

# Chapter 4

## Rhizosphere Bacteria from Coastal Sand Dunes and Their Applications in Agriculture

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### 4.1 Introduction

Coastal sand dunes are a nutrient-limited ecosystem. Their types and vegetation vary from place to place.

#### 4.1.1 Coastal Sand Dune Ecosystem

The word sand dune reflects the images of vast amount of shifting sand barren to plants and hostile to human habitation. Sand dunes are generally of two types. The first type is the extremely dry interior deserts such as Sahara in Africa or Rajasthan in India, and the other type is known as the coastal sand dunes which occur along the coasts of the Atlantic, Pacific, North America, and Australia. In Asia the coastal dunes occur in Japan, India, and several other countries (Desai and Untawale 2002; Boorman 1977; Carter 1998).

##### 4.1.1.1 Sand Dune Vegetation

Vegetation plays a dominant role in determining the size, shape, and stability of fore dunes (Fig. 4.1). The aerial parts of the vegetation obstruct the wind and absorb wind energy. Wind velocity near vegetation is thus reduced below that needed for sand transport and hence the sand deposit around the vegetation. A characteristic of dune vegetation, particularly the grasses growing under these conditions, is its ability to produce upright stems and new roots in response to sand covering.

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**Fig. 4.1** Beach grasses and shrubs growing on coastal sand dunes



The development of vegetation cover on newly formed dunes, if undisturbed, creates conditions which suit the colonization and growth of a wider range of plant species. Dead plants and litter from these plants add humus to the sand. The accumulation of humus results in improved moisture- and nutrient-holding capacity of developing dune soils. Thus, with lower surface temperature and increased moisture and nutrient content, the sand is able to support a great variety of plants (Desai and Untawale 2002).

## 4.2 Rhizosphere as a Site of Plant: Microbe Interactions

The rhizosphere is the portion of the soil under the direct influence of the roots of higher plants. It is considered the most intense ecological habitat in soil in which microorganisms are in direct contact with plant roots. The root system of all higher plants is associated with a distinct, diverse community of metabolically active soil microbiota that carries out biochemical transformations. Rhizosphere microorganisms may have specific associations with plants through which they exert their influence on plant growth. The production of biologically active metabolites, particularly the plant growth regulators by rhizosphere microbiota, is considered one of the most important mechanisms of action through which the rhizosphere microbiota affect plant growth directly after being taken up by the plant or indirectly by modifying the rhizosphere environment. The plant rhizosphere is a dynamic environment in which many factors may affect the structure and species composition of the microbial communities that colonize the roots. Microbial communities associated with the rhizosphere also vary depending on the plant species, soil type, and cultural practices such as crop rotation or tillage (Frankenberger and Arshad 1995; Davison 1988).

Bacteria can form close associations with roots within the root tissue itself, on the root surface (rhizoplane), and within the soil immediately adjacent to the root (rhizosphere). Inhabitants of these sites rely heavily for their energy supply on organic substances provided by the roots, and their growth is therefore related intimately to the metabolic activity of the plants involved (Gaskins et al. 1985). While many bacteria found in soil are bound to the surface of soil particles and are found in soil aggregates, a number of soil bacteria interact specifically with the

roots of plants. In fact, the concentration of bacteria (per gram of soil) that is found around the roots of the plants (i.e., in the rhizosphere) is generally much greater than the bacterial density, or concentration, that is found in the zone around the roots and can be used to support bacterial growth and metabolism (Glick 1995; Alexander 1977). The rhizobacteria respond to plant signals, exchange nutrients with plant cells, suffer damage due to plant defense responses, and colonize or even evade root tissues, creating pathologies or symbiosis as compared to the bacteria present in bulk soil. Mucigel provides the immediate environment for rhizobacteria; it consists of plant mucilage, bacterial exopolymers, and soil particles. Plant roots sheathed with mucigel have higher relative water content than do bare roots, and thus mucigel protects the root and associated microflora from dehydration (Miller and Wood 1996).

The constituents of root exudates play an important role in selecting and enriching the types of bacteria. Depending on the ability of the bacteria to utilize these as sources of energy, the bacterial community develops in the rhizosphere. Plant root exudate components serve as a source of carbon substrate for microbial growth; in addition they also contain chemical molecules that promote chemotaxis of microbes to the rhizosphere. Root exudates are supplemented in maintaining a steady concentration of flavonoids and mineral nutrients in the rhizosphere by the compounds released from the decomposition of organic matter such as dead roots and fallen leaves (Dakora and Phillips 2002). Thus, depending on the nature and concentrations of organic constituents of exudates, and the corresponding ability of the bacteria to utilize these as sources of energy, the bacterial community develops in the rhizosphere. Bacteria living in the soil are called free-living as they do not depend on root exudates for their survival, while rhizospheric bacterial communities have efficient systems for uptake and catabolism of organic compounds present in root exudates. Several bacteria have the ability to attach to the root surfaces (rhizoplane) allowing these to derive maximum benefit from root exudates. Some of these are more specialized, as they possess the ability to penetrate inside the root tissues (endophytes) and have direct access to organic compounds present in the apoplast. It is also known that some of the Plant growth-promoting rhizobacteria (PGPR) strains can colonize inside plant tissues, and bacterial strains that naturally exist in healthy plant tissues are referred to as "endophytes." Hallmann et al. (1997) defined endophytic bacteria as "bacteria that can be isolated from surface disinfested plant tissue or extracted from within the plant, and that do not visibly harm the plant." Most of the endophytes reported previously were isolated by maceration of surface-sterile plant tissues. Various endophytes have been isolated from agronomic crops and prairie plants (Hallmann 1997; Weller 1988), and many of them have been utilized as microbial inoculants to control plant pathogens and promote plant growth. By occupying this privileged endophytic location, bacteria do not have to face competition from their counterparts as encountered in the rhizosphere or in soil. Such bacteria which influence the plant growth either directly or indirectly are termed as plant growth-promoting bacteria (PGPB). They inhabit majority of healthy and symptomless plants, in various tissues, seeds, roots, stems, and leaves (Johri 2006). Plants benefit

