosulfonomonas methylovora*, *Bacillus methanolicus*, and *Amycolatopsis methanolica* do so. Therefore, a factor that might select for co-occurrence of methanol-utilizing species in the same soil community is the different capability to utilize alternative carbon substrates.

Oxygen availability is restricted to a thin surface layer around the rhizosphere of oxygen-releasing plants in wetlands and flooded soils (Conrad 1996; Brune et al. 2000; Conrad and Frenzel 2002). In aerated soils, oxygen availability may be extended to the main soil body (Tiedje et al. 1984), nonetheless, its concentration is dynamic. Steep oxygen gradients establish over the surfaces of soil aggregates (Tiedje et al. 1984; Sexstone et al. 1985; Zausig et al. 1993; Küsel and Drake 1994), and inside, anoxic microzones may occur (Tiedje et al. 1984; Sexstone et al. 1985; Zausig et al. 1993). Variable oxygen concentration in soils might drive differential adaptation: the incubation of subsamples from the same original rice field soil sample under different oxygen concentrations led to divergent methanotrophic community structures (Henckel et al. 2000). Most known soil-derived methanol-utilizing methylotrophs grow under oxygen-saturated conditions but three are microaerophilic species, i.e., *Methylosoma difficile, Methylokorus infernorum*, and *R. sphagnicola* (Kulichevskaya et al. 2006; Dunfield et al. 2007; Rahalkar et al. 2007). Quantitative data on oxygen concentration being utilized by known species are not available in most cases. Analogous to the methanotrophic species, it seems likely that methanol-utilizing methylotrophs have different optima regarding oxygen concentration. The capability of utilizing an alternative electron acceptor

may be advantageous for certain methanol-oxidizing species in soils, because they can remain active when conditions are anoxic. Nonetheless, 75% of methylotrophic species from soil are strictly aerobic. The solely utilized external alternative electron acceptor is nitrate. Some species were identified as denitrifiers, e.g., *Paracoccus denitrificans* and *Hyphomicrobium denitrificans*. Many soil isolates have not been tested for their capability of dissimilatory nitrate reduction. Even some strictly aerobic species (e.g., methanotrophs) harbor genes of nitrite and nitric oxide reductases (Ward et al. 2004), and strains produced N_2O and nitrite when nitrate was present (Knowles 2005). However, growth on methane with denitrification has not been verified till date. In spite of obvious questions, the capability to use nitrate as an alternative electron acceptor is a key factor that determines whether a methanol-utilizing species can thrive under various redox conditions (Kolb 2009).

Similar to other microorganism, methanol-utilizing methylotrophs require nitrogen sources for growth. Most species can assimilate ammonium and nitrate. Even certain genera namely *Xanthobacter* (Jenni and Aragno 1987), *Ancylobacter* (Raj 1989), *Methylosinus*, *Methylocystis*, *Methylococcus*, *Methylocella*, and *Methylocapsa* fix dinitrogen and many strains have genes *nifH* and/or *nifD* for nitrogenase (Boulygina et al. 2002; Dedysh et al. 2004; Lidstrom 2006; Trotsenko and Murrell 2008). In nitrogen-limited forest soils (Bodelier and Laanbroek 2004), diazotrophy is advantageous for the respective species. In agricultural soils with high nitrogen input and turnover (Bodelier and Laanbroek 2004), diazotrophic species might not be favored, unless they can induce root nodules (Jourand et al. 2004; Renier et al. 2008). On the other hand, methanol-oxidizers compete for nitrate and ammonium with other soil organisms. Therefore, even in nitrogen-rich soils, N_2-fixation might be advantageous. The application of this method using unlabeled methane as the sole substrate revealed active diazotrophs of the methanotrophic genera of Methylocystaceae and methanol-utilizing of Rhizobiales (Buckley et al. 2007, 2008). $^{15}N_2$ DNA stable isotope probing is a promising tool to address, to what extent diazotrophy is present in methanol-utilizing species of soil communities. Methane-utilizing species respond differently to inorganic fertilizers containing ammonium and nitrate (Bodelier et al. 2000a, b; Gulledge et al. 2004; Seghers et al. 2005; Mohanty et al. 2006). In methane-rich soils, such as flooded rice fields, selective growth of some indigenous methanotrophic species could be stimulated by adding inorganic fertilizers (Bodelier et al. 2000b; Mohanty et al. 2006; Noll et al. 2008). These observations suggesting that methanotrophic methylotrophs have different optima of ammonium and nitrate concentration. However, different requirements for the external nitrogen source may be regarded as a general issue concerning all methylotrophs, even though ammonium is a competitive inhibitor for methane mono-oxygenases (Conrad 1996; Bodelier and Laanbroek 2004). This should be considered when conclusions from fertilizer experiments with methanotrophic communities are conveyed to methanol-utilizing communities, i.e., ammonium might have a divergent effect on methanotrophs compared with methanol-utilizing methylotrophs. The nitrate concentration may be a key factor for methanol oxidizers, and it is relevant in two aspects: (1) as an

external N-source (2) a potential alternative electron acceptor. Soil pH is an abiotic factor with an effect on prokaryotic community structure in soils (Buckley et al. 2006; Lejon et al. 2007), while 71% of soil-derived methanol-utilizing species are neutrophils, only 29% are acid tolerant, acidophilic, or alkaliphilic. The occurrence of certain methanotrophic genotypes is correlated with soil pH (Knief and Dunfield 2005; Kolb et al. 2005), and it seems reasonable to assume that soil pH may also affect the community structure of other soil methanol oxidizers.

Soil temperature differs spatially and over time. This favors different adaptations to temperature by different species of the same community. As an example, the incubation of rice field and forest soil samples (at 5–35°C) altered methane oxidation rates and also thrive the relative abundance of different species (Mohanty et al. 2007) due to divergent temperature optima. Between soils with larger mean temperature differences, one might expect even more dramatic differences in community structure. Recently, thermophilic methanotrophs affiliating with Verrucomicrobia were isolated from soils with high temperatures (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008; Semrau et al. 2008). Isolation attempts from temperate soils have not revealed methanotrophic isolates of this phylum. Both meso- and thermophilic methanol-utilizing methylotrophs namely *Methylobacterium extorquens* and *B. methanolicus* have been isolated from soil. Hence, also within the group of methylotrophs that cannot utilize methane, different adaptation to temperature occurs. Collectively, these observations indicate that temperature is an important factor for methylotroph niche differentiation, but further research is needed to draw a fruitful conclusion. In several species soil salinity concentrations are measured that a species can tolerate. The concentrations range of NaCl varies from $\leq$0.02% for *B. methanolicus* (Arfman et al. 1989) up to 5% for *Hyphomicrobium vulgare* (Hirsch, 1989). Few studies have addressed the direct interaction of eukaryotes with methylotrophs in soils. *Methylobacterium nodulans* induces root nodule formation in tropical legume *Crotolaria podocarpa* (Sy et al. 2001; Jourand et al. 2004; Renier et al. 2008). Within the root nodule, this species grows exclusively and fixes dinitrogen for its host. The mechanism of the interaction is analogous to the classical root nodule symbiosis (Sy et al. 2001). In tissues of submerged mosses (*Sphagnum cuspidatum*), an endophytic methanotroph affiliating with Alphaproteobacteria was detected (Raghoebarsing et al. 2005). A transfer of carbon between bacterium and plant suggests a close trophic link between the two partners (Raghoebarsing et al. 2005). These two types of symbiotic interactions with plants suggest that methanol-utilizing methylotrophs may be adapted to certain plant species. Grazing by protozoa is another factor that might influence soil methanol-oxidizing communities. Protozoa from rice field soil displayed different preferences for various methanotrophic species (Murase and Frenzel 2007, 2008). Predatory protozoa might have selected for species that were more protected from grazing and have the capability to control species abundances. If methanol-utilizing species in general are subjected to grazing by soil protozoa, this is speculative. Nonetheless, this finding illustrates that niche differentiation of free-living soil methanol oxidizers might be impacted by predatory protozoa (Kolb 2009).

12.4 Plant-Associated *Methylobacterium*

Plant interactions with microorganisms are well-documented phenomena. Symbiotic bacteria that inhabit the rhizosphere and form nodules on the root of legumes are able to assimilate atmospheric nitrogen and provide it to the host plant. Rhizosphere bacteria, including members of the genera *Rhizobium* and *Bradyrhizobium*, however, are dominant players involved in plant–microbe symbiosis. Many bacteria are present on the leaves and there is evidence that these inhabitants have a significant impact on plant growth and development. One such inhabitant is Pink-Pigmented Facultative Methylotrophic bacteria, or PPFMs, which are members of the Genus *Methylobacterium* and are Gram-negative alpha-*Proteobacteria*. These plant-associated bacteria are easily detected by their pink color and ability to utilize one carbon compounds, such as methanol, as sole carbon and energy sources. They are phylogenetically related to both plant-associated bacteria *Agrobacterium* and *Rhizobium* (Bratina et al. 1992) and have more recently been placed in a clade including a *Methylobacterium* strain that is able to nodulate and fix nitrogen in symbiosis with legumes (Sy et al. 2001). PPFMs have been isolated from virtually all land plants examined (Corpe 1985). Although, they do not grow as rapidly as other phylloplane bacteria on multi-carbon sources, they compete well for leaf surface colonization. Hirano and Upper (1991) measured bacterial populations on snap pea throughout a growing season and found PPFMs to be the most abundant organisms in the phylloplane microflora at each sampling date.

Utilizing mutants in the pathway for one-carbon metabolism of *Methylobacterium* together with wild type, Sy et al. (2005) demonstrated that methylotrophy is advantageous to the bacterium colonizing *Medicago truncatula* under competitive conditions. Under non-competitive conditions, these methylotrophy mutants were able to colonize the plants as well as wild type indicating that methanol is not the only carbon source available to *Methylobacterium* while it is associated with the plant (Sy et al. 2005). Populations of *Methylobacterium* on red clover in the field were shown to decrease from the spring towards summer, but then increase again towards the end of the cropping season (Omer et al. 2004). PPFMs, however, are not limited to the phylloplane and also associated with other parts of the plant, concentrated at the actively growing portions. Gourioin et al. (2006) harvested *Methylobacterium* from the roots and the aerial portions of inoculated plants and compared proteins that were up or down regulated during colonization with those from free-living bacteria grown on minimal medium. Among proteins induced during phyllosphere colonization was *PhyR*, a two-component response regulator that was shown to play an essential role in plant colonization. They suggested that it is part of a key regulator for adaptation to epiphytic life of *Methylobacterium* (Gourioin et al. 2006). A *PhyR* disruption mutant exhibited in vitro growth similar to the progenitor isolate. However, during colonization of *Arabadopsis*, *phyR* cell numbers were below the detection limit for 65% of the 3-week old plants. Colonization to wild type levels was restored when the *PhyR* gene was expressed in trans (Gourioin et al. 2006).

PPFMs and other commensal plant-associated bacteria differ from plant pathogens by not eliciting a hypersensitive response and not causing disease in associated tissue. Hirano and Upper (2000) have studied the various phyllosphere inhabitants and used *Pseudomonas syringae* as a model to explain the complex association of bacteria with plants. Depending on the host plant and the environmental conditions, *P. syringae* can act as an epiphyte, an ice nucleus or as a pathogen in the phyllosphere (Hirano and Upper 2000). Recently, a plant growth-promoting *Methylobacterium* isolate was shown to induce defense responses in groundnut (Madhaiyan et al. 2006). The induced systemic resistance activity in *Methylobacterium*-associated groundnut provided protection against rot pathogens suggesting that PPFMs could be useful as a means of biological control of pathogens (Madhaiyan et al. 2006). PPFMs have been studied for their stimulation of seed germination and other aspects of plant growth and development. PPFMs are seed transmitted in soybean (Holland and Polacco 1994) and have been detected intra-cellularly in Scotch pine buds by in situ hybridization (Pirttila et al. 2000).

In general, seed-associated bacteria affect germination. Klincare et al. (1971) showed a correlation between lowered populations of seed microflora and a decline in the germination rates in a variety of species. This observation led to the investigation of a possible role of PPFMs in germination. Heat-cured seeds showed decrease seed germination frequency, by 30–75% depending on seed lot that could be corrected by imbibition with PPFMs, their spent medium, or by addition of cytokinins (Holland and Polacco 1994). Since exogenous cytokinins had an effect on germination similar to PPFMs or their spent media, cytokinin production was investigated in PPFMs (Holland and Polacco 1994; Freyermuth et al. 1996). Four different leaf isolates and a type culture were shown to produce and secrete *trans*-zeatin by the way of tRNA turnover (Koenig et al. 2002). A cytokinin-null mutant (*miaA*-), however, stimulated germination of soybean seeds as well as wild-type bacteria (Koenig et al. 2002). The component(s) in PPFM spent medium were found to be associated for the germination effect. More recently Ryu et al. (2006) demonstrated the production of plant growth regulators, including the auxin indole-3-acetic acid (IAA) as well as the cytokinins *trans*-zeatin riboside (*t*-ZR), dihydrozeatin riboside (DHZR) and isopentenyladenosine (iPA), by two *Methylobacterium* isolates from rice. Inoculation of red pepper and tomato seeds with these two isolates resulted in increased germination percentage as well as increased root length compared to uninoculated controls and plants inoculated with the *miaA*-mutant described above (Ryu et al. 2006). Similarly rice seeds inoculated with these isolates exhibited both an increase in the seed germination percentage and the germination rate. Such enhancement was a result of phytohormones secreted by the PPFMs (Lee et al. 2006). Earlier, PPFMs have been shown to produce vitamin B12 (Toraya et al. 1975). In liverworts, Basile et al. (1985) showed a correlation between exogenous vitamin B12-mediated enhanced growth and development due to PPFM. *Methylobacterium* spp. isolated from *Funaria hygrometrica* were shown to cause an acceleration of bud formation and growth in the protonemata (Zabetakis 1997). Secretions of other interesting secondary metabolites by PPFMs are being reported in the literature, e.g., two character-impact

compounds of strawberry flavor, the furanones 2,5-dimethyl-4-hydroxy-2*H*-furan-3-one (DMHF) and 2,5-dimethyl-4-methoxy-2*H*-furan-3-one (mesifuran) were synthesized by strawberry tissue cultures only after being treated with *Methylobacterium extorquens*. It was demonstrated that the precursor to furanones, 2-hydroxy-propanol (lactaldehyde), was formed by the bacterial oxidation of 1,2-propanediol, present in strawberry cells (Zabetakis 1997). Sugarcane sets inoculated with *Methylobacterium* spp. showed an accelerated and high rate of germination (Madhaiyan et al. 2005). When combining seed inoculation with *Methylobacterium* spp. in sugarcane with a soil treatment and a foliar application, researchers demonstrated an increase in specific leaf area, plant height, number of internodes and cane yield (Madhaiyan et al. 2005). Foliar applications of *Methylobacterium* spp. have resulted in increased growth and yield of cotton as well (Madhaiyan et al. 2006). In vitro regenerated sunflower plantlets from excised hypocotyl segments were inoculated with *Methylobacterium* strain from a field-grown sunflower plant prior to being placed on shoot-induction medium, showed an increase in both the number of shoots and roots while having no effect on the length of the shoots (Koopman and Kutschera 2005). A PPFM strain originally isolated from contaminated rice callus stimulated the growth of recolonized rice callus (Maliti et al. 2005). This callus isolates from green leafy plants, were shown to inhibit plantlet generation in two rice cultures resulting in continual embryo-like cell proliferation (Maliti et al. 2005). However, when rice seeds were inoculated with these isolates and grown in culture, there was a significant increase in growth and development of the seedlings increased biomass, leaf development and shoot growth (Maliti et al. 2005).

12.5 ACC Deaminase Producing *Methylobacterium* and Their Effects on Plant Growth

When plants are exposed to abiotic or biotic stressed environment, they often respond by producing stress ethylene. Various plants respond differently to stress and also have a range of sensitivities to ethylene. In addition, there is a complex web of interactions between ethylene and other plant hormones that varies from one plant to another. Thus, it is difficult to explain the functioning of stress ethylene in one simple model. Nevertheless, plants exposed to various types of stress invariably exhibit increased ethylene levels, as a result to increased damage (Hyodo 1991).

In an apparent paradox, stress ethylene has been suggested to both alleviate and exacerbate some of the effects of pathogen infection, depending upon the plant species, its age and the nature of the pathogen (Abeles et al. 1992; Arshad and Frankenberger 2002; Van Loon and Glick 2004). A model that explains these seemingly contradictory effects of stress ethylene on plants has been proposed (Stearns and Glick 2003; Pierik et al. 2006; Van Loon et al. 2006). In this model, there is an initial small peak of ethylene close in time, usually a few hours after, to

the onset of the stress and then a second much larger peak that appears some time later, usually 1–3 days. The first peak is only a small fraction of the magnitude of the second peak and is thought to initiate a protective response by the plant, such as transcription of pathogenesis-related genes and acquired resistance (Ciardi et al. 2000; Van Loon and Glick 2004). The first small wave of ethylene production is thought to consume the existing pool of ACC within plant tissues (Robison et al. 2001). On the other hand, the second ethylene peak is so large that physiological processes such as senescence, chlorosis and abscission are initiated resulting in, inhibitory effect to plant survival. Thus, following a severe infection by pathogens, a large portion of the plant damage occurs due to autocatalytic ethylene synthesis and not from direct pathogen action (Van Loon 1984). In this regard, not only exogenous ethylene can increase the severity of a pathogen infection, but as well, inhibitors of ethylene synthesis or ethylene action can significantly decrease the severity of a fungal or bacterial infection. The second peak of ethylene production occurs as a consequence of increased transcription of ACC synthase genes triggered by environmental and developmental cues (Yang and Hoffman 1984).

The enzyme ACC deaminase (EC: 4.1.99.4) catalyzes the cleavage of ACC to ammonia and α-ketobutyrate was first discovered in 1978 (Honma and Shimomura 1978). This enzyme has subsequently been detected in a wide range of bacterial strains and fungi (Klee et al. 1991; Sheehy et al. 1991; Honma 1993; Jacobson et al. 1994; Campbell and Thomson 1996; Burd et al. 1998; Minami et al. 1998; Jia et al. 1999; Belimov et al. 2001; Mayak et al. 2004; Ghosh et al. 2003; Ma et al. 2003; Uchiumi et al. 2004; Belimov et al. 2005; Blaha et al. 2006; Madhaiyan et al. 2006, 2007a; Anandham et al., 2008; Poonguzhali et al. 2008a; Yim et al. 2009), and for many of these strains, the ACC deaminase gene has been isolated and characterized. The presence of ACC deaminase is relatively common amongst soil microorganisms. In one study, 27 out of 233 newly isolated *Rhizobium* spp. from various sites in southern and central Saskatchewan contained ACC deaminase activity (Duan et al. 2006). In another study, ACC deaminase activity/genes were found in a wide range of bacterial isolates including *Azospirillum*, *Rhizobium*, *Agrobacterium*, *Achromobacter*, *Burkholderia*, *Ralstonia*, *Pseudomonas,* and *Enterobacter* (Blaha et al. 2006). Moreover, 62 out of 88 *Pseudomonas* strains exhibit biocontrol activity and ACC deaminase activity (Wang et al. 2000). The association of *Methylobacterium* species with plants seems to be symbiotic in which the bacteria utilize methanol, the waste metabolite from plants and in turn produce compounds that promote plant growth. Production of plant growth hormones like indole-3-acetic acid, cytokinins, and ACC deaminase involved in modulation of ethylene levels and plant growth promotion by *Methylobacterium* have been investigated (Madhaiyan et al. 2006, 2007a, b).

ACC deaminase is a member of a large group of enzymes that require the co-factor pyridoxal 5′-phosphate for its activity (Walsh et al. 1981). These enzymes have been classified based on their three dimensional structure, into four folding types: (1) tryptophan synthase, (2) aspartate aminotransferase, (3) D-amino acid aminotransferase, and (4) alanine racemase (Jansonius 1998). According to

this classification scheme, ACC deaminase fits into the tryptophan synthase family. The coenzyme pyridoxal phosphate is a tightly bound cofactor of ACC deaminase in the amount of approximately one mole of pyridoxal phosphate per trimeric subunit (Honma 1985). Interestingly, ACC synthase also requires pyridoxal phosphate for enzyme activity.

A model is proposed by which plant growth-promoting bacteria can lower plant ethylene levels and in turn facilitate plant growth (Glick et al. 1998). In this model the plant growth-promoting bacteria bind to the surface of a plant (usually seeds or roots, although ACC deaminase-producing bacteria may also be found on leaves and flowers). In response to tryptophan and other small molecules in the plant exudates, the bacteria synthesize and secrete IAA, some of which is taken up by the plant. This IAA together with endogenous plant IAA can stimulate plant cell proliferation, plant cell elongation or induce the transcription of ACC synthase, enzyme that catalyzes the formation of ACC. Some of the ACC is exuded from seeds, roots, or leaves (Penrose et al. 2001; Grichko and Glick 2001) along with other small molecules normally present in these exudates and may be taken up by the bacteria and subsequently cleaved by the enzyme, ACC deaminase, to ammonia and α-ketobutyrate. In this model, the bacterium acts as a sink for plant ACC and as a result of lowering either the endogenous or the IAA-stimulated ACC level, the amount of ethylene in the plant is also reduced. As a direct consequence of lowering plant ethylene levels, PGP bacteria possess the enzyme ACC deaminase, reduce the extent of ethylene inhibition of plant growth following a wide range of stresses. Thus, plants grown in association with these bacteria may have longer roots and shoots and be more resistant to growth inhibition by a variety of ethylene-inducing stresses (Saleem et al. 2007).

The question arises, as to how bacterial ACC deaminase can selectively lower deleterious ethylene levels but not affect the small peak of ethylene that is thought to activate some plant defense responses. As discussed later in this chapter, ACC deaminase is generally present in bacteria at a low level until it is induced, and the induction of enzyme activity is a relatively slow and complex process. Immediately following an environmental stress, the pool of ACC in the plant is lower as is the level of ACC deaminase in the associated bacterium. Following the relatively rapid induction of a lower level of ACC oxidase in the plant, it is likely that there is increased flux through this enzyme resulting in the first small peak of ethylene which is of sufficient magnitude to induce a protective/defensive response in the plant. With time, bacterial ACC deaminase is induced (by the increasing amounts of ACC that ensues from the induction of ACC synthase in the plant) so that the magnitude of the second, deleterious, ethylene peak is decreased significantly. The second ethylene peak may be reduced dramatically, but it is never completely abolished since ACC oxidase has a much higher affinity for ACC than ACC deaminase does (Glick et al. 1998). Thus, when ACC deaminase-producing bacteria are present, ethylene levels are ultimately dependent upon the ratio of ACC oxidase to ACC deaminase (Glick et al. 1998; Glick 2005; Saleem et al. 2007).