extensively by harboring these endophytic microbes; they promote plant growth (Compant et al. 2005) and confer enhanced resistance to various pathogens by producing antibiotics. Endophytes also produce unusual secondary metabolites of plant importance. It has been suggested that the presence of a mutualistic endophyte acts as a “biological trigger” to activate the stress response system more rapidly and strongly than nonmutualistic plants (Bandara et al. 2006).

### 4.3 Bacteria Associated with Sand Dune Vegetation

Little is known about the bacterial communities associated with the plants inhabiting sand dune ecosystems. Accelerated coastal erosion threatens private and public property in many areas of the world. Lost sand is replaced with material of compatible physical properties which is shaped to the desired beach profile and planted with pioneer species, such as *Ipomoea* and *Spinifex*, to enhance beach stability and begin the dune-building process. The major factors limiting establishment and early, vigorous growth of dune plants in the face of environmental extremes are infertility and the poor moisture-holding capacity of coarse replenishment materials. Rhizosphere microorganisms may allow beach grasses to overcome these environmental extremes (Will and Sylvia 1990). Plants are known to alter the composition of microbial communities associated with their roots (Grayston et al. 1996; Marschner et al. 2001). Plant roots in the soil represent a four-dimensional region, in space and time, of profuse activity relative to the bulk soil, revolving around pH, nutrient, redox potential, and exudate gradients changing as distance from the root increases (Marschner 1995). This region of gradients in chemical and physical factors strongly influenced by the presence of plant roots and characterized by high rates of microbial population and activity is referred to as the rhizosphere.

In 1904, Hiltner first defined the rhizosphere as “. . . that zone of soil in which the microflora are influenced by plant roots” (Kang and Mills 2004). This rhizosphere effect is primarily due to the influx of mineral nutrients to the plant roots through mass flow and diffusion, alongside the efflux and accumulation of plant root exudates. Microbial communities in the rhizosphere are primarily plant driven, responding with respect to density, composition, and activity to the abundance and diversity of plant-derived exudates, eventually leading to plant species-specific microflora. A substantial portion of the root exudates consist of carbon and energy sources readily available for microbial growth; by now it is clear that plant roots excrete amino acids, proteins, sugars, organic acids, vitamins, and other bacterium-beneficial substances affecting growth, development, and physiology of a microbial population. Low molecular weight plant-derived exudates, mainly amino acids, organic acids, and sugars commonly found in most plants, are rapidly utilized by microorganisms. In addition, high molecular weight root mucilage, consisting of approximately 95 % sugars and 5 % amino acids in the form of heteropolysaccharides and glycoproteins, also serve as a source of energy for rhizosphere bacteria (Somers et al. 2004). The exact composition of the exudates is determined by many

factors, including species and nutritional status of the plant, soil structure, and micronutrient status (Marschner 1995). Depending on the composition of the exudates secreted by a given plants' roots, that plant may be able to alter the physical and chemical properties of the soil, inhibit the propagation or growth of another plant species, withstand underground herbivory, enhance the possibilities and success of symbiotic relationships, and dictate, to some extent, the soil microbial community in the rhizosphere. In fact, most rhizosphere bacteria and fungi are highly dependent on associations with plants that are clearly regulated by root exudates (Bais et al. 2004), and in the rhizosphere numbers of microorganisms can reach  $10^{10}$ – $10^{12}$  organisms  $g^{-1}$  soil (Forster 1979). Plant–microbe symbioses have been exploited in programs of sand dune restoration. Plant-associated bacteria may increase the ability of plants to utilize nutrients from the soil by increasing root development, nitrate uptake, or solubilizing phosphorus and to control soil-borne pathogens (Smith and Read 1997; Whipps 2001).

In order to understand the effects of plant–bacteria interactions, it is essential to study the bacterial diversity associated with plants, and there have actually been a number of studies characterizing the structures and functions of rhizosphere and root bacterial communities (Hallmann et al. 1997; Mahaffee and Kloepper 1997; Maloney et al. 1997; Germida et al. 1998). Plants are known to alter the composition of microbial communities associated with their roots (Grayston et al. 1996; Marschner et al. 2001). Plant communities in sand dunes are controlled by the interaction between biotic and physicochemical components of the sand matrix (Read 1989). Interactions with microbes appear crucial in obtaining inorganic nutrients or growth-influencing substances. In addition, human activities may also be an important factor, as they will certainly affect the vegetation as well as plant–microbe interactions.

Dalton et al. (2004) suggested that the nitrogen-fixing bacteria isolated from the rhizosphere and root of *Ammophila arenaria* may contribute to the prolific success of these plants in nutrient-poor sand. Despite the important role played by bacterial diversity in sand dune plant communities, little is known on the distribution and abundance of root or rhizosphere associated bacteria. Park et al. (2005) first reported on the diversity of culturable bacteria associated with the two major sand dune plant species, *Calystegia soldanella* (beach morning glory) and *Elymus mollis* (wild rye), which are found as the dominant plant species along the coastal sand dune areas in Tae-An, Chungnam Province. While in another study carried out by Lee et al. (2006), bacterial diversity in the rhizosphere of beach morning glory (*Calystegia soldanella*) and wild rye (*Elymus mollis*), two of the major plant species inhabiting the coastal sand dune in Tae-An, Korea, was studied by the analysis of community 16S rRNA gene clones.

In our studies the seasonal variation of rhizosphere and endophytic bacteria associated with *Ipomoea pes-caprae* and *Spinifex littoreus* was studied. Based on the cultural, physiological, and biochemical characteristics, it was observed that among the neutrophiles, majority of the isolates belonged to *Bacillus* genus, while among the alkaliphiles, the majority of the isolates were gram-positive irregular rods belonging to genera such as *Brochothrix*, *Cellulomonas*, *Microbacterium*, and

*Brevibacterium*. Zinniel et al. (2002) identified *Cellulomonas*, *Clavibacter*, *Curtobacterium*, and *Microbacterium* as the most promising colonizing strains with four agronomic crop species. Karp and Nelson (2004) reported that the sand and soil root zones were dominated largely by gram-positive species, e.g., *Arthrobacter*, *Bacillus*, and *Microbacterium* species, as also observed during the present study. Soil rhizosphere communities consisted almost entirely of *Actinobacterium*, *Arthrobacter*, and *Bacillus* isolates, whereas sand root zones contained clones of a few gram-negative genera such as *Aminobacter*, *Chelatobacter*, *Ensifer*, and *Pseudomonas*. Smit et al. (2001) studied the bacterial diversity and dynamics in Lovinkhoeve soil samples the most dominant bacterial genera detected by plating appeared to be *Micrococcus* and *Arthrobacter*. These genera are often found in various soils, such as those of wheat fields, deciduous woodlands, grasslands, and sand dunes. Tiago et al. (2004) investigated the bacterial diversity in a nonsaline alkaline environment and reported that the majority of the isolates were related to *Microbacteriaceae* family members, while another set of isolates represented populations related to different species in the lineage of the *Micrococcaceae*, namely, *Micrococcus luteus*, *Citrococcus muralis*, and *Rothia dentocariosa*, and others were related to various species of the genera *Kocuria* and *Nesterenkonina*. Overall, it was observed that endophytic bacteria counts were higher than rhizosphere bacterial counts among the different bacterial groups. Interestingly, the total viable counts in unvegetated areas of sand dunes were lower than the vegetated areas as seen from the analysis of the samples collected from unvegetated area.

#### 4.4 Plant Growth-Promoting Rhizobacteria

PGPR are naturally occurring, free-living soil bacteria that are capable of colonizing roots and enhancing plant growth when added to seeds or roots (Kloepper and Schroth 1978; Frankenberger and Arshad 1995). There are several ways in which plant growth-promoting bacteria can directly facilitate the proliferation of their host plants. They may fix atmospheric nitrogen and supply it to plants; solubilize minerals such as phosphorus; produce siderophores, which can solubilize and sequester iron and provide it to plants; and synthesize phytohormones, including auxins, cytokinins, and gibberellins, which can enhance various stages of plant growth. Indirect promotion of plant growth occurs when these bacteria decrease or prevent some of the deleterious effects of a pathogenic organism by any one or more of several different mechanisms including improving growth-restricting conditions either via production of antagonistic substances or by inducing resistance against plant pathogens (Kloepper 1993; Tilak et al. 2005). For example, production of antibiotics can interfere directly with growth and activity of deleterious soil microorganisms (Glick and Bashan 1997), whereas induction of resistance in the plant increases the plants defense capacity (VanLoon et al. 1998). In addition,

bacteria may reduce stresses resulting from the presence of toxic wastes by sequestering heavy metals or degrading organic pollutants.