12.6 Conclusions

In the present chapter the methylotrophy, diversity of methylotrophs and their interactions with plants have been discussed. These methylotrophs belong to the Alpha-, Beta-, and Gammaproteobacteria, Verrucomicrobia, Firmicutes, and Actinobacteria. Their ecological niches are determined by oxygen and methanol concentration, temperature, pH, the capability to utilize nitrate as an electron acceptor, and the spectrum of nitrogen sources and utilizable multi-carbon substrates. Recently, discovered interactions with eukaryotes indicate that their ecological niches may not solely be defined by physicochemical parameters. Since methylobacteria produce and secrete cytokinins and auxin, a model of plant–microbe-interaction (symbiosis) is proposed in which the methanol-consuming bacteria are viewed as coevolved partners of the gametophyte that determine its growth, survival, and reproduction (fitness). Methylobacteria are widely distributed in a variety of habitats, such as freshwater, dust, and soils. On leaf surfaces, these methanol-consuming microbes often dominate the "bacterial flora" (microbiota). Quantitative studies led to the conclusion that typically more than 80% of the viable bacteria recovered from healthy leaves are members of the genus *Methylobacterium*. Hence, there should be a futuristic approach to explore the diversity of methylotrophs and their novel traits which will contribute to improve the plant microbe symbiotic relationship.

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Chapter 13
Actinobacteria–Plant Interactions: A Boon to Agriculture

Janice L. Strap

13.1 Introduction

Without question, the control of plant diseases is essential for sustainable agriculture. The application of agrochemicals to control plant diseases is an important method in agricultural practices, but is not without complications such as environmental pollution, development of pathogen resistance, and deleterious effects on nontarget organisms (Gerhardson 2002). Growing demand for ecologically sensible agricultural practices underscores the need for new strategies to augment existing disease control practices.

Saprophytic microflora within the plant rhizosphere can be both deleterious and beneficial to plant growth and to crop yields (Burr and Caesor 1984; Frederickson and Elliott 1985; Gaskins et al. 1985; Schroth and Hancock 1982; Solans 2007; Suslow and Schroth 1982). Plant diseases caused by soil-dwelling microorganisms, especially fungi, are responsible for significant losses of agricultural crops each year. While the traditional approach to controlling the spread of diseases in agriculture has been the use of pesticides, this is a costly practice which poses serious hazards to the environment. The growing demand for alternative approaches to agricultural pest control and fertilization is fueled by the current impetus toward chemical-free agricultural products and a paucity of registered chemicals available for use (Fravel 2005). Therefore, the application of biocontrol and plant-growth-promoting microbes provides a valuable alternative to effectively reduce reliance on potentially harmful chemicals.

Microbial inoculants are a much needed addition to the arsenal required to overcome agroenvironmental problems since they can promote the health and growth of plants, improve nutrient availability and uptake, and attenuate crop

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damage by inhibiting or outcompeting phytopathogens, thereby reducing the need for agrochemicals (Barea et al. 1998; Bonfante 2003; Hodge et al. 2001; Kloepper et al. 2004). Efficient disease control, however, requires a thorough understanding of plant–microbe interactions including the complex relationships between plant pathogens and potential microbial inoculants. Details of the molecular mechanisms that govern the specificity of these interactions remain to be elucidated.

13.2 Actinomycetes

Actinomycetes are a heterogeneous and widely distributed group of bacteria in nature (Srinivasan et al. 1991), which make important contributions to nutrient cycling of natural substrates and have a role in humification (McCarthy 1987). Actinomycetes produce chemically diverse metabolites many of which exhibit antibiotic activity and plant growth regulatory activity. In addition, they produce lytic enzymes which not only facilitate their survival by degrading diverse substrates but which can inhibit the growth of phytopathogens (Gonzalez-Franco et al. 2003; Hoster et al. 2005; Singh et al. 1999). Actinomycetes, especially *Streptomyces*, can degrade diverse, recalcitrant polymers occurring naturally in plant litter and soil, including lignocelluloses (Crawford 1978; Thomas and Crawford 1998), chitin (Gonzalez-Franco et al. 2003; Krsek and Wellington 2001), and pectin (Beg et al. 2000; Hématy et al. 2009). The degradative ability of streptomycetes is important for carbon cycling in the environment and humic formation that can ultimately affect soil nutrition and plant health.

Members of the genus *Streptomyces* belong to the order *Actinomycetales* and are filamentous, Gram-positive, and soil-dwelling organisms of both industrial and agricultural significance. These bacteria undergo both chemical (antibiotic production) and morphological differentiation as part of their complex life cycle (Chater 1993). The unique growth nature of *Streptomyces*, specifically their ability to form desiccant-resistant spores, makes them ideal biological control agents since they can be formulated as dry, stable powdered products (Emmert and Handelsman 1999). Moreover, the streptomycetes are of great interest due to their ability to produce an extensive array of chemically diverse secondary metabolites that serve as commercially important compounds including antimicrobial agents, antiparasitic agents, and agriculturally important herbicides (Bibb 1996). These secondary metabolites are usually produced in a growth-phase-dependent manner (Demain and Fang 1995). The onset of morphological differentiation, which is characterized by the development of aerial hyphae and spores, is often coincidental with the onset of antibiotic production (Challis and Hopwood 2003; Champness and Chater 1994). These antibiotics may serve many functions in the rhizosphere. It has been reported, for example, that some known antibiotics can affect plant growth. Geldanamycin, a benzoquinoid ansamycin antibiotic that inhibits bacteria, protozoa, and fungi (DeBoer et al. 1970), likewise inhibits seed germination of both monocots and dicots but lacks a post emergence effect on larger seedlings (Heisey and Putnam

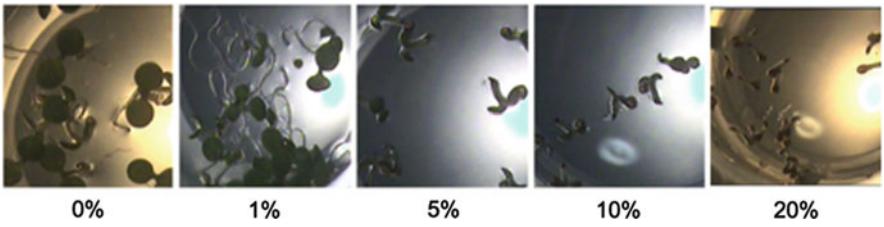

Fig. 13.1 Dose-dependent effect of a putative phytotoxin produced by a *Streptomyces* soil isolate on the growth of *Arabidopsis thaliana*. At low concentrations, the phytotoxic compound stimulates the growth of *A. thaliana*, while plant growth is severely compromised at higher concentrations (photos by J.L. Strap)

1990). Nigericin, a polyether antibiotic that inhibits select phytopathogenic fungi and Gram-positive bacteria (Harned et al. 1951), inhibits radicle growth of germinating seeds (Heisey and Putnam 1990). Interestingly, the herbicidal activity of geldanamycin and nigericin is dose dependent; specific concentrations can stimulate the growth of some plants (Heisey and Putnam 1990). This has been observed for other herbicidal compounds at subtoxic concentrations as well (Fig. 13.1).

13.3 The Rhizosphere

The rhizosphere, defined as the zone of soil directly influenced by plant roots, represents a unique biological niche within the soil environment (Lechevalier 1989). The rhizosphere supports an abundance of diverse saprophytic microorganisms able to decompose polymeric organic matter such as lignocellulose and chitin in the soil (Lynch 1990; Whipps 2001), thereby making important contributions to nutrient cycling and the formation of humic substances (Trigo and Ball 1994).

Plant root exudates directly affect microbial populations within the rhizosphere (Brown 1975; Buyer et al. 2002; Grayston and Campbell 1996; Morgan et al. 2005; Whipps 2001; Yang and Crowley 2000) by providing growth substrates such as sugars, amino acids, organic acids, fatty acids, nucleotides, sterols, vitamins, and other compounds that influence the growth of bacteria and fungi (Broeckling et al. 2008; Fischer et al. 2003; Fries and Forsman 1951; Jaeger et al. 1999; Rovira 1956; Smucker 1993; Welbaum et al. 2004; Yang and Crowley 2000). In turn, the plant is affected by the increased activity of the microorganisms in the rhizosphere. There is evidence that the variety of nutrients secreted by plant roots is both plant species and age specific, thereby influencing the diversity of microbes that inhabit the rhizosphere of specific plant species over the course of plant development (Buyer et al. 2002; Grayston and Campbell 1996; Grayston et al. 1996; King and Wallace 1956; Wallace and Lochhead 1949). The microbial flora present in the rhizosphere greatly impacts the establishment of potential phytopathogens.

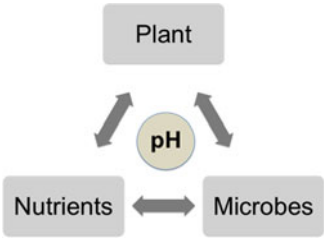

Fig. 13.2 Influence of plant root exudates on microbial communities in the rhizosphere. Plants produce exudates that not only help to solubilize essential nutrients from the soil but also serve as carbon and energy sources for microbial populations within the rhizosphere. Microorganisms that dwell in the rhizosphere in turn affect the availability of essential nutrients such as iron to the plant and to other microbes. Sequestration of bioavailable iron stores by biocontrol strains restricts the growth of phytopathogens

In the rhizosphere, the concentration of bioavailable iron is extremely low (Guerinot and Yi 1994). Plant exudates facilitate the acquisition of essential iron by acidification of the surrounding soil through excretion of protons and organic acids (Jones et al. 1996; Marschner and Romheld 1994), secretion of reductases (Marschner and Romheld 1994), and production of phytosiderophores which chelate Fe^{3+} (Takagi et al. 1984). Humic substances can also increase iron bioavailability in a pH-dependent manner (Robin et al. 2008; Weber et al. 2006). The production of plant root exudates, therefore, influences the resident microbial population capable of humic formation, which in turn affects the bioavailability of iron which subsequently influences not only plant growth but also the microbial community within the rhizosphere (Fig. 13.2).

There is still much to learn about the complex interactions between plant roots and the diverse microbes that dwell in close association with them. The extent to which microbes interact with each other and with plant roots is still relatively understudied. The reason for this lies in the inability to accurately quantify and identify the diverse microbial inhabitants of the rhizosphere.

13.3.1 Diversity in the Rhizosphere

In the past, microbes present in the rhizosphere have been investigated through isolation and cultivation from rhizosphere soils on laboratory media, which has resulted in an underestimation of diversity within rhizosphere communities due to the limited cultivability of environmental microbes. The application of molecular methods to study microbial communities has enhanced our understanding of the unculturable microorganisms within this important ecosystem. This knowledge is crucial to the successful development of actinomycetes as microbial inoculants.

Detection of the actinomycete diversity present in association with plant roots is possible through culture-independent techniques such as repetitive elements-PCR

(repPCR) (Davelos et al. 2004; Schneider and De Brujin 1996), BOX-PCR (Davelos et al. 2004; Lanoot et al. 2004), denaturing gradient gel electrophoresis (DGGE) (Basil et al. 2004; Gonzalez-Franco et al. 2009; Muyzer and Smalla 1998; Williamson et al. 2000), metagenomics (Fierer et al. 2007), use of green fluorescent protein (GFP) (Coombs and Franco 2003), and recently, genome-wide microarrays (Val et al. 2009). The application of molecular techniques in combination with the more traditional culture-based techniques is necessary to gain a better understanding of the microbial diversity and mechanisms governing microbe–microbe and plant–microbe interactions in the rhizosphere. This knowledge is essential for making informed choices for developing biocontrol agents to control phytophathogens and to overcome current limitations to field application of biocontrol agents.

13.3.2 Exudates in the Rhizosphere

As mentioned above, the rhizosphere is a unique environment, where plant exudates are an important nutrient source for root-colonizing microbes. Root exudates selectively influence the growth of bacteria and fungi that colonize the rhizosphere. They alter the chemistry of the soil within the rhizosphere by serving as a selective growth substrate for soil microbes. Since the exudates for a given plant species can be highly specific, they enrich their rhizosphere for microbes that are well adapted to the utilization of the specific organic exudates excreted by that plant (Lynch and Whipps 1990). Thus, specific bacteria that can utilize those exudates as well as antagonize pathogens are likely more abundant in the rhizosphere (Neal et al. 1973a, b). Metabolites from highly adapted root-colonizing actinomycetes are, in turn, an important source of the antibiotics, enzymes, and bioactive products involved in promoting plant growth and preventing invasion by root pathogens (Goodfellow and Williams 1983; Tokala et al. 2002).

13.4 Biological Control of Plant Diseases by Actinobacteria

Biological control of plant diseases uses microorganisms to suppress plant disease (Emmert and Handelsman 1999) and is often accompanied by plant growth promotion. Interest in the biocontrol of plant pathogens has increased considerably over the years, partly as a response to public concern about the use of hazardous chemical fungicides and pesticides. An advantage of biocontrol microbes is that they protect the plant not only from microbial threats but also from insects and weeds (Fravel 2005; Table 13.1).

The interactions between Gram-negative bacteria and plants has been extensively studied compared to plant interactions with Gram-positive bacteria, especially in the area of biocontrol. This is surprising given the ubiquitous nature of

Table 13.1 Examples of agroactive compounds produced by *Streptomyces* species

Organism	Products	Application	References
Streptomyces lydicus strain WYEC 108	Actinovate	Biocontrol	Elliott et al. (2009)
Streptomyces lydicus strain WYEC 108	Actino-Iron®	Biocontrol	Pasura (2008)
Streptomyces griseoviridis strain K61	Mycostop® WP	Biocontrol	Hamdali et al. (2008)
Streptomyces sp. A43 (FJ442821)	Non-commercial	Biocontrol	de Vasconcellos and Cardoso (2009)
Streptomyces avermitilis	Avermectin	Anthelmintic	Miller et al. (1979)
Streptomyces griseus	Streptomycin	Antimicrobial	Leben and Keitt (1954)
Streptomyces griseus IMRU 3570	Candicidin	Antifungal	Campelo and Gil (2002)
Streptomyces grieseochromogenes	Blasticidin-S	Antifungal	Dekker (1963)
Streptomyces griseoviridis	Polyene antibiotic	Fungicide	Raatikainen et al. (1994)
Streptomyces hygroscopicus	Bialaphos	Non-selective herbicide	Murakami et al. (1986)
Streptomyces hygroscopicus	Herbimycins	Herbicide	Omura et al. (1979)
Streptomyces hygroscopicus	Milbemycin	Anthelmintic	Takiguchi et al. (1980)
Streptomyces hygroscopicus	Validamycin A	Antifungal	Endo et al. (1983)
Streptomyces kasugaensis	Thiolutin	Antifungal	Sturdíková et al. (1990)
Streptomyces species No. 638	Anisomycin	Herbicide	Yamada et al. (1972)
Streptomyces thermoarchaensis	Milbemycin	Anthelmintic	Thomas et al. (1991)

actinobacteria, their ability to colonize plant root systems (Alexander 1977; Tokala et al. 2002), and their amenability to agricultural application.

While biological control of phytopathogens shows great promise under laboratory conditions, the results do not consistently translate to field conditions which typically fluctuate with season (Fravel 2005). The use of biocontrol does lend itself readily to greenhouse applications (Paulitz and Belanger 2001) since greenhouse conditions can be optimized for a specific biocontrol agent, whereas field conditions cannot be controlled (Fravel 2005).

Biocontrol of fungal plant diseases has been described using *Trichoderma* sp. (Elad et al. 1980), *Pseudomonas* sp. (Ligon et al. 2000), and *Streptomyces* sp. (Trejo-Estrada et al. 1998a). Actinomycetes, including *Streptomyces* spp., possess several qualities of effective biocontrol agents, including the ability to colonize plant root surfaces (Tokala et al. 2002), antibiosis against plant root pathogens (Basil et al. 2004; Kang et al. 2010), the synthesis of extracellular enzymes (Trejo-Estrada et al. 1998b), and the degradation of phytotoxins (Lewis and Starkey 1969).

Actinomycetes produce a large number of agroactive metabolites (Table 13.1) that play a role as biocontrol agents exhibiting antagonism against a variety of plant pathogens (Bressan 2003; Chamberlain and Crawford 1999; Tahvonen and Avikainen 1987; Trejo-Estrada et al. 1998a; Yuan and Crawford 1995). For

example, *Streptomyces nigrescens* produce phosphazomycins that exhibit in vitro activity against *Botrytis cinerea*, *Rhizoctonia solani*, and *Alternaria kikuchiana* (Tomiya et al. 1990). In addition to antifungal activity, some agroactive metabolites produced by *Streptomyces* also exhibit anthelmintic activity contributing to their biocontrol properties.

A microorganism that colonizes roots is ideal for use as a biocontrol agent against soil-borne diseases (Weller 1988). This property would ensure protection and beneficial effects throughout the growing season. Root colonization is exhibited by many *Streptomyces* (Basil et al. 2004; Crawford et al. 1993; Kortemaa et al. 1994; Tokala et al. 2002; Strap unpublished) underscoring their importance as rhizosphere microorganisms.

The genus *Streptomyces* has been investigated as a potential biocontrol agent against fungal phytopathogens such as *Pythium ultimum* (Castillo et al. 2002; Chamberlain and Crawford 1999; Crawford et al. 1993), *Sclerotinia homeocarpa* (Castillo et al. 2002; Chamberlain and Crawford 1999; Trejo-Estrada et al. 1998a), *Fusarium oxysporum* (Chamberlain and Crawford 1999), *Gaeumannomyces graminis* (Chamberlain and Crawford 1999), and *Phytophthora fragariae* (Valois et al. 1996).

Many streptomycetes have been shown to suppress fungal growth in vitro (Basil et al. 2004; Crawford et al. 1993; Kang et al. 2010; Tokala et al. 2002). In situ biocontrol by *Streptomyces* spp. has been demonstrated using the organisms themselves as well as isolated, bioactive products produced by the organisms (Smith et al. 1990).

As described above, the unique growth strategy of *Streptomyces* makes them ideally suited for use as biocontrol agents: they have the ability to colonize plant root surfaces (Barakate et al. 2002; Basil et al. 2004; Tokala et al. 2002), they exhibit antibiosis against plant root pathogens (Chamberlain and Crawford 1999), and they synthesize extracellular enzymes (Chamberlain and Crawford 2000). The mechanisms that have been proposed for the observed activities of biocontrol strains are briefly outlined below.

13.4.1 Antibiosis

The colonization of a biocontrol agent in the rhizosphere accompanied by the production of one or more substances, usually a secondary metabolite that inhibits or kills a pathogen, is known as antibiosis. A number of streptomycete antibiotics effective against phytopathogens in vitro have been identified (Kim et al. 1999; Trejo-Estrada et al. 1998b). Antibiosis by root-colonizing actinomycetes has also been observed (Chamberlain and Crawford 1999; Crawford et al. 1993; Rothrock and Gottlieb 1984; Trejo-Estrada et al. 1998a).

Antibiosis is an advantage in the biological control of disease since compounds that mediate antibiosis can diffuse readily in nature and therefore direct contact between the antagonist and the pathogen is not necessary (Hajlaou et al. 1994).

There is evidence that antibiotics are indeed produced in soil, and they have been implicated in the biocontrol of pathogens in situ. *Streptomyces violaceusniger* YCED9 produces geldanamicin, nigericin, and a complex of macrocyclic lactone antibiotics which are thought to be involved in the control of *R. solani* and *S. homeocarpa* in greenhouse experiments since nigericin could be recovered from soil (Trejo-Estrada et al. 1998a). Other *Streptomyces* have also been shown to produce antibiotics in soil (Cundliffe 1989; Fravel 1988; Rangaswami and Ethiraj 1962). Chamberlain and Crawford (1999) demonstrated the effectiveness of the lignocellulolytic *Streptomyces* strains YCED9 and WYE53 as biocontrol agents to control pathogenic fungal agents of turfgrass including *R. solani*, *P. ultimum*, and *G. graminis* (Chamberlain and Crawford 1999). While antimicrobial substances are likely to facilitate the biocontrol of plant diseases, it is unlikely that this is the sole mechanism by which biocontrol occurs. Biocontrol is likely to be based on the ecological balance of many competing factors. There have been reports of mutant biocontrol strains deficient in the production of antimicrobial substances that are able to control plant diseases as efficiently as the wild-type strains (Kempf and Wolf 1989; Neeno-Eckwall et al. 2001) and likely due to the ability to colonize and outcompete the pathogen as described below. Another study of the same *Streptomyces* species showed that a mutant of the strain defective in the production of geldanamycin lost the ability to control the disease (Agbessi et al. 2003).

13.4.2 Colonization of Plant Roots and Surfaces

The ability of a biocontrol organism to maintain a sufficient population density in the rhizosphere for a sufficient length of time is critical to the success of the biocontrol method (Weller 1988). Properties of successful colonizers are introduced below.

13.4.2.1 Competition

There are examples where mutants of biocontrol strains that are deficient in the production of antimicrobial substances are almost as effective in biocontrol as the wild-type strains (Kempf and Wolf 1989), suggesting that this mechanism of biocontrol is related to the colonization ability and other competitive traits of the biocontrol agent. A competitive advantage is conferred upon a biocontrol agent that has the ability to use a specific energy source or inorganic compound that not all microorganisms within a given niche are able to use. For example, iron can limit the growth of plant pathogens and is a well-known source for nutrient competition in the rhizosphere (Douling and O'Gara 1994; Robin et al. 2008).

Competition for Iron: Importance of Siderophores

The ability of a biocontrol agent to outcompete a pathogen for nutrients and space within the rhizosphere is an advantageous trait. Sequestration of iron gives a biocontrol strain a competitive advantage.

Iron is an essential element for the growth of all living organisms but is scarce as a consequence of its low solubility in most habitats. Siderophores are low molecular weight compounds secreted by many bacteria that permit the acquisition of ferric ion (Whipps 2001). Due to the importance of iron for growth and metabolism, siderophore-mediated iron acquisition plays an important role in the ability of a microorganism to colonize plant roots, thereby impacting microbial interactions within the rhizosphere (Crowley 2006; Yang and Crowley 2000). Streptomycetes produce diverse siderophores (Hamby-Salove 2002; Muller and Raymond 1984; Tokala et al. 2002; Yang and Leong 1982) that have high affinity for ferric iron, thereby sequestering iron away from pathogens restricting their growth (Loper 1988; Whipps 2001). Furthermore, these siderophores differ in their specificity allowing them to bind other metals further depriving phytopathogens of essential elements. Many plant-growth-promoting bacteria including streptomycetes can use iron from heterologous siderophores (Barona-Gomez et al. 2006; Crowley 2006; Neilands and Leong 1986; Neilands 1984) allowing them to outcompete phytopathogens. Plants are not affected by the resulting localized depletion of iron since most plants can grow at lower iron concentrations than microbes and many plants can utilize microbial siderophores.