There are 20 different biocontrol PGPR strains commercially available in the market at present. Biocontrol of plant pathogens is achieved through antibiotic synthesis; secretion of iron-binding siderophores to obtain soluble iron from the soil and provide it to a plant, thereby depriving fungal pathogens in the vicinity of soluble iron; production of low molecular weight metabolites such as hydrogen cyanide with antifungal activity; and production of enzymes including chitinase,  $\beta$ -1,3-glucanase, protease, or lipase which can lyse some fungal cells, outcompeting phytopathogens for nutrients and niches on the root surface (Penrose and Glick 2003). A particular bacterium may promote plant growth and development using any one, or more, of these mechanisms. For example, following seed germination a PGPR may lower the plants ethylene concentration thereby decreasing the ethylene inhibition of seedling root length. Once the seedling has depleted the resources that are contained within the seed, the same PGPR may help to provide the plant with iron and phosphorus from the soil. The impact of the mechanisms by which the bacteria provides a compound or nutrient such as fixed N, P, or Fe to the plant varies considerably depending upon the soil composition. Thus PGPR often have little or no measurable effect on plant growth when the plants are cultivated in nutrient-rich soil and grown under optimal conditions.

Further root-associated bacteria capable of fixing nitrogen occur regularly in diverse soils which vary widely in nitrogen content. Common genera capable of fixing nitrogen include *Azospirillum*, *Azotobacter*, *Bacillus*, *Clostridium*, *Derxia*, and *Klebsiella*. These are commonly designated “free-living” bacteria, since they are able to exist in the soil and reduce nitrogen without entering into symbiotic association with plants (Gaskins et al. 1985). Denitrification which transforms reduced nitrogen compounds into gaseous nitrogen allows return of nitrogen to the atmosphere from the soil. *Alcaligenes*, *Bacillus*, and *Pseudomonas* spp. are common types of denitrifying bacteria. The removal of soil nitrogen by denitrifying bacteria is normally considered detrimental to crop production, because in most instances nitrogen is the element which most severely limits plant growth. However, these bacteria are useful since they prevent nitrogen compounds from accumulating to toxic levels, particularly in poorly drained areas. Also, denitrification activity beneath the root zone is beneficial, since it reduces the nitrate load in groundwater. Denitrification tends to maintain a balance between soil and atmospheric nitrogen (Gaskins et al. 1985). Also the mechanism most often invoked to explain the various effects of plant growth-promoting bacteria on plants is the production of phytohormones most notably auxin. Auxins are a class of PGPR known to stimulate both rapid (e.g., increases in cell elongation) and long-term (e.g., cell division and differentiation) responses in plants. Diverse soil microorganisms including bacteria, filamentous fungi, and yeasts are capable of producing physiologically active quantities of auxins and which have pronounced effects on plant growth and development. L-Tryptophan (L-TRP) is considered as a physiological precursor of auxin biosynthesis in both higher plants and

microorganisms (Arshad and Frankenberger 1998). Since plants as well as plant growth-promoting bacteria can synthesize indoleacetic acid (IAA), it is important when assessing the consequences of treating a plant with a plant growth-promoting bacterium to distinguish between the bacterial stimulation of plant auxin synthesis on the one hand and auxin that is synthesized by the bacterium on the other. The level of auxin produced by a bacterium in the rhizosphere determines its effect on the host plant; high levels induce developmental abnormalities and stimulate formation of lateral and adventitious roots, while low levels promote root elongation (Van Loon and Glick 2004).

A number of different bacteria considered to be PGPR include *Azotobacter* spp., *Azospirillum* spp., *Pseudomonads*, *Acetobacter* spp., *Burkholderia* spp., *Bacillus*, *Alcaligenes*, *Klebsiella*, *Enterobacter*, *Herbaspirillum*, and *Xanthomonas* (Glick 1995). A number of bacterial species associated with the plant rhizosphere belonging to genera *Azospirillum*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, and *Serratia* are able to exert a beneficial effect on plant growth (Tilak et al. 2005).

The PGPR play a significant role in supporting growth of plants. These bacteria possess traits which help in either improving the availability of the nutrients or inhibiting the pathogenic bacteria. The availability of nutrients is facilitated by production of siderophores, exopolysaccharides (EPS), and polyhydroxyalkanoates (PHAs).

## 4.5 Significant Plant Growth-Promoting Metabolites Produced by Sand Dune Rhizobacteria

Many scientists have evaluated the efficiency of isolated PGPR. Some of their significant mechanisms have been enlisted below.

### 4.5.1 ACC Deaminase

Ethylene, which is produced in almost all plants, mediates a range of plant responses and developmental steps. Ethylene is involved in seed germination, tissue differentiation, formation of root and shoot primordia, root elongation, lateral bud development, flowering initiation, anthocyanin synthesis, flower opening and senescence, fruit ripening and degreening, production of volatile organic compounds responsible for aroma formation in fruits, storage product hydrolysis, leaf and fruit abscission, and the response of plants to biotic and abiotic stresses (Frankenberger and Arshad 1995). In some instances ethylene is stimulatory, while in others it is inhibitory. The increased level of ethylene formed in response to trauma inflicted by temperature extremes, water stress, ultraviolet light, chemicals,



mechanical wounding, insect damage, and disease can be both the cause of some of the symptoms of stress (e.g., onset of epinastic curvature and formation of aerenchyma) and the inducer of responses, which will enhance survival of the plant under adverse conditions (e.g., cell wall strengthening, production of phytoalexins, and synthesis of defensive proteins).