13.4.3 Parasitism and the Production of Extracellular Proteins

Parasitism, the physical destruction of fungal cell walls by the action of extracellular hydrolytic enzymes produced by an antagonist, is yet another mechanism of biocontrol (Adams 1990; Chernin and Chet 2002). Hydrolases involved in mycoparasitism of fungal pathogens include β-1,3 glucanases and chitinases (Berg et al. 2002; Chernin et al. 1995; Cohen-Kupiec et al. 1999; El-Tarabily et al. 2000; Gonzalez-Franco et al. 2003; Inbar and Chet 1995; Mahadevan and Crawford 1997; Valois et al. 1996), which results in degradation of the fungal cell wall reducing the number of fungal pathogens able to cause disease. The importance of chitinases as a mechanism of biocontrol is exemplified by the observation that nonchitinolytic streptomycetes do not protect plants from *Sclerotinia* (El-Tarabily et al. 2000).

13.4.4 Volatile Substances

The synthesis and perception of volatile substances as signaling molecules adds an additional layer of complexity to the interactions between plants and microbes.

Volatile substances are lipophilic, low molecular weight organic or inorganic compounds with high vapor pressure (Vespermann et al. 2007). Plant roots are known to release volatile metabolites (Wenke et al. 2010). Many bacteria including actinomycetes have been reported to produce volatile substances (Carpenter-Boggs et al. 1995; Collins and Gaines 1964; Gerber and Lechevalier 1965; Schulz and Dickschat 2007; Vespermann et al. 2007; Wilkins and Schöller 2009); some of these substances have been implicated as a biocontrol mechanism (Wan et al. 2008). For example, Wan et al. (2008) demonstrated that *Streptomyces platensis* F-1 effectively suppressed the growth of *R. solani*, *Sclerotinia sclerotiorum*, and *B. cinerea* and effectively reduced the severity of leaf and seedling blight of rice caused by *R. solani*, leaf blight of oilseed rape caused by *S. sclerotiorum*, and strawberry fruit rot caused by *B. cinerea* (Wan et al. 2008), suggesting a potential role for this organism as a biofumigant to protect postharvest fruits from decay or for use under greenhouse conditions for control of foliar diseases.

While the importance of volatile compounds in the biocontrol process is undeniable, there is a dearth of information on the mechanisms involved in the signal transduction process and how these compounds mediate interaction between microbes and between plants and microbes.

13.5 Plant Growth Promotion by Actinobacteria

Plant-growth-promoting rhizobacteria (PGPR) colonize the rhizosphere of numerous plant species and confer diverse beneficial effects, such as increased plant growth (Tokala et al. 2002), increased seed germination rate (Glick et al. 1997), tolerance to abiotic stress (Timmusk and Wagner 1999; Yang et al. 2009), and reduced susceptibility to diseases (Kloepper et al. 2004). Additional beneficial effects of PGPR strains include enhancement of phosphorus availability (Gyaneshwar et al. 2002; Rodriguez and Fraga 1999), fixation of atmospheric nitrogen (Gregor et al. 2003), sequestration of iron for plants by production of siderophores (Hamby-Salove 2002; Mondal and Sen 1999; Simeoni et al. 1987; Tokala et al. 2002), and production of phytohormones such as gibberellins and auxins (Glick 1995; Merzaeva and Shirokikh 2010).

Streptomyces and other PGPR can affect plant growth either indirectly or directly. Indirect promotion occurs when PGPR lessen or prevent the harmful effects of one or more deleterious microorganisms. This is biocontrol as described above. The antagonism of soil phytopathogens results due to competitive exclusion by root colonization and/or the biosynthesis of metabolites, which prevent pathogen invasion and establishment. In comparison, direct promotion of plant growth by PGPR occurs when the plant is supplied with a compound that is synthesized by the bacteria or when a PGPR otherwise facilitates plant uptake of soil nutrients (Lynch 1990; Lynch and Whipps 1990).

Despite their enormous potential as plant-growth-promoting rhizobacteria, *Streptomyces* have not been well investigated for their use as PGPR. This is

surprising since streptomycetes account for a significant percentage of typical soil microflora (Alexander 1977; Janssen 2006; Lacey 1973), are particularly effective colonizers of plant root systems (Basil et al. 2004; Tokala et al. 2002), and are able to endure unfavorable growth conditions by forming spores which allows them to persist in the environment (Chater 1993; Goodfellow and Williams 1983). While the beneficial effects of some *Streptomyces* and other PGPR strains have been shown, the mechanisms they utilize for plant growth promotion are not yet fully understood and it is likely that multiple mechanisms are utilized by any given strain (Glick 1995).

13.5.1 Nutrient Uptake

The mechanisms behind plant–PGPR interactions are conceivably complex and appear to be species and context dependent. One proposed mechanism by which PGPR affect nutrient uptake is by enhancing growth and development of plant roots, leading to root systems with larger surface area with increased number of root hairs, which are then able to access more nutrients (Adesemoye et al. 2008; Biswas et al. 2000).

13.5.1.1 Iron Sequestration

Siderophore production by root-colonizing *Streptomyces* species has been shown to be a mechanism for plant growth enhancement (Hamby-Salove 2002; Tokala et al. 2002). In legumes, a siderophore-secreting *Streptomyces* by an unknown mechanism assimilated iron from the surrounding soil and transferred it into root nodules where it was assimilated by bacteroides (Tokala et al. 2002) promoting nodulation. This interesting streptomycete symbiosis resulted in pea plants that had longer shoots, higher average nodule weights, root weights, and greater nitrogenase activity than uninoculated plants. The actinomycete-specific association had a positive influence on the physiology of the host plant.

For many other PGPR, there is also a positive correlation between siderophore production and observed enhancement of plant growth (Aronson and Boyer 1994; Becker and Cook 1988; Hofte et al. 1991). There are multiple proposed mechanisms by which this may occur. First, sequestration of the iron in the rhizosphere by PGPR renders the iron less available to potential pathogens in the rhizosphere (Kloepper et al. 1980) as already discussed. Second, the PGPR's own growth and its ability to promote host plant growth could be enhanced if it is able to utilize the bound, bioavailable iron through specific membrane receptors and uptake transport systems. These receptors and transporters, and their corresponding genes, have been described for several rhizobacteria (Bunet et al. 2006; de Weger et al. 1986; LeVier and Guerinot 1996; Marugg et al. 1985; Reigh and O'Connell 1993). Third, the PGPR may help the host plant assimilate iron when bioavailability

is low in soil. Finally, certain root-colonizing species of rhizobacteria are effective biocontrol agents. Microbial biocontrol is typically achieved through competitive antagonism or parasitism of the plant pathogen, and the capacity of some biocontrol agents to antagonize a plant pathogen appears to be closely related to siderophore production (Becker and Cook 1988; Hofte et al. 1991; Loper 1988; Mondal and Sen 1999; Simeoni et al. 1987).

13.5.1.2 Enhancement of Phosphorus Availability

Another mechanism by which microbial inoculants with plant-growth-promoting activity may exert their effect is in the solubilization of inorganic phosphate and mineralization of organic phosphates (Tawaraya et al. 2006). Phosphorus is an essential macronutrient required for the growth and development of plants (Illmer and Schinner 1992). However, a large proportion of phosphorus applied as fertilizer is in an insoluble form restricting the amount available to plants. Phosphate-solubilizing bacteria within plant rhizospheres have the ability to solubilize inorganic and organic phosphorus (Vazquez et al. 2000), which can lead to increased plant growth and improved health.

Hamdali et al. (2008) investigated select phosphate-solubilizing actinomycete isolates originating from Moroccan phosphate mines for their ability to promote the growth of *Triticum durum*. They found that the strains exhibiting the best phosphorus release also had the best stimulatory effect on shoot and root growth, inhibited phytopathogenic fungi, and colonized the root surfaces (Hamdali et al. 2008). This underscores the importance of phosphate solubilization as a desirable characteristic of biocontrol strains.

13.5.1.3 Phytohormone Production

The rhizosphere is a dynamic habitat rich in microorganisms and in plant–microbe interactions, some of which are beneficial to the microbes and plants, and some of which are detrimental to one and/or the other.

As mentioned above, PGPR can influence the nutritional status of the rhizosphere by profoundly enhancing plant root development. Phytohormones such as auxins, gibberellins, and cytokinins produced by PGPR can function as plant growth regulators (Aldesequy et al. 1998; Glick 1995; Merzaeva and Shirokikh 2010; Strzelczyk and Pokojska-Burdziej 1984; Tsuchiya et al. 1997).

A significant number of rhizosphere-isolated bacteria are able to produce the auxin, indole-3-acetic acid (IAA), which is involved in cell division, root initiation, and cell enlargement (Patten and Glick 1996). Bacterial IAA production typically leads to increased root length, which increases the root surface area facilitating access to nutrients. It should be noted that some phytopathogenic bacteria can also produce IAA; this includes *Streptomyces* species (Manulis et al. 1994).

Gibberellins are phytohormones involved in a number of developmental processes in plants which are also synthesized by a number of bacteria (Atzorn et al. 1988; Bottini et al. 1989). Aldesequy et al. (1998) found that two different *Streptomyces* species, *S. olivaceoviridis* and *S. rochei*, significantly increased the shoot length and shoot fresh mass of wheat plants, respectively. Hormone extraction, purification, and bioassay showed that both species produced substantial amounts of growth regulating substances including auxins, gibberellins, and cytokinins, thus demonstrating that *Streptomyces* species produce at least one class of compounds that directly influence plant growth (Aldesequy et al. 1998). However, some other putative mechanisms of plant growth promotion such as siderophore biosynthesis and phosphorus solubilization were not assessed in that study. El-Abyad et al. (1993) and Merriman et al. (1974) reported plant growth enhancement as a function of *Streptomyces* inoculation under gnotobiotic conditions, although they did not study the possible mechanisms of streptomycete-mediated growth promotion (El-Abyad et al. 1993; Merriman et al. 1974).

Plants commonly synthesize increased levels of the hormone ethylene in response to stress (Morgan and Drew 1997). Synthesis of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase which hydrolyzes 1-aminocyclopropane-1-carboxylic acid, the immediate biosynthetic precursor of ethylene in plant tissues to ammonia and α-ketobutyrate, effectively lowers plant ethylene levels, thereby increasing resistance to environmental stress (Glick et al. 2007; Penrose and Glick 1997). The ability of ACC deaminase producing streptomycetes to enhance plant growth through the reduction of ACC levels in planta has been demonstrated (El-Tarabily 2008).

13.5.2 Current Limitations of PGPR

As discussed above, PGPR have been shown to be effective in laboratory experiments and greenhouse studies, but the desired effects in the field have not been observed consistently (Fravel 2005; Paulitz and Belanger 2001). The lack of consistency in performance between controlled environments such as the laboratory and greenhouse and the more variable field conditions along with microbial community differences may be the explanation. Despite the variability so far observed under field conditions, this avenue of research is an important one. As additional information regarding the mechanisms of plant–microbe interactions and the mechanisms by which the specificity of certain microbial interactions is controlled, it may be possible to overcome many of the current limitations to field application.

13.6 Considerations for Actinomycete Formulations

As discussed above, actinomycetes are important agents in plant growth promotion and for the control of plant diseases. Many of the actinomycetes that have been described for these applications have an advantage over their Gram-negative

counterparts in that they sporulate. Sporulation itself is not the hallmark of a successful biocontrol agent; however, vegetative inoculants do pose challenges, especially for maintaining adequate shelf life. For example, Sabaratnam and Traquair (2002) formulated vegetative hyphae of rhizosphere-competent *Streptomyces* sp. Di-944 for application onto tomato seeds using talcum powder, determined to be the most stable, starch granules, and alginate beads. The latter was found to be the least effective, attributed in part to the limited aeration and delayed hydration (Sabaratnam and Traquair 2002).

The use of actinomycetes as microbial inoculants in the field requires that a suitably large inoculum be prepared that can be stored without losing viability. Growth of actinomycetes and production of desirable biocontrol and plant-growth-promoting characteristics have been shown to vary with a given isolate, the growth conditions employed such as aeration (Clark et al. 1995; Okabe et al. 1992), temperature, pH, and source of carbon, nitrogen, and phosphorus (Aharonowitz 1980; Martin and Demain 1980). Actinomycete inocula have been prepared for field and greenhouse applications in a variety of ways. For example, Soares et al. (2007) describe a method in which streptomycete isolates were grown on sterilized rice (Soares et al. 2007). While this was an effective method, spore production varied with the *Streptomyces* species tested.

The formulation of a biological control agent can greatly impact its practicality in the field or greenhouse. It is quite common to formulate viable agents with inert carriers such as talc or Kaolin clay. Formulation is driven by the nature of the biological control agent, the target pathogen, and delivery mechanism. Zeolite and powdered milk formulations of *S. violaceusniger* YCED9 spores were shown to be effective in reducing crown-foliar disease caused by *S. homeocarpa* (Trejo-Estrada et al. 1998b) in both laboratory and greenhouse conditions. Another zeolite formulation containing *Streptomyces* sp. 385 and chitosan supplementation was shown to suppress *Fusarium* wilt in greenhouse-grown cucumber (Singh et al. 1999). In addition to the biological considerations, dry formulations weigh less and are therefore easier to ship and have reduced risk of contamination (Fravel 2005).

Determining the optimum conditions for preparing microbial inoculants in sufficient quantities to be used in field applications is not a trivial process. Further complicating the implementation of microbial inoculants is the requirement for a carrier suitable to the delivery method for a given application. Overcoming these obstacles requires a thorough knowledge of strain characteristics and is important for generating an economically viable biocontrol product.

13.7 Conclusion

It is clear that the mechanisms of plant growth promotion and biocontrol are not mutually exclusive but interdependent processes. Many of the properties exhibited by plant-growth-promoting actinobacteria are also those exhibited by effective

biocontrol agents which make their use in agriculture an attractive alternative to agrochemicals.

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Chapter 14
Functional Significance of Insect Gut Bacteria and Their Role in Host Insect Processes, Development, and Crop Production

P. Indiragandhi, R. Anandham, and Tong Min Sa

14.1 Introduction

The existence of an intimate relationship between many insect species and large numbers of microorganisms has been known for over a century. The success of insects has created myriad of opportunities for bacteria to occupy niches created by insects. The guts of insects offer such niches where the insects are able to take advantage of the products of bacterial metabolism and the adaptability of the prokaryotes. These insect–host and microbe associations form a dynamic spectrum in relation to the necessity as well as physiological impact toward those involved. The association of insects with a wide variety of bacteria includes beneficial endosymbionts, occasional disease causing entomopathogens, and harmless transient cuticle contaminants acquired from the environment (Wernegreen 2002). Endosymbiotic bacteria may be extracellular or intracellular

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and can be found in the digestive system, hemocoel, reproductive tract, or localized in specialized cells called mycetome. The interplay between insects and the microorganisms inhabiting the gut was recognized as early as 1929. In this year, Wigglesworth (1929) suggested that blood sucking triatomines, which feed solely on blood during its whole life cycle, would have symbionts to provide the insects with complex B vitamins. Bacterial symbionts have also been implicated in furnishing nutrients to sap feeders (aphids, psyllids, whiteflies, mealybugs, etc.). In aphids, the bacterium *Buchnera* was found to utilize nonessential amino acids present in the phloem sap to produce essential amino acids, completing the dietary needs of aphids (Shigenobu et al. 2000; Moran et al. 2003; Wilcox et al. 2003).

Insects living on nutrient-poor diets, where amino acids are very scarce like wood and humus, benefit from symbiotic bacteria that are able to fix atmospheric nitrogen and synthesize amino acids (Nardi et al. 2002). Nevertheless, gut microbes are not limited to insects with unbalanced diets, but could also be involved in insect temperature resistance (Montllor et al. 2002), host plant detoxification (Lauzon et al. 2003), and parasite protection (Olivier et al. 2003). Most of the arthropod symbioses involve highly specific interactions, often between one bacterial species and one insect (Kikuchi et al. 2005). There are a few examples of multispecies communities, such as the associates of the hindgut of termites. Degradation of complex carbohydrates and fixation of nitrogen form the metabolic foundation for the community's function (Nakashima et al. 2002; Bakalidou et al. 2002). The intricacies of managing the carbon and nitrogen economy of the hindgut provide evidence for interdependence and coevolution among the members of the community. The termite system offers one of the most powerful existing models for studying nutritional interactions among community members.

Evidence for a more interactive relationship between microbiota and host was assessed by many researchers (Dillon and Dillon 2004; Genta et al. 2006). However, studies have often been restricted to a catalogue of species present (Sittenfeld et al. 2002; Broderick et al. 2004; Xiang et al. 2006). This might be due to the culture-independent method of analysis of such interrelationships. In order to evaluate the functional significance of the gut bacteria to the host insect, the bacterial isolate should be cultivable under in vitro condition (Mohr and Tebbe 2007).

Thus, the interactions between insects and their microbial symbionts have provided the bases for many principles in symbiosis and have revealed unexpected mechanisms that illustrate how organisms cooperate to perform life functions and gain a competitive edge.

This chapter describes some of the bacteria associated with the class *Insecta* and their functional role in insect host nutrition and development. Furthermore, insight is given into the possible use of insect gut bacteria as potential plant-growth-promoting bacteria (PGPB) for crop production.

14.2 Functional Significance of Insect Gut Bacteria

Numerous studies in the past have described the microbial gut community using classical techniques, but little information was provided on its biological role. A number of reviewers commented on the lack of information on the biological role of the insect microbiota. Brooks (1963) stated that further isolation and characterization of insect intestinal biota were pointless unless they were correlative with either the ability of the host to control its biota or the effect of the biota on the host's physiology. The microbial midgut inhabitants seem likely to benefit the insect host, based on the results of studies conducted with different insect orders by various researchers (Takatsuka and Kunimi 1998, 2000; Lauzon et al. 2003; Dillon and Dillon 2004; Behar et al. 2005; Xiang et al. 2006; Indiragandhi et al. 2007a, 2008a; Figs. 14.1 and 14.2). Gradually, literature related to the biological roles of gut bacteria to the host insect has been increasing. Some of the reported examples of benefits/biological roles of gut bacteria include its assistance in host insect morphogenesis (Zacharuk 1976a, b; Iverson et al. 1984) and food digestion (Borkott and Insam 1990), detoxification of plant allelochemicals and xenobiotics (both synthetic and biopesticides), disease resistance by providing antimicrobial metabolites, and modification of midgut pH by their own metabolism, thereby conferring protection against indigenous and invading pathogenic microorganisms.

14.2.1 Role of Chitinase Produced by Gut Bacteria in Host Insect Processes

Chitin is one of the most important biopolymers in nature. In insects, it supports the cuticles of the epidermis and trachea as well as the peritrophic membrane (PM) lining the gut epithelium. An invertebrate–unique structure, the PM, is irreversibly permeable to secreted digestive enzymes and to the products of digestion (Chapman 1985). Insect growth and morphogenesis are strictly dependent on the capability to remodel chitin containing structures and food materials. For this purpose, insects repeatedly produce chitin synthases and chitinolytic enzymes in different tissues. In the context of morphogenesis, chitinolytic activity of the gut bacteria helps in the emergence of an adult from puparium (Iverson et al. 1984). Similar observations were reported in certain coleopterans in which gut bacteria were found to secrete enzymes capable of digesting portions of the pupal case, thereby weakening the wall and facilitating emergence. Winicur and Mitchell (1974) demonstrated that chitinase was present in the molting fluid of *Drosophila melanogaster* and described about the digestion of endocuticular chitin before larval emergence. It seems that a low level of chitinase activity imparted by the symbiotic bacteria is essential for the host insect throughout its life cycle (Iverson et al. 1984). It has been anticipated that gut microbial symbionts in the intestinal tract can be situated in the gut lumen or associated with the gut epithelium or PM (Buchner 1965). Likewise,

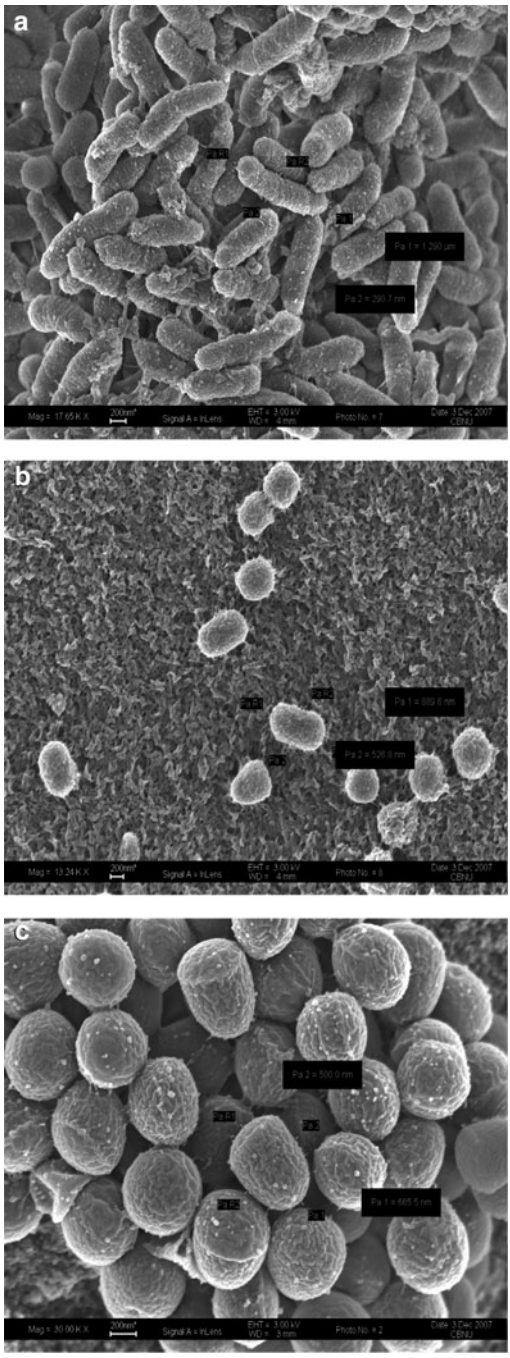

Fig. 14.1 Scanning electron micrograph of *P. xylostella* gut bacterial strains (**a**) *Pseudomonas* sp. PRGB06, (**b**) *Stenotrophomoas* sp. PRGB08, and (**c**) *Brachybacterium* sp. PSGB10

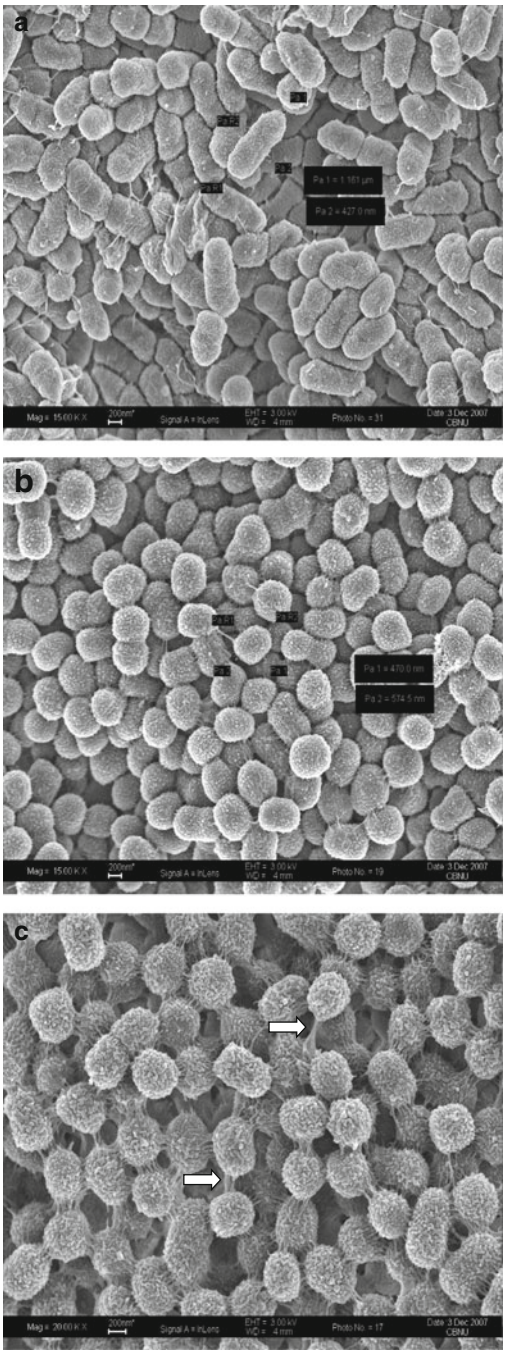

Fig. 14.2 Scanning electron micrograph of *P. xylostella* gut bacterial strains (**a**) *Serratia* sp. PRGB11, (**b**) *Acinetobacter* sp. PSGB03, and (**c**) *arrows* indicates the interlinking pigments among cells of *Acinetobacter* sp. PSGB03

studies on the house cricket (*Acheta domestica*) indicated that part of the bacterial gut microbiota is attached to the PM in the midgut, the gut wall, and cuticular bristles in the hindgut. The ability of insects to digest the chitin containing food resource is rather limited and is often dependent on symbiotic microorganisms. For instance, Brendelberger (1997) observed that the chitinase activity was parallel with the reduction in bacterial numbers in the host gut after antibiotic treatment. It indicates that chitobiase was not synthesized by the host but was taken up by bacteria from the food. This bacterial origin of chitinolytic enzymes was also shown by Genta et al. (2006) for the digestion of cell wall of wheat bran in yellow mealworm, *Tenebrio moliter* (Tenebrionidae), and in some other insects, in the digestion of their own exuvia (Mira 2000).