1-Aminocyclopropane-1-carboxylate (ACC), the cyclopropanoid amino acid, is a precursor in the biosynthetic pathway of the plant hormone ethylene. Plant growth-promoting soil bacteria have been found to contain ACC deaminase (ACCD), a PLP-dependent enzyme that converts ACC to a ketobutyrate and ammonium. Introduction of ACCD in higher plants by gene modification technology reduced the production of ethylene and delayed ripening of fruits. *Pseudomonas putida* UW4, a novel ACCD-containing bacterium, has been shown to promote plant growth under different environmental stresses including flooding, drought, and the presence of heavy metals and phytopathogens. The possibility of a close mutualistic relationship between the plants and the soil bacteria has been suggested and the role of ACCD in ensuring low levels of ethylene at critical stages of root growth has been proposed by Hontzeas et al. (2004a, b). The enzyme ACC deaminase is important as this enzyme can cleave the plant ethylene precursor ACC and thereby lowers the level of ethylene in a developing or stressed plant. A burst of ethylene is required to break seed dormancy for many plants, but following germination a sustained high level of ethylene would inhibit root elongation. PGPR that contain the enzyme ACC deaminase when bound to the seed coat of a developing seedling act as a mechanism for ensuring that the ethylene level does not become elevated to the point where crucial root growth is impaired. By facilitating the formation of longer roots, these bacteria may enhance the survival of some seedlings especially during the first few days after the seeds are planted.

Thus, plant growth-promoting bacteria are supplied with a unique additional source of nitrogen in the form of ACC that enables them to proliferate under conditions in which other soil bacteria may not flourish, for instance, when nitrogen availability is low and competition for nutrients is intense. As a result of lowering the ACC level within the plant, either the endogenous level or the IAA-stimulated level, the amount of ethylene in the plant is also reduced. Plant growth-promoting bacteria that possess the enzyme ACC deaminase and are bound to seeds or roots of seedlings can reduce the amount of plant ethylene and the extent of its inhibition on root elongation. Thus, these plants should have longer roots and possibly longer shoots as well, stem elongation is also inhibited by ethylene, except in ethylene-resistant plants (Van Loon and Glick 2004).

### 4.5.2 Auxins

One of the direct mechanisms by which PGPR promote plant growth is by production of plant growth regulators or phytohormones (Glick 1995). Frankenberger and Arshad (1995) have discussed in detail the role of auxins, cytokinins, gibberellins,

ethylene, and abscisic acids (ABA) which, when applied to plants, help in increasing plant yield and growth. Microbial production of individual phytohormones such as auxins and cytokinins has been reviewed by various authors over the last 20 years (Pilet et al. 1979; Hartmann et al. 1983; Fallik and Okon 1989; Barbieri and Galli 1993; Patten and Glick 1996, 2002). Auxins are a class of plant hormones and one of the most common and well characterized is indoleacetic acid (IAA), which is known to stimulate both rapid (e.g., increases in cell elongation) and long-term (e.g., cell division and differentiation) responses in plants (Glick 1995). Some of the plant responses to auxin are as follows: (a) cell enlargement, (b) cell division, (c) root initiation, (d) root growth inhibition, (e) increased growth rate, (f) phototropism, (g) geotropism, and (h) apical dominance (Frankenberger and Arshad 1995; Leveau and Lindow 2005). Most notably, exogenous auxin production by bacteria has been associated with altered growth of the roots of plants on which they were inoculated. While many plant growth-promoting bacteria, which stimulate the growth of roots, can produce at least small amounts of the auxin indole-3-acetic acid (IAA), high IAA producers are inhibitory to root growth (Lindow et al. 1998). Bacterial IAA producers (BIPs) have the potential to interfere with any of these processes by input of IAA into the plant's auxin pool (Leveau and Lindow 2005).

IAA is a common product of L-tryptophan metabolism by several microorganisms including PGPR. Promotion of root growth is one of the major markers by which the beneficial effect of plant growth-promoting bacteria 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 (Patten and Glick 2002).

Bacteria belonging to the genera *Azospirillum*, *Pseudomonas*, *Xanthomonas*, and *Rhizobium* as well as *Alcaligenes faecalis*, *Enterobacter cloacae*, *Acetobacter diazotrophicus*, and *Bradyrhizobium japonicum* have been shown to produce auxins which help in stimulating plant growth (Patten and Glick 1996).

### 4.5.3 Hydrogen Cyanide

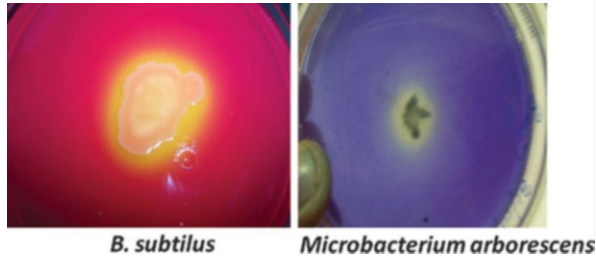
Cyanide is a potential inhibitor of enzymes involved in major plant metabolic processes including respiration, CO<sub>2</sub> and nitrate assimilation, and carbohydrate metabolism and may also bind with the protein plastocyanin to block photosynthetic electron transport (Grossman 1996). HCN is a potent inhibitor of cytochrome c oxidase and of several other metalloenzymes—some of them involved in respiratory processes. HCN biosynthesis is catalyzed by HCN synthase, from glycine, with stoichiometric production of CO<sub>2</sub>. HCN affects sensitive organisms by inhibiting the synthesis of ATP mediated by cytochrome oxidase and is highly toxic to all aerobic microorganisms at picomolar concentrations (Pal and McSpadden 2006). No role is known for HCN in primary bacterial metabolism, and it is generally

considered as a secondary metabolite (Blumer and Haas 2000). HCN-producing bacteria can help plants in their defense against fungal pathogens (Voisard et al. 1989; Blumer and Haas 2000). This property was predominantly described among *Pseudomonas* strains (Kremer and Souissi 2001). Therefore depending on the target organisms, HCN-producing microorganisms are regarded as harmful when they impair plant health and beneficial when they suppress unwanted components of the microbial community (Bellis and Ercolani 2001).

Hydrogen cyanide production is a physiological activity which is energetically dependent on the availability of organic carbon sources and low oxygen pressure, i.e., conditions which commonly prevail in the rhizosphere (Tarnawski et al. 2006). It has been shown that cyanide released by *Pseudomonas fluorescens* suppresses the growth of microorganisms (e.g., phytopathogenic bacteria and fungi) sharing the same ecological niche (e.g., the rhizosphere), thereby acting as a biocontrol metabolite (Voisard et al. 1989). Hence, cyanide production would increase the biological fitness by providing cyanogenic species with a selective advantage over competitors (Haas and Défago 2005; Voisard et al. 1989). The production of HCN by certain fluorescent pseudomonads is believed to be involved in the suppression of root pathogens. *P. fluorescens* CHA0 produces antibiotics, siderophores, and HCN, but suppression of black rot of tobacco caused by *Thielaviopsis basicola* appeared to be primarily due to HCN production (Pal and McSpadden 2006).

#### 4.5.4 Ammonia

Biological N<sub>2</sub>-fixation (BNF) by soil microorganisms is considered one of the major mechanisms by which plants benefit from the association of micropartners. One of the benefits that diazotrophic microorganisms provide to plants is fixed nitrogen in exchange for fixed carbon released as root exudates (Glick 1995). Many of the PGPR described to date are free-living diazotrophs that can convert molecular nitrogen into ammonia in a free state by virtue of the nitrogenase enzyme complex (Postgate 1982; Saikia and Jain 2007). Raj Kumar and Lakshmanan (1995) suggested that ammonia excretion seems to be the result of nitrogenase activity in symbiotic associations where relatively large amounts of atmospheric N reach the plant as ammonia released by the bacteroids. By contrast, most of the ammonia produced in PGPB by the nitrogenase-catalyzed N<sub>2</sub> fixation would be assimilated by the rhizobacteria through the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. Also plant growth-promoting bacteria contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase and this enzyme can lower the ethylene precursor ACC to  $\alpha$ -ketobutyrate and ammonia and thereby lower the level of ethylene in developing or stressed plants (Hontzas et al. 2004a, b).



**Fig. 4.2** Plate assays of P solubilizers *Bacillus subtilis* (Pikovskaya medium incorporated with phenol red dye) and *Microbacterium arborescens* (Pikovskaya medium incorporated with bromothymol blue dye) isolated from rhizosphere of coastal sand dune plants

### 4.5.5 Phosphate Solubilization

Bacteria isolated from the rhizosphere are capable of increasing availability of phosphorus to plants either by mineralization of organic phosphate or by solubilization of inorganic phosphate by production of acids (Lifshitz et al. 1987). These bacteria referred to as phosphobacteria and have been considered to have potential use as bioinoculants.

Many soil microorganisms are able to solubilize “unavailable” forms of calcium-bound P through their metabolic activity by excreting organic acids which either directly dissolve rock phosphate or chelate calcium ions to bring P into solution (Fig. 4.2). The production of microbial metabolites results in a decrease in soil pH, which probably plays a major role in solubilization. Besides changes in pH, chelation by organic acids which bind phosphate anions also brings about phosphate in soil solution. Soil inoculation with phosphate solubilizing bacteria has been shown to improve solubilization of fixed soil P and applied phosphates resulting in higher crop yields (Nautiyal et al. 2000).

### 4.5.6 Exopolysaccharide

Exopolysaccharide is a term first used by Sutherland to describe high molecular weight carbohydrate polymers produced by marine bacteria. EPS can be found as capsular material that closely surrounds a bacterial cell or as a dispersed slime in the surrounding environment with no obvious association to any one particular cell (Sutherland 1982; Decho 1990). In the natural environment bacteria occur mostly in aggregates whose structural and functional integrity is based on the presence of a matrix of extracellular polymeric substance. Thus EPS production seems to be important for their survival (Sutherland 1982).