One more example for the evidence of chitinase produced by gut bacteria on host insect process is with the tsetse fly and its gut bacteria, *Sodalis glossinidius*. *S. glossinidius* has been shown to play a role in potentiating its host's susceptibility to trypanosome infection by influencing the efficacy of the tsetse immune system (Welburn and Maudlin 1991, 1999). In the tsetse fly, it is known that trypanosomes can only be established in the fly midgut if these parasites can successfully evade the action of an *N*-acetyl-D-glucosamine (GlcNAc), a specific trypanocidal lectin which is secreted during feeding (Maudlin and Welburn 1987). *Sodalis* is thought to influence lectin activity through the production of lectin-inhibitory sugar (GlcNAc), which is known to accumulate during pupal development as a result of this bacterium's chitinolytic activity (Welburn et al. 1993). More recently, it has been shown that the chitinase-producing gut bacteria (Fig. 14.1) isolated from *P. xylostella* increased the host insect consumption index (CI), relative growth rate (RGR), approximate digestibility (AD), efficiency of conversion of ingested food (ECI), and efficiency of conversion of digested food (ECD) (Table 14.1) (Indiragandhi et al. 2008a).

14.2.2 Gut Bacteria Mediated Detoxification of Allelochemical and Xenobiotics

Miyazaki et al. (1968) documented the significant role of gut bacteria *Rhagoletis* to detoxify harmful plant compounds, thereby protecting the host fly. Bacterial culture of *Pseudomonas* spp. could break down a variety of pesticides, apparently through the hydrolytic action of strong esterases, and might synthesize amino acids essential for growth of the maggot. Furthermore, gut microbes are reported for their detoxification of allelochemicals such as flavanoids, tannins, and alkaloids (Lauzon et al. 2003). There has been a report on bacterial modification of plant phenolics leading to the production of volatile phenolics in the locust *Schistocerca gregaria* to give guaiacol, a component of the locust aggregation pheromone (Dillon and Dillon 2004). *Pseudomonas aerugenosa* was reported for its detoxification of the plant secondary metabolite polyketide known as pederin in *Paederus* beetle (Piel 2002). Participation of gut bacteria *Pantoea* (=*Enterobacter*) *agglomerans* (a common

Table 14.1 Quantitative food-use efficiency measures for third instar larvae of *Plutella xylostella* after treatment with chitinase-producing *Serratia marcescens* PSGB12 and FLGB16

	Milligram of ingested food mg^{-1} avg. larval weight day^{-1}				
Treatment	CI	RGR	AD (%)	ECI (%)	ECD (%)
A. Cell pellet + buffer	0.62 ± 0.029^{c}	0.068 ± 0.007^{d}	23.17 ± 1.56^{cb}	3.71 ± 0.98^{d}	2.42 ± 0.23^{d}
B. Cell pellet + crude enzyme	1.63 ± 0.226^{b}	0.283 ± 0.062^{b}	30.88 ± 0.91^{abc}	8.45 ± 0.34^{a}	12.83 ± 1.08^{c}
C. Crude enzyme	0.61 ± 0.066^{c}	0.032 ± 0.004^{d}	24.66 ± 0.26^{bc}	0.81 ± 0.03^{f}	1.86 ± 0.37^{de}
D. Cell pellet + buffer	0.34 ± 0.036^{d}	0.179 ± 0.007^{c}	32.53 ± 0.73^{ab}	4.72 ± 0.37^{c}	40.0 ± 0.75^{b}
E. Cell pellet + crude enzyme	3.06 ± 0.036^{a}	0.579 ± 0.066^{a}	46.8 ± 22.72^{a}	5.6 ± 0.45^{b}	65.05 ± 1.96^{a}
F. Crude enzyme	0.22 ± 0.022^{de}	0.177 ± 0.015^{c}	13.8 ± 1.80^{cb}	2.95 ± 0.32^{e}	1.58 ± 0.25^{de}
G. Buffer	0.06 ± 0.015^{e}	0.016 ± 0.003^{d}	11.5 ± 1.32^{c}	0.97 ± 0.16^{f}	0.47 ± 0.073^{e}
LSD ($P \geq 0.05$)	0.18	0.06	20.0	0.74	1.62

Treatment details: A–C cell pellet, crude enzyme of PSGB12; D–F cell pellet, crude enzyme of FLGB16; G – buffer alone (0.05 M phosphate buffer); CFU ml^{-1} for PSGB12 $= 2 \times 10^{9}$ and for FLGB16 $= 5.75 \times 10^{9}$; 1 ml of supernatant gave 5.03 and 5.01 mU for PSGB12 and FLGB16, respectively. *CI* consumption index, *RGR* relative growth rate, *AD* approximate digestibility, *ECI* efficiency of conversion of ingested food, and *ECD* efficiency of conversion of digested food. Values are mean of $\pm$ SE of five replications per treatment. Means followed by the same letter are not significantly different from each other at 5% (LSD) (Indiragandhi et al. 2008a)

inhabitant of fruit fly and its host plant apple) in biochemical transformation of plant allelochemical dihydrochalcone phloridzin has been explored for *Rhagoletis pomonella*. Since the extent to which insects catabolize harmful compounds in their digestive tracts or on their host plant is of considerable importance to their survival (Lauzon et al. 2003), it would be possible that when insects feed on plant materials that contain plant-produced defensive toxins or/are exposed to insecticides or other pesticides. Therefore, it is likely that the gut bacteria are also exposed to these toxins and may actually contribute to detoxification of these compounds (Davidson et al. 2000; Genta et al. 2006), since gut microbes can adapt rapidly to changes in the diet of the insect by changing the population profiles and induction of requisite enzymes (Santo-Domingo et al. 1998). Therefore, the ability of microbes to modify or detoxify plant allelochemicals is an additional property that may be important in certain insect–microbe relationships (Dillon and Dillon 2004).

Most insects detoxify chemicals by converting lipophilic compounds to more water-soluble compounds that are more easily excreted. A well-known enzyme responsible for this detoxification action is glutathione-S-transferase (GST), which is widely distributed in all living organisms. So far, reports indicate that the action of GST in the insect gut is the well-explored enzyme in the detoxification of insecticides sprayed against the insects (Mohan and Gujar 2003a). Some studies reported that the bacterially produced enzymes were responsible for the detoxification of toxic substances in host insects. For instance, field-collected strains of *Spodoptera frugiperda* (J.E. Smith) showed high activity of detoxification enzymes, namely, microsomal oxidases, glutathione S-transferases, and hydrolase (Yu 1992). Interestingly, the enzyme phosphotriesterase (PTE) from microorganisms in soil

seems to be associated with the detoxification of organophosphorous pesticides and organophosphate resistance mechanism in insects. PTE was first detected in the soil microoganisms, *Pseudomonas diminuta* and *Flavobacterium* sp., which are capable of hydrolyzing paraoxon and parathion at a high catalytic rate (Munnecke and Hsieh 1974). Gut isolates of *P. xylostella* which are resistant to prothiofos were shown to have increased activity of GST than that of the same but susceptible field population (Indiragandhi and Sa, unpublished data). The high reliance to insecticides for the control of insect pests and the repeated use of the same insecticides may result in significant reduction of their biological efficacy. This loss of efficacy might be attributed to the rapid microbial degradation of insecticides by a gut symbiotic microflora as reported in soil applied nematicides (Karpouzas et al. 2005). Therefore, the enzymes of gut bacteria might contribute to the detoxification of insecticides in insects, consequently giving rise to resistance development.

14.2.3 Gut Bacteria Mediated Disease Resistance in Host Insect

The insect intestinal tract is generally inhospitable to fungi and infection rarely occurs via the gut. This is surprising as both foregut and hindgut are lined with unsclerotized cuticle, which is actively invaded, for example, in locust by an adapted fungal pathogen. It was generally thought that anaerobiosis, digestive enzymes, adverse pH, speed of food throughput, and protection from the peritrophic membrane are primarily responsible for the gut barrier to fungi (Dillon and Charnley 1991). However, none of these factors can account for the failure of the conidia of *Metarhizium anisopliae* to germinate in the guts of locusts (Dillon and Charnley 1991). Since the guts of germfree locusts do not have such inhibitory properties, it was concluded that an antifungal toxin was produced by the gut microbiota of the locust. Subsequently, antimicrobial phenolic compounds were purified from the gut and fecal pellets of conventionally reared locusts. Three phenolics were identified using GC-MS, namely, hydroxyquinone, 3,4-dihydroxybenzoic, and 3,5-dihydroxybenzoic acids. These compounds were not detected in fractions from germfree insects. A solution of authentic phenols inhibited germination of *M. anisopliae* at concentrations estimated to occur in the fecal pellets. When *Pantoea agglomerans*, a prominent member of the microbial population in the locust gut, was introduced into germfree insects the antifungal compounds reappeared in the feces in proportion to the size of the gut bacterial population (Dillon and Charnley 1995, 1996). In Hymenopteran social insect honeybees, in addition to the genetically determined hygienic behavior (uncapping of cells and removal of diseased and dead larvae) toward the Chalkbrood disease caused by *Ascosphaera apis*, gut bacteria of worker bees such as *Paenibacillus alvei*, *Bacillus circulans*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, and *B. subtilis* were reported for the host insect resistance to disease (Gilliam 1997). The phenolics produced by the gut microbiota are toxic to a wide range of insect and plant

Table 14.2 Antagonistic activities of *P. xylostella* gut bacterium *Pseudomonas* sp. PRGB06 against phytopathogenic fungi

Phytopathogenic fungi	Host	Disease	Inhibition zone diameter (cm)
Botrytis cinerea	Tomato	Gray mold	1.8 ± 0.4^{bc}
Colletotrichum acutatum KACC 41932	Strawberry	Leaf spot	–
C. capsici KACC 40978	Red pepper	Anthracnose	–
C. coccodes KACC 40008	Red pepper	Anthracnose	1.2 ± 0.1^{c}
C. gloespoiroides KACC 40690	Red pepper	Anthracnose	3.6 ± 0.1^{a}
Fusarium oxysporum f. sp. *niveum* KACC 40902	Muskmelon	Wilt	–
Rhizoctonia solani KACC 40106	Rice	Sheath blight	3.0 ± 0.4^{a}
Sclerotia sclerotiorum	Ginseng	Wilt	2.3 ± 0.2^{b}
Phytophthora capsici KACC 40483*	Red pepper	Anthracnose	–
LSD ($P \geq 0.05$)			0.7

– Absence of antagonism

Values are the mean ± SE of three replications. In the same column, significant differences according to the LSD at 0.05% levels are indicated by different letters (Indiragandhi et al. 2008a)

*Growth of *Phytophthora capsici* was inhibited by *Serratia* sp. PRGB11 (1.4 ± 0.3), *Acinetobacter* sp. strains PRGB15 (1.8 ± 0.6), PRGB16 (1.6 ± 0.4), PSGB03 (1.6 ± 0.5), PSGB04 (1.6 ± 0.2), and PSGB05 (1.6 ± 0.3); plus values in parentheses following strain names indicate diameter of inhibition zone (cm)

pathogenic fungi and insect pathogenic bacteria (Dillon and Charnley 2002). Antifungal activity of gut bacteria isolated from *P. xylostella* was reported for entomo- and phytopathogenic fungi under in vitro condition (Table 14.2 and Fig. 14.3) (Indiragandhi et al. 2007a, 2008a). The gut bacterial isolates showed significant activity of biocontrol determinants such as siderophore (Fig. 14.4), chitinase, and β-1,3 glucanase (Tables 14.3 and 14.4). Consequently, the antifungal activity of gut bacteria toward entomopathogenic fungi might be due to the action of individual or combination of all the biocontrol determinants. For the former case, it could be explained that the antibiotic activity might be due to the siderophore and its cross-utilization of siderophores produced by the fungal pathogens (Indiragandhi et al. 2008a, b) (Table 14.4).

14.2.4 Gut Bacteria Mediated Protection Against Biopesticides

The soil bacterium, *Bacillus thuringiensis* (*Bt*), is an important component of insect pest management program all over the world. Insecticidal proteins of *Bt* are effective for controlling many insect species, but insect resistance to *Bt* threatens the long-term effectiveness of these toxins (Oppert et al. 1997; Mohan and Gujar 2003b). The salient feature of this species is the accumulation of crystalline parasporal inclusions during sporulation. These inclusions are composed of one or more protoxins, known as δ-endotoxins, each of which is specific primarily at the level of insect orders, particularly Lepidoptera, Diptera, and Coleoptera. In

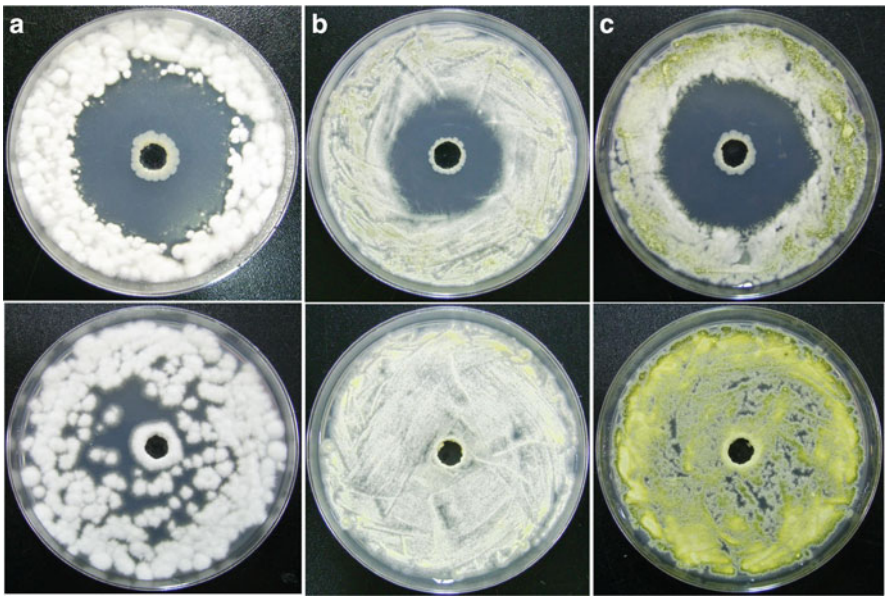

Fig. 14.3 Antagonistic activity of *P. xylostella* gut associated *Pseudomonas* sp. PRGB06 against the entomopathogens (**a**) *Beauveria bassiana* KACC 40039, (**b**) *Metarhizium* sp. KACC 400217, (**c**) *Metarhizium anisopliae* KACC 40029; their respective controls were shown at bottom panel

Lepidoptera, specificity is in part due to the extremely alkaline midgut environment that is required to solubilize the protoxin into the active form (Broderick et al. 2004). In vivo, protoxins of 120 kDa undergo proteolytic conversion to active toxins of 60–65 kDa through the action of gut protease activity at high pH in the larval midgut. Several mechanisms of insect resistance to *Bt* toxin have been proposed, such as reduced binding of toxin to larval brush border membrane vesicle which results in increased resistance in the Indian meal moth – *Plodia interpunctella*, tobacco budworm, *Heliothis virescens*, and beet armyworm, *Spodoptera litura* (Oppert et al. 1997). However, in some insect species the development of resistance to *Bt* has been linked to altered gut protease activity that interacts with *Bt* toxins. Possibly, these protease enzymes in the gut juice would originate from the gut bacterial genera present in the host insect. Few studies have indicated the inhabitation of the lepidopteran insect gut by different phylotypes of bacteria and their role in determining the insecticidal activity of *Bt* (Takatsuka and Kunimi 2000; Broderick et al. 2004). Midgut pH is important from the perspective of both the microbes and the insect. A predominant member of the midgut microbial community, *Enterobacter faecalis*, is commonly found at higher pH (8–9) and acidifies its environment through its metabolism (Manero and Blanch 1999). This could confer some advantage to the insect host, as well-known microbial toxins of Lepidoptera such as *Bt* toxin are activated only in alkaline conditions (Wilson and Benoit 1993). Thus, *E. faecalis* might protect the insect from *Bt* toxin by decreasing the midgut pH as evidenced by the smaller population of *E. faecalis* in the larvae of

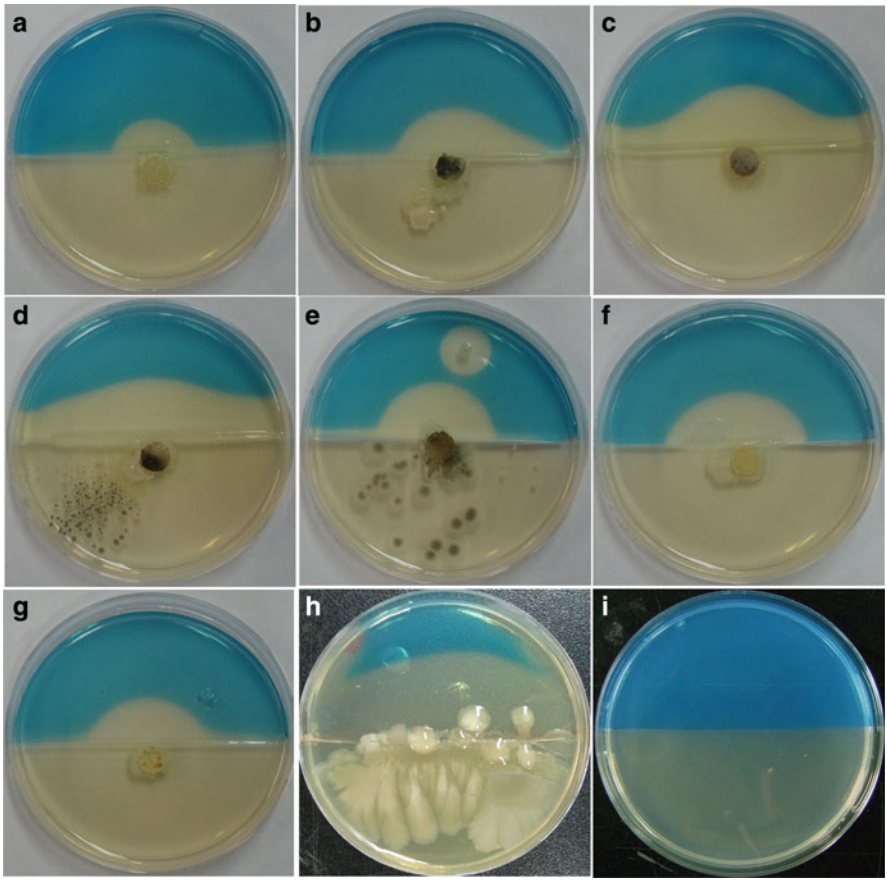

Fig. 14.4 Modified CAS agar plate assay for testing siderophore production by bacterial and fungal entomopathogens. Agar plate containing CAS-blue agar (*top half*) and MEA for fungi (**a**–**g**) and NA for *Bt* (**h**) media (*bottom half*) inoculated with (**a**) *Beauveria bassiana* KACC 40227, (**b**) *Hirsutella thompsoni* KACC 40023, (**c**) *Metarhizium anisopliae* KACC 40230, (**d**) *Metarhizium* sp. KACC 40229, (**e**) *Metarhizium* sp. KACC 40217, (**f**) *Paecilomyces tenuipes* KACC 40503, (**g**) *Paecilomyces* sp., (**h**) *Bacillus thuringiensis* KACC 10168, and (**i**) control (Indiragandhi et al. 2008a)

more susceptible insects to *Bt* toxin (Broderick et al. 2003). The bacteria may also contribute to their own survival by managing midgut pH to enhance their own growth or exclude competing microorganisms. This was demonstrated in the reduction of *Bt* growth to about 20 times lesser than that of larvae without true gut bacteria *Staphylococcus* sp. and *Streptococcus* sp. of lepidopteran oriental tea tortrix, *Homona magnanima* (Takatsuka and Kunimi 2000). On the other hand, it was shown that the siderophore produced by the gut bacteria cross-utilizes the siderophore produced by other bacteria and fungi (i.e., entomopathogenic fungi and bacteria), such as *Bt* through their outer membrane receptor protein (OMRP) (Table 14.4 and Fig. 14.5) (Indiragandhi et al. 2008a).

Table 14.3 Plant-growth-promoting characteristics of bacterial strains isolated from larval guts of *P. xylostella*

	Direct plant-growth promotion through							Indirect plant-growth promotion through		
	Mineral solubilization/oxidation			Production of enzymes and phytohormones				Production of biocontrol determinants		
Bacterial strains[†]	Soluble P (μg^{-1} ml^{-1})	Zn SE (%)	Sulfate content (μg^{-1} ml^{-1})	ARA (nmol ethylene/h/mg protein)	ACCD (nmol α-KB/min/mg protein)	IAA (μg^{-1} ml^{-1})	Salicylic acid (μg^{-1} ml^{-1})	Siderophore	Chitinase	β-1,3-glucanase (μg glucose/min/mg protein)
Acinetobacter sp. PSGB03	458 ± 15^{b}	286 ± 5^{d}	7.7 ± 1.3^{ab}	2.7 ± 0.2^{bc}	60.4 ± 0.6^{d}	7.9 ± 0.2^{e}	5.8 ± 0.5^{d}	+	+	1.3 ± 0.1^{c}
Acinetobacter sp. PSGB04	468 ± 6^{b}	300 ± 3^{c}	2.4 ± 0.9^{c}	2.0 ± 0.4^{e}	572 ± 1.2^{a}	8.3 ± 0^{dc}	4.7 ± 0.3^{e}	+	–	0.7 ± 0.1^{d}
Acinetobacter sp. PSGB05	479 ± 35^{b}	271 ± 2^{e}	6.5 ± 0.6^{b}	2.8 ± 0.1^{bc}	133 ± 1.4^{b}	8.0 ± 0.1^{e}	6.0 ± 0.3^{d}	+	–	–
Acinetobacter sp. PRGB15	540 ± 20^{a}	300 ± 4^{c}	–	2.8 ± 0.2^{ab}	86.5 ± 1.2^{c}	8.2 ± 0^{d}	3.1 ± 0.3^{f}	+	+	–
Acinetobacter sp. PRGB16	547 ± 59^{a}	314 ± 10^{b}	–	2.2 ± 0.2^{de}	–	8.2 ± 0^{dc}	7.2 ± 0.6^{c}	+	–	2.4 ± 0.2^{b}
Pseudomonas sp. PRGB06	250 ± 35^{c}	171 ± 1^{g}	10.0 ± 3.4^{a}	3.0 ± 0.4^{ab}	–	10.0 ± 0.1^{a}	6.8 ± 0.4^{c}	+	–	8.0 ± 0.4^{a}
Serratia sp. PRGB11	216 ± 5^{c}	250 ± 3^{f}	8.0 ± 1.2^{ab}	2.4 ± 0.3^{cd}	–	8.7 ± 0^{b}	8.0 ± 0.4^{b}	+	+	–
Serratia sp. PSGB13	120 ± 18^{d}	514 ± 3^{a}	2.4 ± 0.9^{c}	3.3 ± 0.4^{a}	–	8.4 ± 0.1^{c}	10.1 ± 0.7^{a}	+	+	–
LSD ($P \geq 0.05$)	43	7.0	3.3	0.4	3.5	0.2	0.7			0.4

ARA acetylene reduction assay, *IAA* indole-3-acetic acid, *ACCD* 1-aminocyclopropane-1-carboxylate deaminase, *α-KB* α-ketobutyrate, + presence of trait, – absence of trait. The values indicate the mean ± SE of three replications. In the same column, significant differences according to the LSD at 0.05% levels are indicated by different letters (Indiragandhi et al. 2008b)

[†]All the strains were positive for ammonia production and negative for HCN and pectinase production

Table 14.4 Cross-utilization of homologous and heterologous siderophores by *P. xylostella* gut bacteria

Bacterial strain[a]	MIC[b] (μM)	Gut bacterial isolates whose siderophores (homologous) were utilized	Entomopathogenic bacteria and fungi, and phytopathogenic fungi (heterologous) whose siderophores were utilized[c]
Brachybacterium sp. PSGB10	200	A, B, F, G	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17
Pseudomonas sp. PRGB06	500	B, C, E, F	1, 2, 4, 5, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17
Serratia marcescens FLGB16	500	A, B, E, F	1, 3, 8, 11, 12, 13, 14

[a]*Acinetobacter* sp. PRGB16 (MIC-1,000 μM), *Stenotrophomonas* sp. PRGB08 (500 μM), and *Serratia* sp. PRGB11 (500 μM) did not cross-feed either homologous or heterologous siderophores
[b]Minimum inhibitory concentration of 2,2′-dipyridyl (DPD)
[c](1) *Bacillus thuringiensis* KACC 10168, (2) *Beauveria bassiana* KACC 40218, (3) *B. bassiana* KACC 40224, (4) *B. bassiana* KACC 40039, (5) *Cordyceps millitans* KACC 40665, (6) *Metarhizium* sp. KACC 40217, (7) *Metarhizium* sp. KACC 40230, (8) *M. anisopliae* KACC 40029, (9) *Hirsutella thompsoni* KACC 40023, (10) *Paecilomyces amarosiensis* KACC 41779, (11) *P. tenuipes* KACC 40503, (12) *Botrytis cinerea*, (13) *Colletotrichum acutatum* KACC 40218, (14) *C. coccodes* KACC 40218, (15) *C. gleosporoides* KACC 40218, (16) *Fusarium oxysporum* f. sp. *niveum* KACC 40902, and (17) *Phytophthora capsici* KACC 40483 (Indiragandhi et al. 2008a)

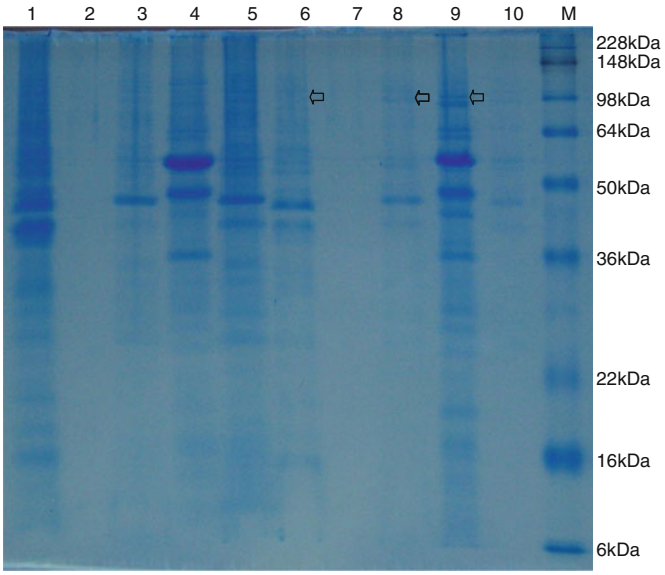

Fig. 14.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) (SDS-PAGE) analysis of OMRP fraction isolated from bacterial cells of *Pseudomonas* sp. PRGB06, *Stenotrophomonas* sp. PRGB08, *Brachybacterium* sp. PSGB10, *Serratia marcescens* FLGB16, and *Serratia* sp. PRGB11 grown under normal and iron-deficient conditions (NA + MIC of 2,2′-DPD for respective strain), shown in the order of lanes 1–5 and 6–10, respectively. 10 μg of protein was loaded in each lane. Standard marker is shown in lane M. The *arrowhead* shows 96–98 kDa outer membrane protein (Indiragandhi et al. 2008)

14.2.5 Gut Bacteria Mediated Nitrogen Fixation in Insects

Nitrogen, although abundant in the atmosphere, is paradoxically a limited resource for multicellular organisms. In the class Insecta, biological nitrogen fixation has been greatly demonstrated in termites. Even though termite gut cells are now known to be capable of digesting lignocellulose without direct assistance from gut symbionts (Bignell 2000), no cells of arthropod guts are competent to fix nitrogen or to convert carbon dioxide to acetate. These two anaerobic activities are the sole province of the gut microbes. Microbial acetogenesis in termite guts has been demonstrated to provide at least one third of the energy requirements of termites (Breznak 2000). Anaerobic prokaryotes provide their arthropod hosts with important nutritional benefits that cannot be provided by eukaryotic cells alone. Behar et al. (2005) found that all individuals of field-collected mediterranean fruit flies, *Ceratitis capitata*, harbor large diazotrophic enterobacterial populations that express dinitrogen reductase in the gut. Moreover, nitrogen fixation was demonstrated in isolated guts and in live flies. This may significantly contribute to the fly's nitrogen intake. The presence of similar bacterial consortia/activity in additional insect orders such as Lepidoptera (Table 14.3) (Hameeda et al. 2006b; Indiragandhi et al. 2008b) suggests that nitrogen fixation occurs in vast pools of terrestrial insects. On a large scale, this phenomenon may have a considerable impact on the nitrogen cycle (Behar et al. 2005). Insect gut bacteria associated with various orders of insects and their functional role in insects are summarized in Table 14.5.

14.3 Potential of Antibiotic Therapy for Insect Pest Management

The literatures reviewed under the various topics highlighted the importance of gut bacteria to the host insect species. The loss of gut bacteria often results in detrimental fitness costs for the host, such as growth impairment, shortened life span, and sterility, while their presence contributes to insect morphogenesis, digestion, nutrition, protection against toxic substances, pheromone production, and even reproduction (Brand et al. 1975; Buchner 1965; Nolte 1977; O'Neill et al. 1992). Evidently, the reduction or elimination of the gut bacteria in insects by antibiotic therapy would certainly cause deleterious effects on the host insect. For instance, Costa et al. (1997) evaluated the effect of different substances on the growth and development of whiteflies. They found significant negative effects on growth and development of whiteflies due to the bacterial protein synthesis affected by the test substances such as tetracycline and rifampicin. Likewise, the removal of *Buchnera* with antibiotics severely debilitates aphid performance and fecundity (Koga et al. 2007). Broderick et al. (2000) observed that lepidopterous larvae fed on antibiotics in various combinations display numerous signs of reduced health. For example,

Table 14.5 Gut bacterial phylotypes identified from different orders of class Insecta

Sl. no.	Insect pests	Gut bacterial phylotypes	Functional significance	References
Coleoptera				
1	Pine bark beetle *Ips paraconfusus* (Scolytidae)	*Bacillus cereus*	Pheromone production	Brand et al. (1975)
2	Rice weevil *Sitophilus* spp. (Dryopthoridae)	*Aeromonas haywardensis*, *Erwinia carotovora*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Providencia stuartii*, *Serratia liquefaciens*, *Escherichia coli*, *Paracoccus* sp., *P. fluorescens*	Not determined	Lefévre et al. (2004)
Diptera				
3	Crucifer root maggot *Delia radicum* (Anthomyiidae)	*Erwinia amylovora*, *E. carotovora*, *K. pneumoniae*, *E. coli*, *Helicobacter canadiensis*, *Mycobacterium avium*, *Pseudomonas aeroginosa*, *P. cichorii*, *Rhodothermus marinus*, *Serratia ficaria*, *Serratia marcescens*	Nutritional role	Lukwinski et al. (2006)
4	Mediterranian fruit fly *Ceratitis capitata*	*Enterobacter* spp., *Klebsiella* spp.	Nitrogen fixation	Behar et al. (2005)
5	Apple fruit fly *Rhagoletis pomonella* (Tephritidae)	*Pseudomonas* spp., *Enterobacter* sp., *Micrococcus* sp., *Flavobacterium* sp., *Bacillus* sp.	Dihydrochalcone phloridzin degradation, detoxification of Azinphosmethyl	Lauzon et al. (1994, 2003)
6	Mexican fruit fly *Anastrepha ludens* (Tephritidae)	*Enterobacter*, *Listeria*, *Povidencia*, *Serratia*, *Staphylococcus* spp. *Citrobacter*, *Streptococcus*, *Aerococcus*, *Listeria*	Not determined	Kuzina et al. (2001)
7	Queensland fruit fly *Bactrocera tryoni* (Tephritidae)	*Klebsiella oxytoca*, *E. cloacae*	Nitrogen fixation	Murphy et al. (1994)
8	Sugar beet root maggot *Tetanops myopaeformis* (Otitidae)	*Acinetobacter calcoaceticus*, *S. liquefaciens*, *S. marcescens*, *P. maltophilia*, *P. fluorescens*	Morphogenesis	Iverson et al. (1984)

(*continued*)

Table 14.5 (continued)

Sl. no.	Insect pests	Gut bacterial phylotypes	Functional significance	References
Hemiptera				
9	Pentatomid bug *Leptocorisa chinensis*, *Riptortus clavatus*, *R. linearis* (Alydidae)	*Burkholderia*	Not determined	Kikuchi et al. (2005)
10	Pink sugarcane mealybug *Saccharicoccus sacchari* (Pseudococcidae)	*Gluconacetobacter sacchari*	Not determined	Franke et al. (1999)
11	Whitefly *Bemisia argentifolii*, *B. tabaci* (Aleyrodidae)	*Acinetobacter lwoffsii*, *A. baumannii*, *Agrobacterium* sp., *Bacillus megaterium*, *B. amyloliquefasciens*, *B. licheniformis*, *Cellulomonas turbata*, *Citrobacter* sp., *Enterobacter cloacae*, *Flavomonas oryzihabitans*, *Staphylococcus aureus*, *S. epidermidis*	Honey dew production	Davidson et al. (2000)
12	Cotton bollworm *Helicoverpa armigera* (Noctuidae)	*Acinetobacter anitratus*, *A. venetianus*, *Aquamonas fontana*, *Flavobacterium*, *Enterobacter avium*, *E. casseliflavus*, *E. gallinarum*, *Leuconostoc citreum*, *Lactococcus lactis*, *Pseudomonas mevalonii*, *Sphingomonas* sp., *Stenotrophomonas maltophilia*	Not determined	Xiang et al. (2006)
13	Pink bollworm (PBW) *Pectinophora gossypiella* (Gelechidae)	*Aeromonas salmonicida* sp. *salmonicida*, *Chromobacterium violaceum*, *Citrobacter koseri*, *Enterococcus faecium*, *Enterobacter gergoviae*, *Klebsiella oxytoca*	Not determined	Kuzina et al. (2002)
14	Automeris *Automeris zugana* (Saturnidae)	*Bacillus* sp., *B. thuringiensis*, *Corynebacterium durum*, *C. pyogenes*, *Citrobacter* spp., *Curtobacterium* spp., *Enterobacter gergoviae*, *E. aerogenes*, *E. agglomerans*, *K. pneumoniae*, *Listeria monocytogenes*, *Micrococcus* spp., *P. diminuta*, *Serratia marcescens*, *S. liquefaciens*, *Staphylococcus* spp.	Not determined	Sittenfeld et al. (2002)

15	Gypsy moth *Lymantria dispar* (Noctuidae)	*Bacillus* sp., *E. faecalis*, *P. putida*, *Staphylococcus xylosus*, *S. cohnii*, *S. lentus*, *Pantoae agglomerans*, *Serratia marcescens*, *Paenibacillus*, *Micrococcus*, *Rhodococcus*	Enhances insecticidal activity of *Bt*	Broderick et al. (2004)
16	Beet armyworm *Spodoptera exigua* (Noctuidae)	*Myroides odoratus*, *Ochrobactrum* sp.	*N*-Acylamino acid production	Spiteller et al. (2000)
17	Cut worm *Agrotis segetum* (Noctuidae)	*Providencia rettigeri*	*N*-Acylamino acid production	Spiteller et al. (2000)
18	Cabbage moth *Mamestra brassicae* (Noctuidae)	*Acinetobacter* sp.	*N*-Acylamino acid production	Spiteller et al. (2000)
19	Oriental tea tortrix *Homona magnanima* (Tortricidae)	*Streptococcus* spp., *Staphylococcus* spp.	Inhibits *Bt* germination in host gut	Takatsuka and Kunimi 2000
20	Leek moth *Acrolepiopsis assectella* (Yponomeutidae)	*Bacillus* spp., *Klebsiella oxytoca*	Kairomone production	Thibout et al. (1995)
21	Diamondback moth *Plutella xylostella* (Yponomeutidae)	*Pseudomonas* sp., *Stenotrophomonas* sp., *Acinetobacter* sp., *Serratia marcescens*, *Brachybacterium* sp. (Figs. 14.1 and 14.2)	Chitinase and siderophore production	Indiragandhi et al. (2008a, b)
Others				
22	Desert locust *Schistocerga gregaria* (Acrididae)	*P. agglomerans*, *K. pneumoniae*, *Enterobacter agglomerans*, *E. cloacea*, *E. casseliflavus*	CR by phenolics and cohesion pheromone production	Dillon et al. (2005)
23	Western flower thrips *Frankliniella occidentalis* (Thripidae)	*Erwinia* spp.	Not determined	de Vries et al. (2001)
24	Formosan subterranean termite *Coptotermes formosanus* (Termitidae)	*Serratia marcescens*, *Enterobacter aerogens*, *E. cloacae*, *C. farmeri*	Scavenge oxygen and create anaerobic condition for anaerobes	Adams and Boopathy (2005)
25	Leaf cutting ants *Atta sexdans*	*Burkholderia* spp.	Antibiotic production	Santos et al. (2004)

a cocktail of antibiotics including tetracycline reduced larval survival by 50% over the first 20 days after hatching, and the surviving larvae weigh one tenth of the untreated control larvae. Tetracycline alone has a similar effect on development, whereas gentamicin, rifampicin, and penicillin have no effect on survival or growth. Another antibiotic, zwittermicin A, dramatically increases larval sensitivity to the insecticidal toxin produced by *Bt* and also alters the population size of more than one member of the gut community. It is possible that a change in the gut microbiota may inhibit larval growth, instar development, and make the larvae more sensitive to pathogens and toxins. Thus, gut bacteria provide a potential target for insect control with systemic antibacterial materials, or transgenic plants that produce antibacterial proteins (Jaynes et al. 1987). With reference to *P. xylostella*, antibiotic-treated leaf-fed larvae showed malformed adult development and failed to successfully develop into pupae (Indiragandhi and Sa, unpublished data).

14.4 Insect Gut Bacteria as Biocontrol Agents and PGPB

Genetic modification of gut isolates which are mildly pathogenic to their host may be exploited as biocontrol agent. For example, *Enterobacter cloacae* WFA73, a mild pathogen to *Bemisia argentifolii*, has the ability to penetrate whitefly gut cells, which suggests that this microorganism could be genetically modified to enhance its effectiveness as a biological control agent. A similar report has been documented for soil microarthropods by Thimm et al. (1998).

Simultaneously, it is possible to exploit the insect gut bacterial isolates for the enhancement of growth and development of crop plants. The transformation of a phyllosphere bacterium, *Bacillus megaterium*, with *Bt* toxin genes for the control of Lepidoptera is an example of such manipulation (Bora et al. 1994). Similar modification of gut bacteria in other insects, to alter their ability to vector plant viruses or other characteristics of these insects, is also an interesting possibility (Richards 1993).

It could be noticeable that there is a tritrophic interaction between plant–insect–bacteria. As well documented, insect guts are known for their microbial population, which is able to fix atmospheric nitrogen, have the ability for mineral solubilization and oxidation and possess hydrolytic enzymes such as chitinase and indole derivatives.

Most reported PGPB are rhizosphere, phyllosphere, and soil isolates, except for a few from milk and cow dung (Nagarajkumar et al. 2004; Swain and Ray 2007). The microbial population in insect guts (4×10^5 to 2×10^{11} ml^{-1} of gut suspension) is greater than that in the phyllosphere (3×10^5 cm^{-2}) (Jacques et al. 1995), representing a well-explored niche for beneficial bacterial isolation. It is assumed that many insect species derive their microbiota from the surrounding environment, for instance, the phylloplane of plants (Dillon and Dillon 2004; Kremer and Souissi 2001). Presumably, the nutrient cycling rates in the gut environment are much higher and the potential disturbances are more dramatic than in soil and plants. Crop plants generally immobilize the nutrients in their leaves. Insects also have well-developed

mouth parts that are used to grind and shred organic substances, making insects more accessible for microbial colonization. Insect gut bacterial strains are also more metabolically versatile than isolates from elsewhere (Dillon and Dillon 2004). Spiteller et al. (2000) reported that the metabolic products produced by gut bacteria of *Spodoptera exigua*, *Mamestra brassicae*, and *Agrotis segetum* play a role in triggering a range of systemic responses with the result of larval feeding. Synthesis of *N*-acyl amino acid by the gut bacteria of *Spodoptera exigua* attracts the predators of the herbivores, when the host insect regurgitates its oral secretions. Findings of this study indicated that the involvement of bacteria in the biosynthesis of compounds which play a pivotal role in the interaction of plants, herbivores, and their predators adds a new trophic level to this complex network of interactions.

The gut passage may also enhance rates of decomposition by inoculating the organic material with bacteria, which might continue to grow outside the gut in the feces. After getting into the soil, the bacteria in the feces can establish in the rhizosphere. Living plant roots exude organic compounds, e.g., water-soluble sugars, amino acids, and organic acids into the rhizosphere, which in turn utilize the products of bacterial metabolism for plant growth. Bacterial features such as the ability to produce ACC (1-aminocyclopropane-1-carboxylate) deaminase, indole derivatives (IAA), and salicylic acid (SA) could benefit plants by restraining the inhibition of root elongation by reducing the level of stress ethylene (Fig. 14.6). SA also acts as (1) a signaling compound in inducing systemic resistance against insect and disease attack and (2) antimicrobial agent (Indiragandhi et al. 2007b). By using the sugar compound as growth substrate, bacterial strains could produce some organic acids, which lead to solubilization of insoluble nutrients such as P and Zn and make them available to crop plants. This may be the reason behind the hypothesis that the insect herbivores can accelerate nutrient mobilization in soil. A number of bacterial isolates colonizing insect gut area have been reported for its potential for plant-growth promotion. Bacterial isolates from the macrofauna and *P. xylostella* were reported for their antagonistic activity against various phytopathogenic fungi, solubilization of rock phosphate by the gluconic acid production, siderophores, ACC deaminase, and hydrolytic enzyme production (Hameeda et al. 2006a, b; Indiragandhi et al. 2008b). Siderophores and indole derivatives synthesized by the insect gut bacterial strains were documented for their antagonistic activity against soil-borne pathogen (Ciche et al. 2003). Bacterial strains of *P. xylostella* significantly inhibit the phytopathogenic fungal growth and enhanced the seedling growth and vigor in economically important crops such as canola, tomato (Indiragandhi et al. 2008b) (Table 14.6), rice, chilli, and maize (Indiragandhi and Sa, unpublished data).