Production of exopolymeric substances especially EPS by bacteria is one of the mechanisms to overcome desiccation. The rate of drying within the colony micro-environment is slower with EPS and helps increase bacterial survival by increasing

**Fig. 4.3** Viscous exopolymer produced by sand dune rhizobacteria *M. arborescens*

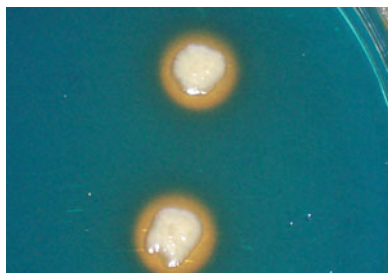


the time available for metabolic adjustment. Further an EPS matrix provides another advantage to bacteria living within it as decreasing water content of soil restricts diffusion of nutrients to microorganisms. Polysaccharides being hygroscopic maintain higher water content in the colony microenvironment than in the bulk soil as water potential declines. This increase in water content could increase nutrient availability within the bacterial colony. Roberson and Firestone (1992) revealed that bacteria respond to desiccation by channeling energy and nutrients into polysaccharide production. Soil is an extremely heterogeneous environment, and wetting and drying may not proceed uniformly throughout it, and any microbial processes in soil depend on this heterogeneity. Godinho and Bhosle (2009) studied the aggregation of sand dune soils by exopolysaccharide-producing *Microbacterium arborescens*, a sand dune rhizobacterial isolate (Fig. 4.3). It was observed that the rhizosphere and endophytic bacteria associated with the sand dune plants may be playing an important role in aiding in the survival of these plants in the sand dunes. Coastal sand dunes is a previously unexplored habitat for EPS-producing bacteria. These exopolymeric substances might be involved in ecological roles, protecting the cells against desiccation especially in nutrient-limited environments such as the coastal sand dunes more so in the extreme conditions of pH. Such polysaccharides may be helping the bacteria to adhere to solid substrates and survive during nutrient limited conditions.

#### 4.5.7 Siderophores

Iron is the fourth most abundant element on earth, but in the presence of oxygen and at neutral pH, it is not sufficiently available to microbes due to the rapid oxidation of  $\text{Fe}^{+2}$  to  $\text{Fe}^{+3}$  and the formation of ferric hydroxides and oxyhydroxide polymers (Neilands 1995). Concentration of free iron in soil under these conditions is as low as  $10^{-17}\text{M}$ , which is much less than that required for optimal growth of soil microflora (Guerinot et al. 1990). A large number of proteins require iron for their activity, which underlines the importance of iron for living organisms. The iron that is present in proteins can exist in several different forms: heme, iron-sulfur, iron-nickel, di-iron, and mononuclear iron (Andrews 1998).

Iron is made biologically available by iron-chelating compounds called siderophores that are synthesized and secreted by many bacteria and fungi under conditions of iron limitation (Fig. 4.4) (Neilands 1995). Siderophores are water-soluble, low molecular weight molecules that are secreted by bacteria and fungi. The term



**Fig. 4.4** The *yellow orange halo* surrounding the bacterial colony is indicative of the production of an Fe-binding compound such as siderophore, which removes Fe(III) from the Fe(III)–CAS–HDTMA complex in the plate and turns the *blue dye* to *yellow color*. The bacteria were isolated from the rhizosphere of sand dune plants

siderophore stands for “iron carriers” or “iron bearers” in Greek. This is an appropriate term because the siderophore binds iron with an extremely high affinity and is specifically recognized by a corresponding outer membrane receptor protein, which in turn actively transports the complex into the periplasm of the cell (Braun and Braun 2002; Gomez and Sansom 2003) or which imparts specificity of uptake and works in association with periplasmic iron-binding proteins and cytoplasmic membrane-associated proteins (Gomez and Sansom 2003). The molecular weights of siderophores range from approximately 600 to 1,500 Da, and because passive diffusion does not occur for molecules greater than 600 Da, siderophores must be actively transported (Ishimaru 1993). The role of these compounds is to scavenge iron from the environment and to make the mineral, which is almost always essentially present available to the microbial cell (Neilands 1995). There are more than 500 different types of siderophores produced by bacteria, yeasts, and fungi. Siderophores are produced and secreted only when the amount of iron is low in the growth environment. The genes involved in siderophore production regulate siderophore production based on the concentration of iron in the environment. That is, siderophore production is shut off when iron is present at sufficient concentration and vice versa.

Siderophores specifically bind to ferric ion with high affinity. The binding power of the siderophore for iron has a stability constant range from  $10^{22}$  to  $10^{50}$  (Ratledge and Dover 2000). This range is sufficiently high for the removal of iron attached to molecules like ferritin and transferrin by siderophore, but not high enough for the removal of iron present in heme proteins. Siderophore molecules display considerable structural variation but can be classified as either hydroxamates or catechols. Structurally, 20 siderophores are ring- or semiring-shaped structures containing oxygen atoms. Siderophores show high affinity for ferric ion, since the oxygen atoms present can form coordination bonds with a single Fe(III) ion (Neilands 1995). The production of siderophores has been reported in aerobic and facultative anaerobic microbes, but their production has not yet been reported in strict anaerobes, lactic acid bacteria, or in higher organisms such as plants and animals. The main function of siderophores is involved in the high affinity acquisition and receptor-dependent transport of ferric ion. Siderophores are also associated with growth or germination factors and virulence factors.