14.5 Future Thrust

This review of earlier studies is indicative of the possibility of utilizing the insect gut bacterial strains as biocontrol agent and PGPB. Therefore, isolation, characterization, and exploitation of microbes from such specialized dynamic environments

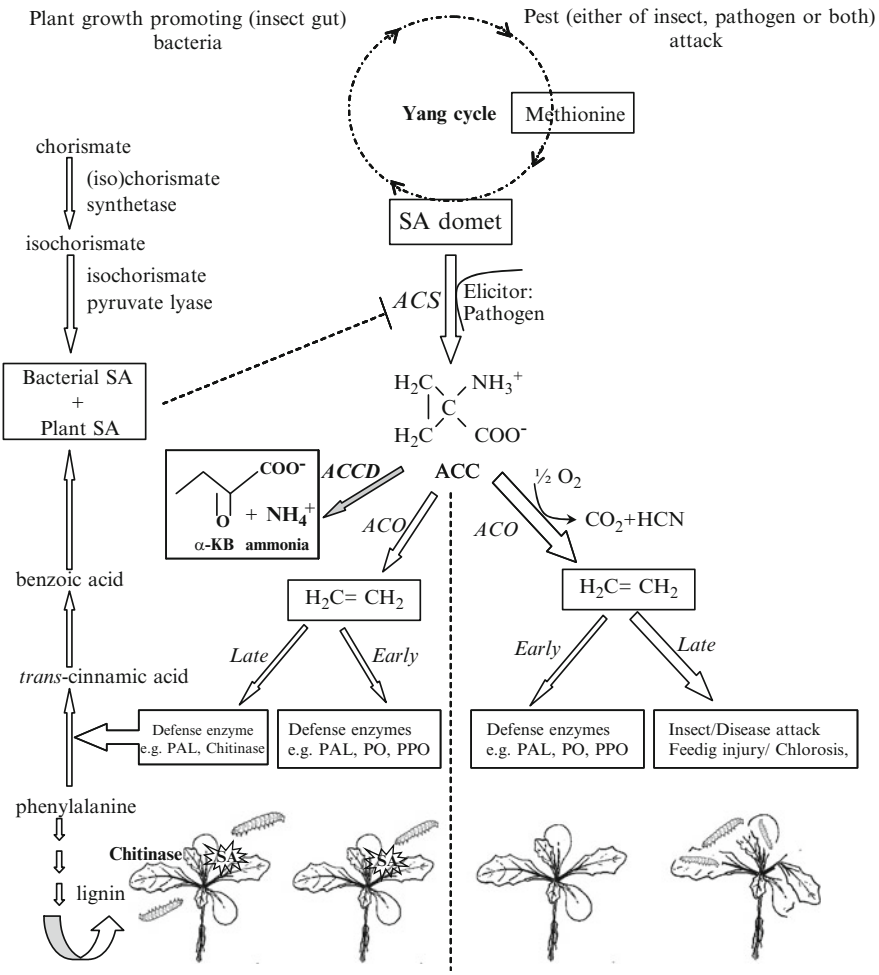

Fig. 14.6 A proposed model for plant-growth-promoting gut bacteria. The eventual increase in defense enzymes activity is a consequence of induction of systemic resistance against insect pests. *SAdomet* S-adenosylmethionine, *SA* salicylic acid, *ACS* 1-aminocyclopropane-1-carboxylate synthase, *ACC* 1-aminocyclopropane-1-carboxylate, *ACO* 1-aminocyclopropane-1-carboxylic acid oxidase, *ACCD* 1-aminocyclopropane-1-carboxylate deaminase, *α-KB* α-ketobutyrate. *Perpendicular* symbol shows the inhibition of ACS by salicylic acid (SA) (Indiragandhi et al. 2007a)

represent a step forward in the development of bioinoculants for increased crop production through effective crop protection.

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Table 14.6 Effect of DBM larval gut bacterial strain seed treatment on early growth of canola and tomato under gnotobiotic conditions

	Root length (cm)		Seedling vigor		Dry biomass (mg)	
Treatment	Canola	Tomato	Canola	Tomato	Canola	Tomato
Acinetobacter sp. PSGB03	8.0 ± 0.2^{a} (39)	5.7 ± 0.3^{c} (9)	$1{,}048 \pm 4^{a}$	892 ± 2^{c}	136 ± 6^{bc} (17)	96 ± 0.5^{b} (22)
Acinetobacter sp. PSGB04	8.0 ± 0^{a} (41)	5.5 ± 0.3^{d} (3)	$1{,}007 \pm 4^{b}$	835 ± 9^{d}	151 ± 2^{a} (30)	100 ± 2.8^{a} (26)
Acinetobacter sp. PSGB05	7.7 ± 0^{ab} (35)	6.4 ± 0.1^{a} (21)	957 ± 6^{d}	937 ± 9^{b}	150 ± 1^{a} (30)	83 ± 1.8^{c} (5)
Acinetobacter sp. PRGB15	7.9 ± 0.6^{a} (38)	5.6 ± 0.2^{dc} (7)	995 ± 9^{c}	819 ± 5^{e}	128 ± 1^{c} (11)	89 ± 1.4^{c} (13)
Pseudomonas sp. PRGB06	7.2 ± 0.1^{b} (27)	6.5 ± 0.1^{a} (23)	881 ± 4^{g}	982 ± 2^{a}	136 ± 7^{bca} (17)	100 ± 1.0^{a} (26)
Serratia sp. PRGB11	8.0 ± 0.1^{a} (41)	6.1 ± 0.2^{ba} (16)	930 ± 7^{e}	934 ± 7^{b}	138 ± 3^{b} (19)	91 ± 1.0^{c} (15)
Control	5.7 ± 0.5^{c}	5.3 ± 0.6^{d}	920 ± 6^{f}	811 ± 8^{f}	116 ± 11^{d}	79 ± 1.7^{c}
LSD ($P \geq 0.05$)	0.6	0.4	5.0	7.0	9.0	2.0

The values indicate the mean ± SE of three replications. In the same column, significant differences according to the LSD at 0.05% levels are indicated by different letters. Values in parentheses indicate the percentage increase over the control (Indiragandhi et al. 2008b)

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Chapter 15
Potentials for Biological Control of Plant Diseases by *Lysobacter* spp., with Special Reference to Strain SB-K88

Md. Tofazzal Islam

15.1 Introduction

Our ability to provide adequate pest control to increase crop yields and reduce land requirements cannot pace with the growing demand of the world's population using conventional agriculture. This has led to higher chemical inputs to control insect pests and plant diseases (Agrios 1997). Consequently, this has placed an undue burden on the planet's ecosystems. The costs associated with the rapid development of modern pesticides include environmental contamination, unpredicted human health consequences, and deleterious effects on wildlife and other nontargeted organisms in the food chain (Carson 1962). The realization that sustainable industrial processes must be compatible with environmental concerns has developed into the philosophy of Green Chemistry. Biological control of plant pests represents a natural overlap between sustainable agriculture and green chemistry (Baker 1987; Bais et al. 2004; Harman et al. 2004; Islam et al. 2005a, b; Haas and Defago 2005).

Biological control has been developed as an academic discipline since 1970s and is now a mature science (Baker 1987; Weller et al. 2002). Most broadly, it can be defined as the suppression of damaging activities on one organism by one or more other organisms, often referred to as natural enemies. However, member of the US National Research Council took into account modern biotechnological developments and referred to biological control "as the use of undesirable organisms and to favor desirable organisms such as crops, beneficial insects, and microorganisms." The organism that suppresses the pest or pathogen is referred to as the biological control agent (BCA). Biological control of notorious plant pathogens by plant-associated beneficial bacteria has been considered as a safe, cost-effective, and an alternative strategy to the synthetic chemical pesticides

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(Whipps 2001; Schmalenberger and Tebbe 2003; Haas and Defago 2005). A large body of literature indicated that bacterial genera such as *Bacillus*, *Pseudomonas*, *Agrobacterium*, *Streptomyces*, and *Burkholderia* can suppress plant diseases through antibiosis, high plant colonization, competition for nutrients, hyperparasitism, and induced systemic resistance (Heungens and Parke 2000; Gardner and Fravel 2002; Hogan and Kolter 2002; Haas and Defago 2005; Islam et al. 2005a, b; Deora et al. 2006; Islam 2008).

The species of the genus *Lysobacter* have ability to lyse other microorganisms, including fungi, peronosporomycetes, and nematodes (Christensen and Cook 1978; Tsfasman et al. 2007). They uniquely display a number of traits that distinguish them from other taxonomically and ecologically related microorganisms, such as (1) show gliding motility (Christensen and Cook 1978; Lin and McBride 1996; McBride 2001; Sullivan et al. 2003; Islam et al. 2005a, b) (2) possess high genomic G + C content generally ranging from 65 to 72%; and (3) lack flagella but contain polar brush-like fimbriae (Islam et al. 2005b). This genus have gained broad interest for several reasons such as (1) rich source for production of a variety of novel antibiotics, such as lysobactins or katanosins (Bonner et al. 1988; O'Sullivan et al. 1988), cephabacins (Harada et al. 1984; Demirev et al. 2006; Lee et al. 2008), tripropeptins (Hashizume et al. 2001, 2004a, b), and macrocylic lactams such as xanthobaccins (Hashidoko et al. 1999; Nakayama et al. 1999; Yu et al. 2007); (2) production of a wide variety of extracellular cell wall degrading enzymes such as β-lytic proteases (Epstein and Wensink 1988; Ahmed et al. 2003), endopeptidase (Wright et al. 1998; Muranova et al. 2004), keratinases (Allpress et al. 2002), β-1,3 glucanases (Palumbo et al. 2003), cellulase (Ogura et al. 2006), and lysoamidase (Riazanova et al. 2005); (3) ability to suppress plant diseases and colonize plant surfaces (Martin 2002; Islam et al. 2004a, 2005b; Islam 2008, 2010); and (4) exhibition of wolf-pack-like micropredatory behavior (Martin 2002; Islam 2010). Some of these unique features of *Lysobacter* spp. are advantageous for using them as BCAs against phytopathogens. Meanwhile, some strains of *Lysobacter* spp. have been reported as effective agents for biocontrol of fungal, bacterial, nematode, and peronosporomycetal diseases in plants (Zhang et al. 2001; Folman et al. 2004; Islam et al. 2005a, b; Kobayashi et al. 2005; Ji et al. 2008). As the genus *Lysobacter* has close similarities to *Xanthomonas* and *Stenotrophomonas*, they were often erroneously identified as *Xanthomonas* or *Stenotrophomonas* sp. by morphological and biochemical tests (Homma et al. 1997; Hashidoko et al. 1999; Nakayama et al. 1999; Zhang and Yuen 1999; Sullivan et al. 2003; Islam et al. 2005a). However, recent development of molecular techniques for identification of bacteria makes it possible to place wrongly identified *Lysobacter* spp. phylogenetically in right place (Nijhuis et al. 2010; Yin 2010) and also easily identify new species in very high numbers (Sullivan et al. 2003; Islam et al. 2005b; Tindall and Euzéby 2006; Kawamura et al. 2009; Aslam et al. 2009; Postma et al. 2010). As a result, more than nine new species have been discovered in the last couple of years and many more new species will be identified in the near future (please see Table 15.1).

Although 23 *Lysobacter* species have already been identified, only a few of them have been tested for their biocontrol efficacy (Nakayama et al. 1999;

Table 15.1 Species/strains of *Lysobacter* spp. isolated from various sources in the environment

Species/strain	Source	References
Lysobacter brunescens	Water	Christensen and Cook (1978)
L. enzymogenes C3	Turf grass leaf surface (grass foliage)	Kobayashi and El-Barrad (1996)
L. enzymogenes 3.1T8	Root tips of hydroponic cucumber plants	Folman et al. (2003)
Lysobacter sp. SB-K88	Rhizoplane of sugar beet	Homma et al. (1993)
L. antibioticus	Rhizosphere of rice	Ji et al. (2008)
L. lactamgenus YK90	Soil	Ono et al. (1984)
L. enzymogenes N4-7	Agric soil	Kobayashi and El-Barrad (1996)
L. concretionis Ko07	Anaerobic sludge blanket reactor of waste water	Bae et al. (2005)
L. daejeonensis	Greenhouse soils	Weon et al. (2006)
L. yangpyeongensis	Greenhouse soils	Weon et al. (2006)
L. koreensis	Ginseng field	Lee et al. (2006a)
L. defulvii	Municipal solid waste	Yassin et al. (2007)
L. niabensis	Greenhouse soils	Weon et al. (2007)
L. niastensis		
L. spongiicola KMM329	Deep-sea sponge	Romanenko et al. (2008)
L. capsici	Rhizosphere of pepper	Park et al. (2008)
L. gummosus	Skin surface of salamander (amphibian)	Brucker et al. (2008)
L. antibioticus HS124	Rhizosphere	Ko et al. (2009)
L. ximonensis	Soil	Wang et al. (2009)
L. oryzae	Rhizosphere of rice	Aslam et al. (2009)
L. dokdonensis	Soil	Oh et al. (2010)
L. ruishenii	Contaminated soils	Wang et al. (2010)
L. penacitarrae	Soil of ginseng field	Ten et al. (2009)
Lysobacter sp. strain OC7	Sea water	Maeda et al. (2009)
L. xinjiangensis	Gamma-irradiated sand soil	Liu et al. (2010)
Lysobacter sp. E4	Soil	Iwata et al. (2010)
L. soli	Ginseng field soil	Srinivasan et al. (2009)
L. enzymogenes OH11	Rhizosphere of green pepper	Qian et al. (2009)
L. daecheongensis	Sediment stream	Ten et al. (2008)
Lysobacter sp.	Rhizosphere of coastal sun dune plants	Lee et al. (2006b)
L. korlensis *L. bugurensis*	Arid soils	Zhang et al. (2010)

Zhang et al. 2001; Folman et al. 2001, 2003; Islam et al. 2005a; Ji et al. 2008). Among them, two strains, namely, *L. enzymogenes* strain C3 (Zhang and Yuen 1999; Yuen and Zhang 2001; Zhang and Yuen 2000; Zhang et al. 2001; Palumbo et al. 2003, 2005; Kilic-Ekici and Yuen 2003, 2004) and *Lysobacter* sp. SB-K88 (Homma et al. 1993, 1997; Nakayama et al. 1999; Islam et al. 2004a, 2005b, Islam 2008, 2010) have been studied extensively. Both these strains have shown high promise as biocontrol of fungal and peronosporomycetal plant diseases. *Lysobacter*

spp. have also been found in disease-suppressive soils and a correlation between the abundance of *Lysobacter* spp. and disease suppression has been claimed (Postma et al. 2008, 2010). The mechanism of biocontrol of plant diseases by *Lysobacter* spp. has not been fully elucidated (Islam et al. 2005a; Islam 2008, 2010). However, several lines of evidence suggest that they exert antagonistic effects toward phytopathogens through secretion of novel antibiotics (Kato et al. 1997; Hashidoko et al. 1999; Nakayama et al. 1999; Folman et al. 2003; Islam et al. 2005a, b; Yu et al. 2007) and lytic enzymes (Zhang and Yuen 2000; Zhang et al. 2001; Palumbo et al. 2003), high plant colonization (Islam et al. 2004a, 2005b), induction of systemic resistance in host plants (Kilic-Ekici and Yuen 2003, 2004), hyperparasitism involving type III, IV, and VI secretion systems (Reedy and Kobayashi 2003; Blackmoore et al. 2009; Patel et al. 2009), and exhibition of micropredatory behaviors (Islam 2010).

Considerable interests have been given in recent years to the genus *Lysobacter* as new members of BCAs, which advances our understanding on ecology, behavior, and mode of action of these potential bacterial BCAs. A recent review titled "*Stenotophomonas* and *Lysobacter*: ubiquitous plant-associated γ-proteobacteria of developing significance in applied microbiology" has recently been published (Hayword et al. 2010). This review surveys the advances of research on biocontrol of plant diseases by bacterial antagonists *Lysobacter* spp., with special reference to *Lysobacter* sp. strain SB-K88. Mode of antagonism and potential use of *Lysobacter* spp. as biological control agents of plant diseases are also discussed.

15.2 Habitat and Taxonomic Position

Christensen and Cook proposed that *Lysobacter* spp. are ubiquitous inhabitants in soil and water (Christensen and Cook 1978). Literature survey revealed that strains of *Lysobacter* were found in diverse environment in different parts of the world, including sugar beet rhizoplane in Hokkaido, Japan (Homma et al. 1993); Kentucky blue grass foliage in Nebraska, USA (Giesler and Yuen 1998); root tips of hydroponics cultured cucumber in the Netherlands (Folman et al. 2003); groundwater of a basement tile drain in Michigan, USA (Sullivan et al. 2003); plant rhizosphere soils in China (Jiang et al. 2005); Kartchner Caverns limestone cave in Arizona, USA (Ikner et al. 2007); soils of ginseng field near Daechung lake in Korea (Lee et al. 2006a); greenhouse soils of Daejeon and Yangpyeong regions in Korea (Weon et al. 2006); deep-sea sponge in Philippines (Romanenko et al. 2008); arid soils in northwest China (Zhang et al. 2010); and rhizosphere soils of rice in Korea (Aslam et al. 2009). A list of *Lysobacter* strains and their sources of isolation is given in Table 15.1.

Lysobacter as an independent genus was first proposed by Christensen and Cook in 1978 with four species, namely, *L. enzymogenes*, *L. antibioticus*, *L. brunescens*,

and *L. gummosus*, where *L. enzymogenes* is the typed species. Originally grouped with myxobacteria, the genus *Lysobacter* belongs to the family Xanthomonadaceae within the γ-proteobacteria under the order Xanthomonadales and family Xanthomonadaceae that includes 23 named species so far (Table 15.1). However, this genus is phylogenetically related to the genera of *Xanthomonas*, *Stenotrophomonas*, *Pseudoxanthomonas*, *Thermonas*, and *Xylella*. *Lysobacter* spp. differ from these related species by some unique features such as (1) no flagella but show gliding motility; (2) high G + C contents generally ranging from 65 to 72%; (3) oxidase activity; (4) 28°C optimum growing temperature; and (5) varying cell length (2–70 mm). Recently, a new order and a family, Lysobacterales and Lysobacteraceae, respectively, have been proposed for the *Lysobacter*.

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Xanthomonadales/Lysobacterales
Family: Xanthomonadaceae/Lysobacteraceae
Genus: *Lysobacter*
Typed species: *Lysobacter enzymogenes*

Recently, several closely related species under the genus *Lysobacter* have been found in clay soils cultivated with different crops in the Netherlands (Postma et al. 2010). DNA fingerprinting and phenotypic characterization revealed that there were considerable diversification and niche differentiation among the strains belonging to *L. capsici*. The *L. capsici* strains strongly inhibit *Rhizoctonia solani* AG-2 and originate from *Rhizoctonia*-suppressive soils where also population of *L. antibioticus* and *L. gummmosus* was present. These results confirmed the presence of combined populations of closely related *Lysobacter* spp. within agricultural soils (Postma et al. 2010). *Lysobacter* spp. have also been found in extreme environmental conditions. For example, *L. xinjiangenesis* has been isolated from abandoned gold mine in the gamma-irradiated sandy soils in Xinjiang in China (Liu et al. 2010). This species is a moderately thermotolerant and alkalitolerant bacterium (Liu et al. 2010). Similarly, a chlorothalonil degrading bacterium, *L. ruishenii* strain CTN-1T, was isolated from a long-term chlorothalonil-contaminated soil in China (Wang et al. 2010). On the other hand, *L. spongiicola* KMM329 (Romanenko et al. 2008) and *L. gummosus* (Brucker et al. 2008) were isolated from deep-sea sponge and the skin surface of salamander (amphibian), respectively. Although small in number in comparison to other bacteria, *Lysobacter* spp. is distributed widely in diverse environments (Yin 2010). *L. gummosus* isolated with other bacteria has recently known to produce polyhydroxyalkanoates in the rhizosphere of sugar beet (Gasser et al. 2009). A novel nitrogen-fixing *Lysobacter* sp. strain E4 has been isolated from soil, which can accumulate high amounts of ammonium (~1.6 mM NH_4^+) in culture broth containing glucose as energy source (Iwata et al. 2010). These findings suggest that *Lysobacter* spp. are cosmopolitan and they inhabit in quite versatile environments.

15.3 Suppression of Plant Diseases by *Lysobacter* spp.

Lysobacter spp. suppress growth of the fungi, peronosporomycetes, unicellular algae, bacteria, and nematodes by secreting various lytic enzymes and/or antibiotics (Christensen and Cook 1978; Nakayama et al. 1999; Folman et al. 2003; Islam et al. 2005b). Suppression of both soil-borne and foliar plant diseases by some strains of *Lysobacter* spp. has been demonstrated by many reports (Homma et al. 1993; Kobayashi and El-Barrad 1996; Giesler and Yuen 1998; Zhang and Yuen 1999; Nakayama et al. 1999; Yuen et al. 2001; Folman et al. 2003, 2004; Islam et al. 2004a, 2005a, b; Islam 2008, 2010; Kobayashi et al. 2005; Kobayashi and Yuen 2005; Ji et al. 2008; Qian et al. 2009) (Table 15.1). Among them, only two strains, namely, *L. enzymogenes* strains C3 and *Lysobacter* sp. SB-K88 have extensively been studied for their biocontrol efficacy against several economically important plant diseases. Other species or strains that have shown biocontrol efficacy against plant diseases include *L. enzymogenes* strain 3.1T8 (Folman et al. 2003, 2004; Postma et al. 2008), *L. antibioticus* strain HS124 (Ko et al. 2009), and *L. enzymogenes* OH11 (Qian et al. 2009).

15.3.1 Suppression of Plant Diseases by *Lysobacter enzymogenes* Strain C3

On isolation from the leaf surface of turfgrass, the *L. enzymogenes* strain C3 suppresses many important plant diseases, including brown patch disease on tall fescue caused by *R. solani* (Giesler and Yuen 1998); bean rust caused by *Bipolaris sorokiniana* (Zhang and Yuen 1999, 2000; Yuen et al. 2001; Kilic-Ekici and Yuen 2004); Fusarium head blight of wheat by *Fusarium graminearum* (Yuen et al. 2003; Jochum et al. 2006), damping-off disease sugar beet by *Pythium ultimum* (Kobayashi et al. 2005; Palumbo et al. 2005); common bean rust caused by *Uromyces appendiculatus* (Yuen et al. 2001); and summer patch of Kentucky bluegrass by *Magnaporthe poae* (Kobayashi and El-Barrad 1996; Kobayashi and Yuen 2005). This strain (C3) also showed antagonistic effects against several species of nematodes including *Caenorhabditis elegans*, *Heterodera schachtii*, *Meloidogyne javanica*, *Pratylenchus penetrans*, and *Aphelenchoides fragariae* (Chen et al. 2006).

Exposure of *C. elegans* to strain C3 on agar resulted in almost complete elimination of egg production and death of 94% of hatched juveniles after 2 days. Similarly, hatch of *H. schachtii* eggs was about 50% on a lawn of strain C3 on agar as compared to 80% on a lawn of *Escherichia coli*. Juveniles that hatched on a lawn of strain C3 on agar died due to disintegration of the cuticle and body contents. *M. javanica* juveniles died after 4 days exposure to a 7-days-old chitin broth culture of strain C3. Immersion of *A. fragariae*, *M. javanica*, and *P. penetrans* juveniles and adults in a nutrient broth culture of *L. enzymogenes* strain C3 led to rapid death and disintegration of the nematodes. *L. enzymogenes* strain C3 cultures also caused

rapid immobilization of *H. schachtii* juveniles in nutrient broth and completely lysed after 3 days coincubation. These results suggest that antibiotics and enzymes produced by strain C3 are active against a range of nematode species (Katznelson et al. 1964; Chen et al. 2006). Other strains of biocontrol *Lysobacter* spp. are also needed to be tested for their biocontrol performances against phytopathogenic nematodes.

15.3.2 *Lysobacter* sp. SB-K88 Suppresses Damping-Off Diseases

Lysobacter sp. strain SB-K88 was isolated from the fibrous roots of sugar beet cultivated in Hokkaido Prefecture of Japan (Homma et al. 1993, 1997). The SB-K88 showed antimicrobial activities against many fungi and peronosporomycetes but were practically inactive against bacteria (Nakayama et al. 1999; Islam et al. 2005b) (Fig. 15.1). Application of SB-K88 as seed inoculants gave suppression of rhizomania of sugar beet (Homma et al. 1993) and damping-off diseases in sugar beet and spinach caused by *Pythium* sp. (Nakayama et al. 1999) and *Aphanomyces cochlioides* (Islam et al. 2004a, 2005b). The tetramic acid-containing macrocyclic lactam antibiotics, xanthobaccins produced by this strain have been found equally effective in the suppression of damping-off disease in sugar beet at less than micromolar concentration (Nakayama et al. 1999; Islam et al. 2004a, 2005b). In greenhouse conditions, sugar beet and spinach seedlings from seeds previously inoculated with SB-K88 (ca. 10^9 CFU/ml) were protected from damping-off disease almost equivalent to those of commercial fungicides (Islam et al. 2005b). However, a large-scale field experiment is needed for assessing practical use of this bacterial strain as biocontrol agent against damping-off diseases in sugar beet and spinach.

15.3.3 Disease Suppression by *L. enzymogenes* Strain 3.1T8 and Others

Folman et al. (2003, 2004) demonstrated that *L. enzymogenes* 3.1T8 suppresses root and crown rot diseases in cucumber plants grown in hydroponics. However, efficacy of the biocontrol of cucumber disease by this strain was somewhat unstable (Folman et al. 2003). Recently, biological control of *Pythium aphanidermatum* in cucumber grown in recirculating nutrient solution in the greenhouse was successfully achieved with the combined application of *L. enzymogenes* strain 3.1 T8 and chitosan (Postma et al. 2009). Application of strain 3.1T8 in combination with chitosan (the decayed derivative of chitin) reduced the member of diseased plants by 50–100% in four independent experiments relative to *Pythium* control. Application of chitosan or the bacterial inoculant alone was not effective. Washed bacterial cells plus chitosan inhibited *Pythium*-induced disease, but the supernatant without bacterial cells

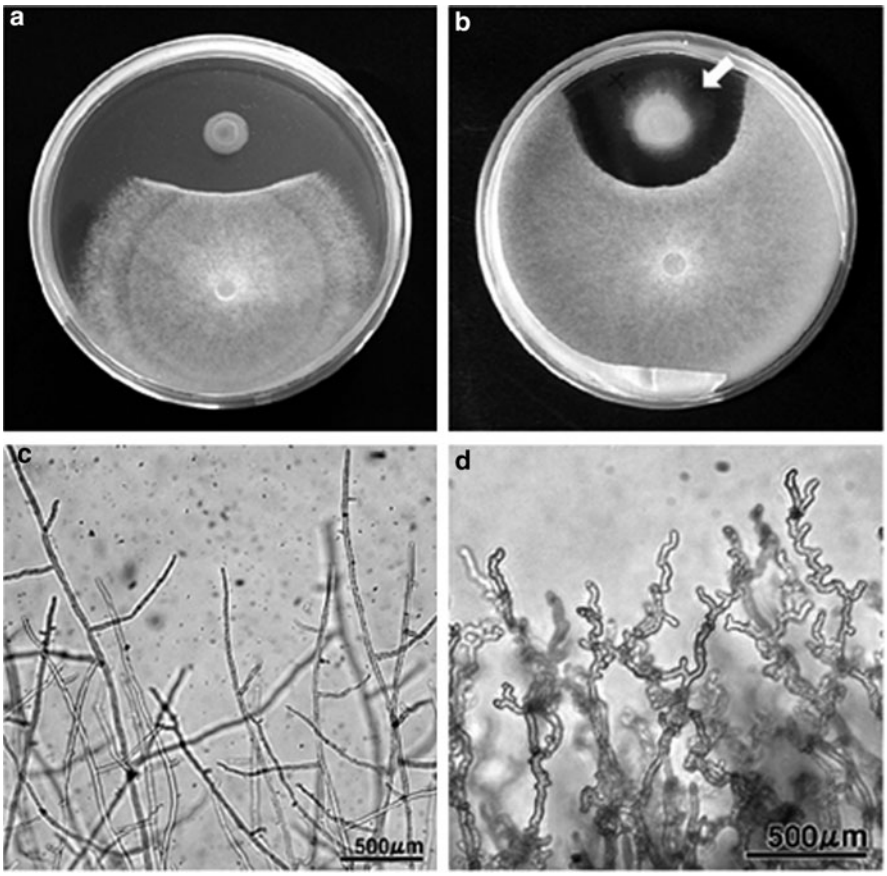

Fig. 15.1 In vitro interactions between *Lysobacter* sp. strain SB-K88 and *A. cochlioides* AC-5 in a dual culture on PDA (Islam et al. 2005). (**a**) Inhibition of AC-5 mycelial growth in the presence of SB-K88 (4 days). (**b**) Gliding motility of SB-K88 (*arrow*) on PDA (10 days) and changes in the hyphal density at the edge of the AC-5 colony. (**c**) Normal hyphal growth in a control. (**d**) Curly growth of AC-5 hyphae approaching an SB-K88 colony

combined with chitosan was not effective. Application of chitosan at the range of 0.1 and 0.03 g/plant resulted in the highest numbers of *L. enzymogenes* 3.1 T8 present in roots, i.e., 10^8–10^9 cells/g root (Postma et al. 2009). These results suggest that chitosan enhances the biocontrol efficacy of *L. engymogenes* 3.1 T8. The efficacy of oligochitosan on plant disease control has been reported (Yin et al. 2010).

L. antibioticus, which was isolated from the rhizosphere of rice, suppresses bacterial leaf blight of rice caused by *Xanthomonas oryzae* (Ji et al. 2008). Another strain (HS124) of the same species displayed suppression of late blight disease in pepper caused by *Phytophthora capsici* (Ko et al. 2009). Postma et al. (2009) claimed a correlation between the presence of *Lysobacter* spp. in disease-suppressive soils and suppression of fungal diseases. A bunch of new species of *Lysobacter*

Table 15.2 Suppression of plant diseases by species/strains of *Lysobacter*

Species/strain	Source	Suppression of disease	Name of pathogen	References
Lysobacter enzymogenes C3	Turf grass leaf surface (grass foliage)	Leaf spot of tall fescue Bean rust Fusarium head blight of wheat Brown patch of turfgrass Damping-off of sugar beet Summer patch of Kentucky bluegrass	*Bipolaris sorokiniana* *Uromyces appendiculatus* *Fasarium* sp. *Rhizoctonia solani* *Pythium ultimum* *Magnaporthe poae*	Kobayashi et al. (1996) Zhang and Yuen (1999) Yuen et al. (2001) Jochum et al. (2006) Giesler and Yuen (1998) Kobayashi et al. (2005) Kobayashi and Yuen (2005)
L. enzymogenes 3.1T8	Root tips of cucumber	Root and crown rot of cucumber	*Pythium aphanidermatum*	Folman et al. (2003, 2004)
Lysobacter sp. SB-K88	Rhizosphere of sugar beet	Rhizomania of sugar beet Damping-off of spinach and sugar beet	*Pythium* sp. *Rhizoctonia solani* and *Aphanomyces cochlioides*	Homma et al. (1997) Nakayama et al. (1999) Islam et al. (2004b, 2005a, b, 2008, 2010) Islam (2008, 2010)
L. antibioticus	Rhizosphere of rice	Bacterial leaf blight of rice	*Xanthomonas oryzae*	Ji et al. (2008)
L. antibioticus HS124	Rhizosphere	Late blight of pepper	*Phytophthora capsici*	Ko et al. (2009)
L. enzymogenes OH11	Rhizosphere of green pepper	Damping-off and root rot diseases	*Rhizoctonia solani* *Sclerotinia* sp.	Qian et al. (2009)

have been discovered in the last couple of years (Table 15.1); some of them might have potentials for biocontrol of plant diseases (Table 15.2). A comparative study including all known species of *Lysobacter* is needed to clarify their diversity in the production of antimicrobial compounds and suppression of phytopathogens.

15.4 General Active Principle and Mechanisms of Disease Suppression

The widely recognized mechanisms of biocontrol mediated by plant growth promoting rhizobacteria are competition for an ecological niche or a substrate, production of inhibitory allelochemicals (such as enzymes and antibiotics), and induction

of systemic resistance in host plants to a broad-spectrum of pathogens and/or abiotic stresses. This section reviews the advances of the active principle and mechanisms of disease suppression by the biocontrol bacteria *Lysobacter* spp.

15.4.1 Secretion of Lytic Enzymes as Mean of Biocontrol

Many bacteria including *Lysobacter* spp. produce and secrete lytic enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemicellulose, glucans, and DNA. Secretions of cell wall degrading enzymes by BCAs often result in the suppression of plant diseases. Production and secretion of a wide variety of lytic enzymes is one of the important systems contributing to broad-spectrum antagonism of *Lysobacter*. Proteases, chitinases, glucanases, endopeptidase, lipases, lysoamidase, phospholipases, keratinases, lactamases, and phosphatases produced by *Lysobacter* can degrade cell wall of many phytopathogens (von Tigerstrom 1980, 1984; von Tigerstrom and Stelmaschuk 1987; Au et al. 1991; Boras et al. 1993; Chohnan et al. 2002, 2004; Allpress et al. 2002; Ahmed et al. 2003; Palumbo et al. 2005). For example, a β-1,3-glucanase contributes significantly to biocontrol activities of *L. enzymogenes* strain C3 against fungal and peronosporomycetal diseases (Palumbo et al. 2005). Proteases were the earliest recognized enzymes that were thought to be responsible for biocontrol of nematodes (Katznelson et al. 1964). Proteases were also active against some gram-positive and gram-negative bacteria (Ensign and Wolfe 1966). However, it is unclear how much of the lytic enzyme activity that can be detected in the natural environment represents specific response to microbe–microbe interactions. Survey of literature revealed that *Lysobacter* and Myxobacteria are known to produce copious amounts of lytic enzymes and thus some of them have been known to be effective in suppressing fungal, peronosporomycetal, and bacterial plant pathogens (Kobayashi and El-Barrad 1996; Bull et al. 2002; Stepnaia et al. 2004).

Palumbo et al. (2003) have characterized three β-1,3-glucanase genes (*gluA*, *gluB*, and *gluC* genes) of *L. enzymogenes* N4-7 at molecular level. In fact, strain N4-7 produces multiple biochemically distinct extracellular β-1,3-glucanase enzymes (Holtman 1998). Suppression of late blight disease in pepper through secretion of lytic enzymes such as chitinase, β-1,3-glucanase, lipase, and protease in concert with the release of antibiotic compound 4-hydroxyphenylacetic acid by *L. antibioticus* strain HS124 has also been demonstrated (Ko et al. 2009). In vivo experiments in the greenhouse revealed that the growth of pepper plants treated with *L. antibioticus* culture was enhanced, resulting in greater protection from the blight disease. Optimum growth and protection was achieved when culture were grown in the presence of Fe (III). Additionally, the activities of pathogenesis-related proteins such as chitinase and β-1,3-glucanase decreased in roots, but increased in leaves with time after treatment compared to control.

15.4.2 Antibiosis as a Means of Biocontrol

Besides enzyme activity, antibiotics produced and secreted by *Lysobacter* are also thought to play significant role in the biological control of plant diseases. A broad-spectrum phenazine antibiotic, myxin, produced by a strain of *Lysobacter* (which was classified as *Sorangium*), has been reported in the mid-1970s (Peterson et al. 1966). *Lysobacter* spp. produce diverse antibiotics with novel mode of actions. Christensen (2001) isolated and identified a broad-spectrum antibiotic, namely, 1-hydroxy-6-methoxyphenazine from *L. antibioticus*. Kato and coworkers (1998) identified macrocyclic peptide antibiotics that were very active against MRSA *Staphylococcus aureus*. Similarly, a polyketide antibiotic, 2,4-diacetylphloroglucinol, has been identified from a *Lysobacter* sp. isolated from the skin of salamander (*Plethodon cinereus*) (Brucker et al. 2008). Among the antibiotics isolated from *Lysobacter* spp., the tetramic acid-containing macrocyclic lactams are found predominantly in some strains that have high potential for biocontrol of plant and fungal diseases (Fig. 15.2).

15.4.2.1 Secretion of Macrocyclic Lactam Antibiotics by SB-K88

The *Lysobacter* sp. SB-K88 suppresses damping-off diseases in sugar beet and spinach. EtOAc extracts of culture supernatants of SB-K88 displayed potent antimicrobial activities against many fungi and peronosporomycetes. Chromatographic separation followed by spectroscopic identification revealed that SB-K88 produces at least three novel antibiotics: xanthobaccin A, B, and C (Hashidoko et al. 1999; Nakayama et al. 1999) (Fig. 15.2). The R_f values of xanthobaccin A, B, and C on silica gel thin layer plates with a solvent system $CHCl_3$–MeOH–H_2O (65:25:4) were 0.49, 0.43, and 0.67, respectively (Nakayama et al. 1999). These compounds have a characteristic 5,5,6-tricyclic skeleton and a tetramic acid chromophore (Hashidoko et al. 1999). Detailed chemical structure of xanthobaccin A has been elucidated by Hashidoko and coworkers in 1999. The plane structure of xanthobaccin A is the same as that of a known antibiotic maltophilin, which was isolated from a rhizobacterium of rape *Stenotrophomonas maltophilia* R3089 (Jacobi et al. 1996). Both xanthobaccins and maltophilin belong to a group of tetramic acid-containing macrocyclic lactam antibiotics. This group of rare metabolites also includes other structures such as the marine natural products, cylindramide A (Kanazawa et al. 1993), discodermide (Gunasekera et al. 1991), and alteramide (Fig. 15.2) (Shigemori et al. 1992).

Due to the unique features and biological activities such as antibiotics, anticancer, antiprotozoal, and antioxidant activities, there have been extensive research interests in total synthesis of these scarce natural products (Boeckman et al. 1989; Cramer et al. 2005, 2006). Although the chemical structures of xanthobaccin B and C have not yet been fully elucidated, spectroscopic analyses revealed that XB-B has a hydroxyl group substitution at position 27 in the six-member ring of XB-A

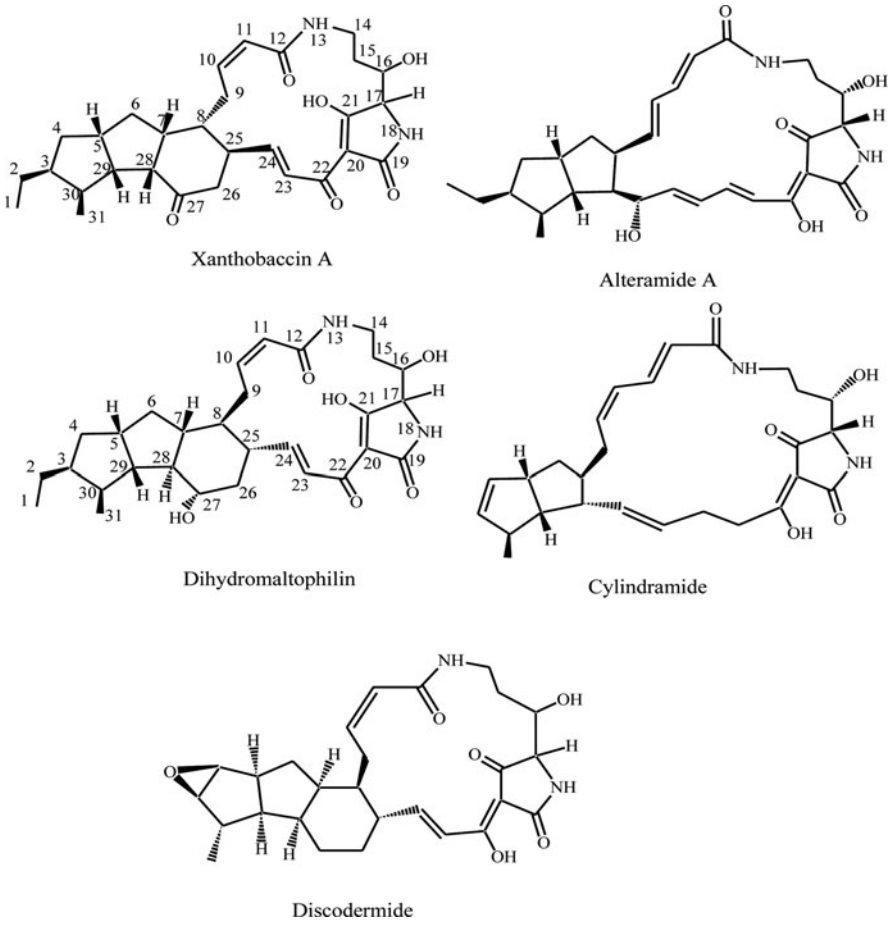

Fig. 15.2 Structure of some biologically active macrocyclic lactam antibiotics from *lysobacter* spp. and other microorganisms

(Nakayama et al. 1999). Literature survey also revealed that the structures of xanthobaccins and dihydromaltophilin (Yu et al. 2007) are closely related to the structure of ikarugamycin, an antibiotic which was produced by *Streptomyces phaeochromogenes* subsp. *ikaruganensis*. However, both xanthobaccins and maltophilin displayed potent antifungal and antiperonosporomycetal activities in vitro but no antibacterial activity, whereas ikarugamycin has been reported as an antibacterial metabolite against gram-positive bacteria (Ito and Hirata 1977). Although detailed structure–activity relationships have not yet been established, however, the differences of antimicrobial spectra of these antibiotics may be associated with the structural differences in their tricyclic skeletons.

The macrocyclic lactam antibiotics, xanthobaccins, displayed multifarious antagonistic effects against *A. cochlioides* and *Pythium* sp. In vitro dual culture

on PDA medium, SB-K88, or xanthobaccin A demonstrated *A. cochlioides* hyphal growth inhibition. Microscopic observation of *A. cochlioides* hyphae growing close to SB-K88 colonies revealed marked alterations in hyphal morphology, including excessive branching, irregular swelling, and curling of hyphal tips and loss of apical growth (Fig. 15.1) (Islam et al. 2005b). Confocal laser scanning microscopy (CLSM) using rhodamine-conjugated phalloidin revealed that excessive branching and curling of hyphae of *A. cochlioides* induced by SB-K88 are linked to the disruption of filamentous actin (F-actin) organization in the hyphal cells (Fig. 15.3) (Islam 2008). The major secreted xanthobaccin A from SB-K88 might be involved in disruption of F-actin in the *A. cochlioides* cells. However, these organizational changes of the dynamic cytoskeletal protein, actin, were reversible. The cytoskeleton is considered as a regulator and target of biotic interactions in the rhizosphere (Tekemoto and Hardham 2004). This study provides convincing evidence that a rhizoplane bacterium, *Lysobacter* sp. SB-K88, has direct inhibitory effect on the growth and development of damping-off pathogen *A. cochlioides* living in the same ecosystem. This activity is attributable to a combination of cytoskeletal F-actin disruption in the zoospores and hyphal cells.

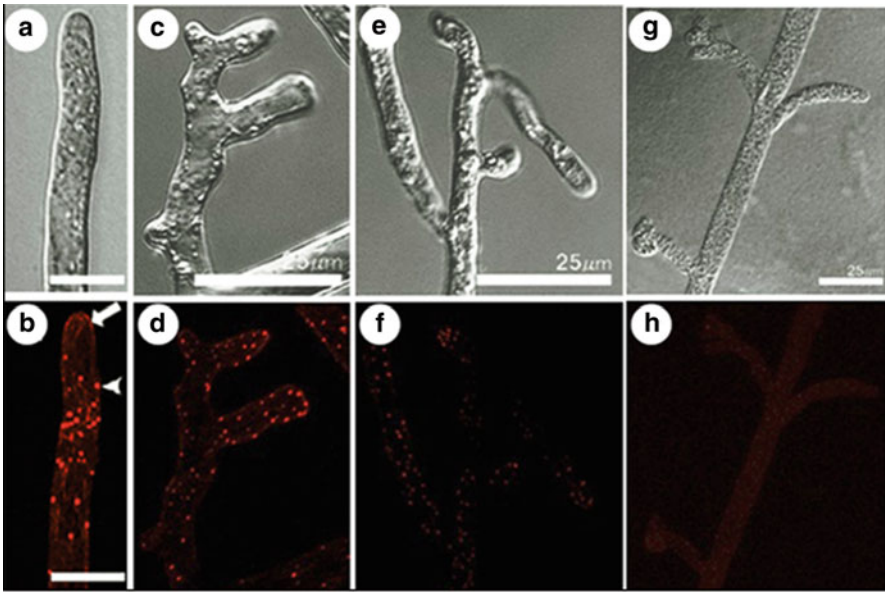

Fig. 15.3 Differential interference contrast (DIC) micrographs (**a**, **c**, **e**, **g**) and their corresponding confocal images (**b**, **d**, **f**, **h**) showing F-actin organization in control and excessively branched and curled hyphae (induced by *Lysobacter* sp. SB-K88) of *Aphanomyces cochlioides* (adapted from Islam (2008)). (**b**) Normal organization of F-actin in *A. cochlioides* hyphae (control). *Arrow* and *arrowhead* indicate distinct cap (arrays only) and a plaque of F-actin, respectively. (**d**, **f**, **h**) Disorganized and disrupted F-actin in the excessively branched and curled hyphae. The plaques became smaller and the arrays were disorganized and concentrated irregularly. *Bars* in (**a**, **b**) indicate 10 μm

Disruption of cytoskeletal protein in cells of many organisms by a marine natural product, latrunculin B, has been reported (Heath et al. 2000). A parallel TEM analysis also convincingly proved that the excessive branching and curling that occurred were due to marked ultrastructural alterations, including invagination of membrane, disintegration or necrosis of cell wall, accumulation of excessive lipid bodies, enlarged electron-dense vacuoles, and disintegration of cytoplasm (Fig. 15.4) (Islam 2008). CLSM analysis revealed that the number of lipid bodies and activities of mitochondria were markedly increased in the affected hyphae as visualized by vital stains (Fig. 15.5) (Islam 2010). It is evident that these ultrastructural changes occurred in hyphal cells were largely due to the effects of secreted macrocylic lactam antibiotics, xanthobaccins in concert with lytic enzymes (Islam et al. 2005b).

Peronosporomycete phytopathogens infect plants through production of biflagellat motile zoospores (Islam et al. 2001, 2002). The zoospores are believed to locate host plants guided by host-specific signal(s) and then differentiate into round cystospores by shedding flagella (Islam and Tahara 2001; Islam et al. 2003, 2004b). The cystospores subsequently germinate to form germ tubes to invade host tissues for infection. Disruption of any stage of preinfection eliminates the chance of infection by the peronosporomycetes (West et al. 2003).