In gram-negative bacteria,  $\text{Fe}^{+3}$  siderophores bind to highly specific receptor proteins and are then transported into the cytoplasm (Faraldo-Gomez and Sansom 2003), while in gram-positive bacteria, which lack an outer membrane, the receptors are binding proteins that are anchored to the cytoplasmic membrane by a covalently linked lipid. A periplasmic transport protein and several inner membrane-associated proteins complete the transport of iron into the cell. This arrangement of proteins from periplasm to cytoplasm is similar to other bacterial periplasmic protein-dependent systems, termed ABC transporters (for ATP-binding cassette-type transport), which transport amino acids, peptides, and sugars into the cell (Braun and Killman 1999; Clarke et al. 2001; Fatht and Kolter 1993).

#### 4.5.8 *Resting Bodies*

Bacteria have also evolved numerous mechanisms of resistance to stress conditions and nutrient limitations. For example, many microorganisms have an inherent ability to form resting stages (e.g., cysts and spores). Even without the formation of such elaborately differentiated cells, bacteria enter starvation-induced programs that allow them to survive long periods of nongrowth and to restart growth when nutrients become available again. This often leads to the formation of metabolically less active cells that are more resistant to a wide range of environmental stresses. This adaptation to starvation conditions is often accompanied by a change in cell size as well as the induction of genes and the stabilization of proteins that are essential for long-term survival. The best-studied examples of starvation–survival in nondifferentiating bacteria are *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio* sp. strain S14, which show qualitative similarities in their survival responses (Madison and Huisman 1999).

#### 4.5.9 *Nutrient Availability*

Nutrients may become available locally, for example, in decaying plant and animal material or via plant roots, which are one of the major sites of carbon input into soil. The rhizosphere therefore is a soil region with a transiently high availability of carbon in a form readily available to soil bacteria. Soil bacteria that have evolved in close association with plants, such as rhizobia and pseudomonads, benefit from being able to quickly escape the starvation state and colonize the plant root. The accumulation of intracellular storage polymers is another bacterial strategy that increases survival in a changing environment. Poly(3-hydroxyalkanoates) (PHAs) are accumulated as discrete granules to levels as high as 90 % of the cell dry weight and are generally believed to play a role as a sink for carbon and reducing equivalents. The bacterial origin of PHAs makes these polyesters a natural material,

and microorganisms have evolved ability to degrade these macromolecules (Madison and Huisman 1999).

In bacteria, PHAs constitute a major carbon and energy storage material, which accumulates when a carbon source is provided in excess and another nutrient (such as nitrogen, sulfur, phosphate, iron, magnesium, potassium, or oxygen) is limiting. The polymerization of soluble intermediates into insoluble molecules does not change the osmotic state of the cell, thereby avoiding leakage of these nutrient-rich compounds out of the cell. In addition, PHA-producing bacteria have the advantage of nutrient storage at a relatively low maintenance cost and with a secured return of energy (Berlanga et al. 2006). PHAs produced by these bacteria are important due to their biodegradability, water resistance, and oxygen permeability. Their applications are varied; they are used for all sorts of biodegradable packaging materials (Thakor et al. 2006).

In our study since the bacteria were isolated from coastal ecosystem, we evaluated their growth-promoting ability under a similar ecosystem. Eggplant was selected as a model plant as it is popularly grown in Goa. The four sand dune bacterial isolates chosen for the study were *B. subtilis*, *M. arborescens*, *K. rosea*, and *B. subtilis* sp. *MF-4* shows good ACC deaminase activity, HCN production, IAA and siderophore production, and phosphate solubilization. Results of pot studies indicated that *K. rosea* and *B. subtilis* increased shoot length and weight of the plants consistently up to 44 DAS. However *Bacillus* sp. *MF-A4* increased the growth significantly from 37 DAS after sowing, while *M. arborescens* was effective in the latter stages (at 44DAS). The study confirmed the bioprospects of using the sand dune bacteria as biofertilizers for agricultural crops (Godinho et al. 2010).

## 4.6 Concluding Remarks and Future Perspectives

This chapter contributes significantly to the knowledge of the wide occurrence of effective PGPR bacteria associated with sand dune vegetation in the ecosystem. A large number of bacteria are associated with rhizosphere and as endophytes with vegetation growing on coastal sand dunes. Such organisms are shown to play a role in promoting growth of plants by making the soils available with nutrients. Plant growth-promoting characteristics of promising isolates studied indicated that native plant growth-promoting microorganisms with properties such as phosphate solubilization, disease control potential, and rhizosphere colonization would seem ideal for selection as a suitable bioinoculant. The cultures were found to produce siderophores, solubilize inorganic phosphates, ammonia, hydrogen cyanide, and indole-3-acetic acid. All these metabolites are important for plant growth promotion. They were all found to utilize ACC as a sole source of nitrogen further confirming the presence of ACC deaminase enzyme.

Plant growth-promoting sand dune rhizobacteria therefore present an alternative to the use of chemicals for plant growth enhancement in many different applications. This research work has demonstrated that sand dune rhizobacteria



could have an important role in agriculture and horticulture in improving crop productivity. Among the four sand dune bacterial isolates, *B. subtilis*, *K. rosea*, and *M. arborescens* were found to have a significant effect on plant growth promotion of eggplant, an agriculturally important crop.

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