Interestingly, zoospores of a damping-off pathogen *A. cochlioides* (peronosporomycete) became immotile within 1 min after exposure to a SB-K88 cell suspension, a cell-free supernatant of SB-K88, or pure xanthobaccin A (MIC, 0.01 μg/ml) (Islam et al. 2004a, 2005b). In all cases, lysis followed in most of the spores within 30 min in the presence of the inhibiting factor(s). This low inhibitory concentration of xanthobaccin A appears within the reasonable range of an antimicrobial agent and may meet the requirements for application in rhizosphere settings (Nakayama et al. 1999). In an experiment involving SB-K88-inoculated sugar beet seeds that were germinated and grown under hydroponic conditions, the level of xanthobaccin A in the rhizosphere was estimated to be 3 μg/plant (Nakayama et al. 1999). Thus, it is possible that at the root surface the levels of dissolved xanthobaccin A are substantially higher than the MIC for *A. cochlioides* zoospore motility and survival in vitro, suggesting that the biocontrol exhibited against this pathogen is linked to the production of a *Lysobacter* sp. strain SB-K88-derived antibiotic at the root surface.

Zoospores that were halted but not lysed due to the action of lower concentration of EtOAc extracts of SB-K88 culture filtrates became round cystospores. These cystospores had diminished F-actin plaques compared to untreated control (Islam 2008). On the other hand, cystospores that were able to germinate in the presence of low concentration of EtOAc extracts or known actin inhibitor, latrunculin B, had disrupted F-actin and malformed germ tubes. As radial F-actin is known to participate in initiation and maintenance of tip formation and growth (Bachewich and Heath 1998), it is now clear that SB-K88 extracts contain actin disrupting compounds. Disruption of F-actin by several marine natural products has also been reported. For example, F-actin disruption in human cells by marine natural products such as pectenotoxin-6 (Leira et al. 2002) and okadaic acid (Fiorentini et al. 1996)

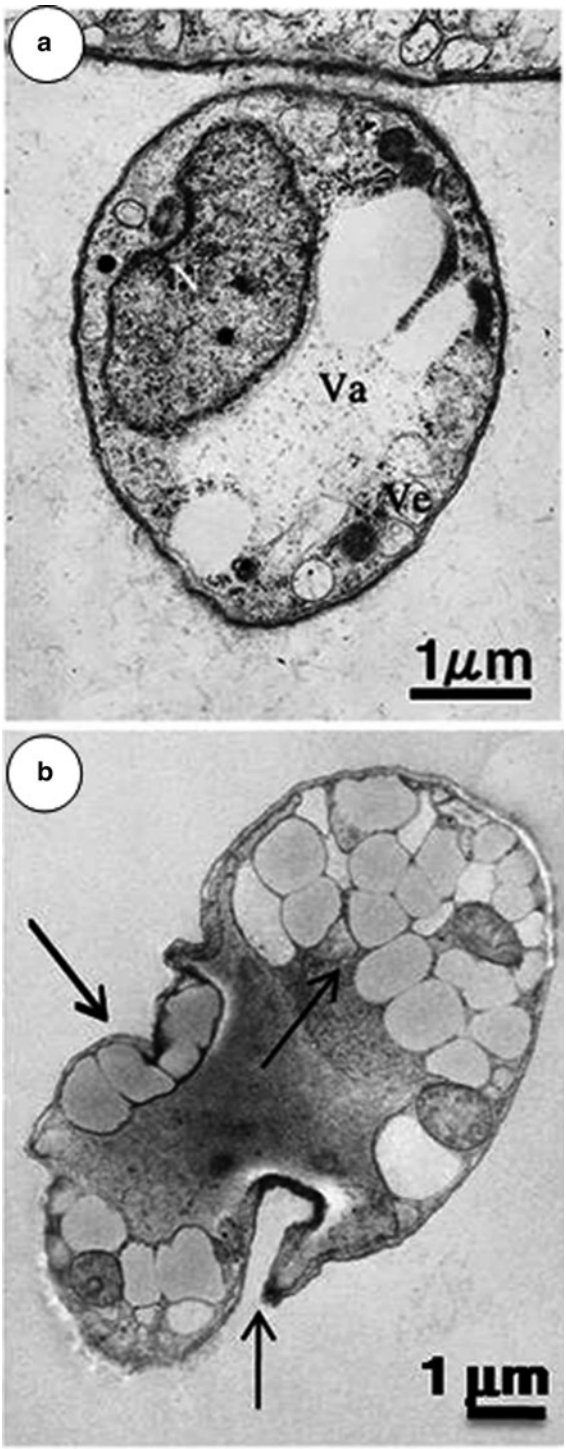

Fig. 15.4 Transmission electron micrographs of cross sections of *Aphanomyces cochlioides* AC-5 hyphae growing toward colonies of *Lysobacter* sp. SB-K88 at day 5 of interactions on PDA medium (adapted from Islam (2008)). (**a**) A cross section of a control hypha. (**b**) Excessive lipid bodies and cytoplasmic disintegration in a longitudinal section of the affected hyphae

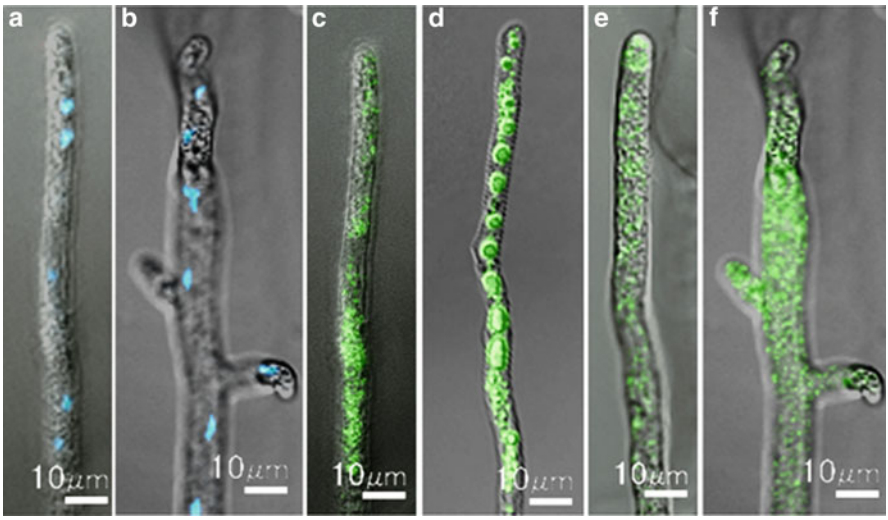

Fig. 15.5 Confocal laser scanning micrographs showing intracellular changes of nuclei, lipid bodies, and mitochondrial activity in *Apahnomyces cochlioides* hyphae challenged with *Lysobacter* sp. SB-K88 on PDA medium (Islam 2010). (**a**) Control hypha, nuclei oval-shaped. (**b**) Affected hypha with nuclei spindle-shaped. (**c**) Control hypha with lipid bodies organized into the cytoplasm. (**d**) Affected hypha with lipid bodies sank into the vacuoles. (**e**) Control hypha, mitochondrial density lower. (**f**) Affected hypha with higher mitochondrial density near the affected area

has also been reported. Further bioassay-guided chromatographic separation and spectroscopic identification of F-actin disrupting molecule(s) secreted from SB-K88 are needed. Cocultivation of *A. cochlioides* and SB-K88 in liquid broth medium revealed that SB-K88 substantially inhibited growth and decomposed AC-5 mycelia and suppressed release of zoospores. The excised root tips of sugar beet seedlings were less attractive to AC-5 zoospores (Islam 2010). However, mechanism of this deleterious effect to phytopathogen is poorly understood.

15.4.2.2 Antibiosis as Mode of Action of Biocontrol by *L. enzymogenes* Strain C3 and Others

An analogue of xanthobaccins, dihydromaltophilin, has recently been identified as a heat stable antibiotic factor (HSAF) from another extensively studied biocontrol bacterium *L. enzymogenes* strain C3 (Yu et al. 2007; Li et al. 2008). The HSAF is a colorless powder. HR-EI-MS of HSAF gave an *m*/*z* 513.2954 for $[M\ (C_{29}H_4O_6N_2) + H]^+$ (calculated 513.2959). This compound exhibits a wide range of antifungal activities and shows a novel mode of action by disrupting the biosynthesis of a distinct group of sphingolipids (Giesler and Yuen 1998). Bioassay revealed that, at a concentration of 0.3–1.5 μM, HSAF arrested spore germination *B. sorokiniana* and caused reduced hyphal extension or profuse branching of

F. graminearum and *F. verticilloides* (Yu et al. 2007). Reduction of germination of spores and polar growth and induction of excessive branching in fungal hyphae by HSAF might be linked to the disruption of filamentous actin organization in the hyphal cells as demonstrated by SB-K88/xanthobaccins in *A. cochlioides* (Islam 2008). In both strains C3 and SB-K88, nutritionally limited media were found suitable for the production of macrocyclic lactam antibiotics. Similarly, *L. antibioticus* HS124 suppresses blight diseases in pepper caused by *Phytophthora capsici* through the production of an antibiotic compound 4-hydroxyphenylacetic acid in concert with various lytic enzymes (Ko et al. 2009). The purified antibiotic 4-hydroxyphenylacetic acid strongly inhibited the hyphal growth of *P. capsici*.

The concept that environmental sensing mechanisms modulate production of many antifungal factors is well understood. Nakayama et al. reported that the amount of xanthobaccin A produced per SB-K88 cell was approximately 1×10^4- to 1×10^5-fold larger in the rhizoplane near attached SB-K88 than in liquid cultured cells (Nakayama et al. 1999). Further research is required to elucidate the mechanisms underlying sensing of the rhizoplane environment and simultaneous production of macrocyclic lactam antibiotics by *Lysobacter* spp. Quantitatively large production of heat stable, tetramic acid-containing macrocyclic lactam antibiotics structurally close to maltophilin is a shared trait by *Lysobacter* spp. that implies the ecological importance of *Lysobacter* biocontrol strains since these antibiotics are known to be effective on inhibition of fungal and peronosporomycetal diseases.

15.4.2.3 Biosynthetic Origin of Macrocyclic Lactam Antibiotics

The genetic locus responsible for the biosynthesis of HISAF in strain C3 has also been identified (Yu et al. 2007). DNA sequencing followed by antifungal bioassay of the developed mutants revealed that a hybrid polyketide synthase-nonribosomal peptide synthase (PKS-NRPS) gene is responsible for biosynthesis of the unique macrocyclic lactam system that is found in many biologically active natural products isolated for marine organisms (Shigemori et al. 1989; Gunasekera et al. 1991; Kanazawa et al. 1993). The analyses of the chemical structure of HSAF also suggest that an ornithine residue is incorporated into the lactam functionalities and that the biosynthesis of HSAF could involve both polyketide and nonribosomal peptide mechanisms, as found in bleomycins and other natural products (Du et al. 2003). Recently, two novel compounds in this group, namely, frontalamide A and frontalamide B have been discovered from a *Streptomyces* strain associated with the southern pine beetle (see structures in Fig. 15.2) (Blodgett et al. 2010). Genome mining efforts revealed strikingly conserved frontalamide-like biosynthetic clusters in the genomes of phylogenetically diverse bacteria ranging from proteobacteria to actinomycetes. These findings suggest the common biosynthetic origins for polyketide tetramate macrolactams from phylogenetically diverse bacteria.

15.4.3 Plant Colonization and Biofilm Formation

Both in vitro and in vivo experiments revealed that *Lysobacter* can colonize both leaves and roots of plants (Sullivan et al. 2003; Islam et al. 2005a, b; Postma et al. 2010). Islam et al. (2005a, b) investigated plant colonization behavior of a *Lysobacter* sp. SB-K88 by the aid of scanning electron microscopy (SEM) (Fig. 15.6). Their SEM analyses of 2-week-old sugar beet and spinach seedlings from seeds previously inoculated with SB-K88 revealed dense colonization on both the root and leaf surfaces in a characteristic perpendicular fashion. In colonized

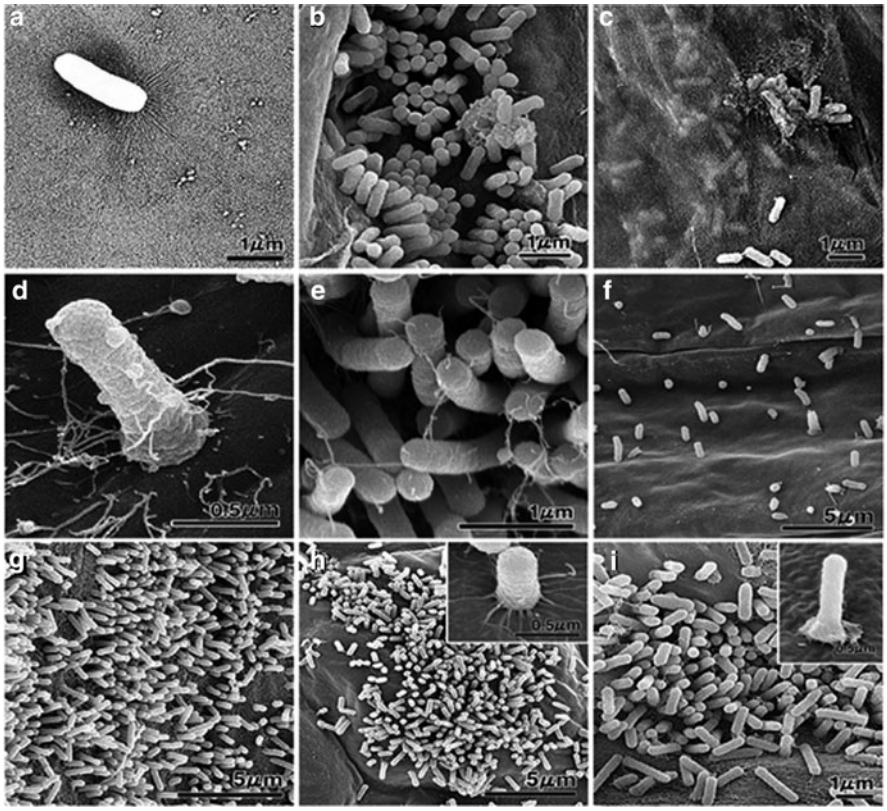

Fig. 15.6 TEM (**a**) and SEM (**b–i**) micrographs illustrating the morphology of *Lysobacter* sp. strain SB-K88 (**a**) and colonization of SB-K88 (**b–i**) on plant surfaces upon inoculation of seeds and seedlings grown in the gellan gum-based medium (**b–d**, **g–i**) or soil (**f**) (Islam et al. 2005b). (**a**) TEM micrograph of a sessile SB-K88 bacterial cell having large, brush-like fimbriae at one end. (**b**) Colonization on sugar beet root by perpendicular attachment. (**c**) Bacterial biofilm that developed under a semitransparent film of sugar beet root mucigel. (**d**) Typical perpendicular attachment of a bacterial cell to a sugar beet cotyledon. (**e**) High-density perpendicular attachment and colonization on the sugar beet leaf surface after immersion into an SB-K88 bacterial suspension (ca. 10^5 CFU/ml). (**f**) Colonization of sugar beet root. (**g**) Colonization of tomato root. (**h**) Colonization of *Arabidopsis thaliana* leaf. (**i**) Colonization of *Arabidopsis thaliana* root

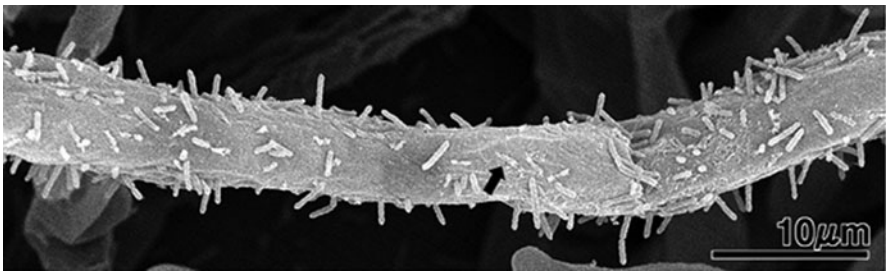

Fig. 15.7 SEM micrograph showing perpendicular attachment of *Lysobacter* sp. strain SB-K88 to a hypha (Islam et al. 2005b). The *black arrow* indicates unidentified granular deposits on the surface of the *A. cochlioides* hyphae colonized by SB-K88. No such granular deposits were observed on the surfaces of untreated control hyphae (not shown)

regions, a semitransparent film apparently enveloping the root and microcolonies were observed on the sugar beet root surface (Islam et al. 2005a). This *Lysobacter* strain also efficiently colonized the roots of several plants, including spinach, tomato, *Arabidopsis thaliana*, and *Amaranthus gangeticus*. Interestingly, the SB-K88 also colonized *A. cochlioides* hyphal surface in the same perpendicular manner when grown together on liquid medium (Fig. 15.7). To the best of my knowledge, perpendicular attachment of biocontrol bacterium to both plant and hyphal cell walls has not been reported (Islam et al. 2005b; Islam 2010). How a rod-shaped bacterium stands on plant and hyphal cell walls? Detailed transmission electron microscopic analysis revealed that the SB-K88 has long (~6 μm) brush-like, fragile fimbriae at one pole of the dividing bacterial cells (Fig. 15.6a).

As fimbriae are known to function in bacteria to adhere to the substrates, it is reasonable to assume that brush-like fimbriae help SB-K88 to attach perpendicularly on plant and hyphal cell walls as well as for gliding motility. Presence of fimbriae appears to be characteristic structural features of bacteria having gliding motility (Spormann 1999). A further study is needed to confirm whether perpendicular mode of attachment is a generic character of *Lysobacter* or not. Biofilm formation accompanying this perpendicular mode of attachment in theory could support a larger population of rod-shaped bacteria on a given plant surface (Islam et al. 2005a, b). Also, establishing a clearer understanding of the SB-K88's mechanism of perpendicular attachment to plant and hyphal surfaces may be important in realizing its potential as a biocontrol agent. Determining whether this phenomenon is characteristic of the genus *Lysobacter* in general may also help workers find other useful species (Islam et al. 2005b). Effective colonization of both foliar and subterranean plant parts by *L. enzymogenes* C3 has also been demonstrated (Sullivan et al. 2003).

15.4.4 Induced Systematic Resistance

Several species of bacterial and fungal biocontrol agents (BCAs) have been shown induced systemic resistance in host plants against the pathogens. One of the strains

of *Lysobacter* spp., i.e., strain C3, has also been shown induced systemic resistance in the host plants (Kilic-Ekici and Yuen 2003). The authors compared strain C3 in growth chamber experiments with other strains of *L. enzymogenes*, strains of plant growth promoting rhizobacteria (PGPR) that induce systemic resistance in dicot plants, and the synthetic elicitor 1,2,3-benzothiadiazole-7-thiocarboxylic acid-*S*-methyl-ester (BTH). They assessed and compared the effects of treatments (1) for induction of localized or systemic resistance against *B. sorokiniana* when applied to leaves and roots, and (2) the effects of induced resistance on pathogen's conidial germination on the phylloplane. None of the bacterial or chemical treatments induced any systemic resistance when applied to a leaf. Strains of *L. enzymogenes* differed in their ability to cause localized disease inhibition following foliage treatment and to induce systemic resistance in leaves when applied to roots. In contrast to strain C3, two other strains of *L. enzymogenes* were ineffective in inducing systemic resistance, indicating that this trait may not be a generic character of *Lysobacter*. The PGPR strains varied in effectiveness in causing localized disease inhibition when applied to the leaves. Most of the bacterial strains increased peroxidase activity in the treated leaves, providing evidence that localized disease inhibition may have been plant mediated. The involvement of localized induced resistance was confirmed in *P. fluorescens* WCS417r, which did inhibit *B. sorokiniana* conidial germination or hyphal growth in vitro. Soil drenches with nearly all PGPR strains resulted in systemic resistance in leaves, but the treatments varied as to the timing and strength of induced systemic resistance. BTH induced localized resistance when applied to leaves but did not activate resistance in leaves when applied to roots. All cases of induced resistance were associated with an inhibition of conidial germination on leaf surfaces and, thus, this reaction appears to be a hallmark of induced resistance in the *B. sorokiniana*–tall fescue pathosystem.

Further work with more species and strains of biocontrol *Lysobacter* spp. is needed for the better understanding of this important trait of biocontrol *Lysobacter* spp. Inoculation of SB-K88 in vitro enhanced vegetative growth (above ground) of sugar beet seedlings; however, root morphology was remarkably changed. Development of root hairs and polar growth of primary and lateral roots were shorted and thicker than that of uninoculated seedlings. A detailed study is needed to confirm whether the secreted chemicals and/or colonization of SB-K88 cause any effect on the root morphology of plants.

15.4.5 Wolf-Pack Predation

One of the unique features of *Lysobacter* spp. is that they exhibit micropredatory behavior like Myxobacteria (Martin 2002). Islam (2010) demonstrated that *Lysobacter* sp. SB-K88, which suppresses damping-off diseases in sugar beet and spinach, aggregates around the dead cystospores of *A. cochlioides*. As a result, the cystospores lysed within few hours probably by secretion of macrocylic lactam antibiotics in concert with lytic enzymes from the bacterial cells (Islam 2010).

Lysobacter spp. and members of the bacteria, such as *Myxococcus xanthus*, tend to attack prey as groups, which is known as wolf-pack predation (Burnham et al. 1981; Lueders et al. 2006). Such group predation can be accomplished remotely via the secretion of diffusible compounds that kill and decompose hapless neighboring prey. Alternatively, some predatory compounds may be normally envisaged as a predator swarm that invades and decimates a prey colony on a solid surface; some pack predators (e.g. *Myxococcus* spp.) can surround and entrap their prey as a group even in aquatic environment. Although wolf-pack predation of bacteria against other prokaryotic organisms has been reported, predatory behavior of a prokaryotic against a eukaryotic organism is scarce. Therefore, molecular details and the sensing mechanism of *Lysobacter's* wolf-pack predation are needed to be clarified. Micropredatory behavior of *Lysobacter* may be a useful trait for biocontrol of plant diseases by these bacterial antagonists (Islam 2010).

15.4.6 Production of Allelochemicals and Growth Promoting Effects

Although *Lysobacter* is famous for production and secretion of antibiotics and lytic enzymes, however, reports on direct plant growth promoting effects of *Lysobacter* are scarce. Recently, Iwata and coworkers (2010) demonstrated that *Lysobacter* sp. strain E_4 can fix nitrogen under free-living conditions. It produces higher amounts of ammonia (~1.60 mM NH_4^+ conc.) in media containing glucose (Iwata et al. 2010). Therefore, this strain can be used as a biofertilizer as an alternative to synthetic nitrogenous fertilizers such as urea. As new species are being discovered every year, it is not unlikely to get similar biofunctional *Lysobacter* spp. in the days to come. It may be interesting to know whether the nitrogen fixer *Lysobacter* sp. can also produce antimicrobial substances or not.

15.5 Conclusion and Perspective

Lysobacter spp. are ubiquitous inhabitants in nature that possess some characteristic features, including gliding motility and high genomic G + C ratio, and produce lytic antibiotics and enzymes. Some strains of *Lysobacter* spp. have shown high promise in biocontrol of economically important plant diseases through multifaceted mechanisms including antibiosis, high plant colonization, induced systematic resistance, and micropredatory activity. The full potentials of *Lysobacter* spp. have not yet been evaluated for biocontrol of plant diseases in the field conditions. As a large number of new species have been discovered very recently, a growing research interest will certainly be given to judge the potentials of *Lysobacter* as biocontrol agents in the practical field of agriculture.

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