Chapter 4
Rhizosphere Colonization: Molecular Determinants from Plant-Microbe Coexistence Perspective

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4.1 Introduction

Inoculation of plants with beneficial microbes can be traced back for centuries. Although bacteria were not proven to exist until in 1683 von Leeuwenhoek discovered microscopic 'animals' under the lens of his microscope, their utilisation to stimulate plant growth in agriculture has been exploited since ancient times. Theophrastus (372–287 BC) suggested the mixing of different soils as a means of 'remedying defects and adding heart to the soil' (Vessey 2003). Colonisation of plant root system is the very first step in nearly all interactions between plants and soil borne microbes. The importance of this compartment for plant growth and soil microbiology had already been realised in the very pioneer times of microbiology in the late 19th century. In 1888, Hellriegel and Wilfarth proved the special case of nitrogen nutrition of legumes through their root nodule bacteria, which Beijerinck finally isolated in 1889. In 1887–1888 Hiltner was certainly fascinated by the findings of Hellriegel and Wilfarth about the special case of nitrogen nutrition in legumes. Together with Professor Nobbe, intensive studies were conducted about the nature of the symbiotic interaction of nodule bacteria and legume roots (Nobbe and Hiltner 1896). Apparently during these studies, Hiltner became aware of the importance of the ecological interactions in the root zone. Together with his teacher he developed the first inoculant based on root nodule bacteria for agricultural practice which they called 'Nitragin' in 1890. As agricultural practice was in close contact to the new achievements of basic sciences, the challenge quickly arose whether at all, and how, these discoveries of the – at that time – very young science 'soil bacteriology' could be applied in the field. Region of contact between root and soil where soil is affected by roots was designated as "rhizosphere" by Hiltner (1904).

The rhizosphere of plants is one of the most fascinating microbial habitats for basic and applied studies in the field of microbiology, as it is shaped by the soil, the

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plant and the microorganisms. Given the tremendous diversity of soil microbes, soil fauna, and plants, it is virtually impossible to investigate the intricacies of every potential rhizosphere interaction in every environmental circumstance. However, an understanding of controls over belowground function is becoming increasingly important as natural and agro-ecosystems around the globe are exposed to anthropogenic pressures. In addition, the chemistry and development of soil present today have been strongly affected by the actions of rhizospheres over evolutionary time frames and the evolution of true plant roots and their extension deep into substrate is hypothesised to have led to a revolution in planetary carbon and water cycling during the Devonian period (Beerling and Berner 2005; Cardon and Gage 2006). What is the biogeochemical function of the rhizosphere on Earth today? In what major ways is rhizosphere function belowground similar across terrestrial ecosystems, and in what fundamental ways can it differ? However, since the microbial inoculations would mainly be performed in soils before the plant is grown up, the strains should also be able to survive in the soil and show a good saprophytic ability. To fulfill these requirements, progress must be made in our knowledge of which bacterial traits affect the soil- and rhizosphere-colonising ability of microbes (Nautiyal 2006; Nautiyal et al. 2006a,b).

This review focuses on methods to improve root colonisation by introduced bacteria using molecular biology strategy and tools to enhance plant-microbe coexistence. The plausible mechanisms adopted by these rhizobacteria in root colonisation, though abundantly documented but still remains to be fully explored. It is thus presumed that better understanding of the molecular basis of plant-rhizobacteria coexistence will enable researchers to make more informed decisions in designing inoculants that will successfully colonise host rhizosphere and consistently promote the growth of host plants, biological control and bioremediation.

4.2 Genetic Regulation of Plant–Microbe Association

For most of the past century, microbiology has been devoted to studying the physiology and genetics of bacteria in vitro. This means that we now understand a great deal about the lives of bacteria on agar plates, but have little understanding of the determinants of ecological success in the wild. Without an understanding of the causes of ecological success our understanding of the biology of bacteria will remain incomplete and the full potential of these organisms in biotechnology will remain unrealised. Bottom-up (genes to population) and top-down (population to genes) approaches are both useful. The bottom-up approach is commonly employed for studies of bacteria, although is rarely pursued to the population level. Bottom-up positive selection approaches open the door to understand ecological performance in bacteria at a mechanistic level in the wild. In vivo expression technology (IVET) strategies, despite their tremendous power, have been little used, except in studies of animal pathogenicity. Rainey and Preston (2000) have reviewed IVET strategies, their development, and potential in biotechnology. Using 22,800

genechip probe array of FPT9601-T5-treated *Arabidopsis* plants in comparison to control plants, Tosa et al. (2005) reported on up- or down-regulation of 95 and 105 genes respectively. Up-regulated ones include genes involved in metabolism, signal transduction, and stress response, putative auxin-regulated genes and nodulin-like genes and some ethylene-responsive genes were down-regulated. Isabel et al. (2005) identified 28 *rap* genes (*r*oot-*a*ctivated *p*romoters) preferentially expressed in the maize rhizosphere during rhizosphere colonisation by *P. putida* KT2440. IVET is a powerful tool to identify promoters and genes that are expressed specifically under certain conditions of interaction. Among modern techniques for the study of rhizosphere colonisation in inoculated and uninoculated controls microarray analysis besides IVET, differential display (DD) technique is also very useful. Differential display of genes is mRNA based technique where differentially expressed genes of plants infected with rhizobacteria resulted in enhanced expression of PR proteins (Hassan et al. 2003). In another cDNA-amplified fragment length polymorphism technique, 81 transcript derived fragments showing differential expression during exponential growth phase in an aggregation-inducing medium containing high C:N ratio. RT-PCR analyses confirmed the differential patterns observed by cDNA-AFLP of *nodQ*, involved in sulfation; *narK*, involved in nitrite/nitrate transport, and flp, involved in auto aggregation; as well as genes encoding a biopolymer transport protein, and the signal recognition particle (Valverde et al. 2006).

Correlations have been reported between rhizosphere competence and growth rate of a plant root adhered bacteria and it has a prerequisite to multiply using organic compounds and other physiological traits which may further contribute to their rhizosphere competence and these rhizosphere competent bacteria helps in increasing the plant productivity by multiple changing in the gene expression.

Auxin produced by bacteria in the rhizosphere can stimulate the activity of the 1-aminocyclopropane-1-carboxylate (ACC) synthase, an enzyme normally used by plants to form ethylene (Xie et al. 1996) and transcription of *ipdC*, an *Erwinia* IAA biosynthetic gene, is induced in response to bean and tobacco compounds (Brandl and Lindow 1997) and the bacterial auxin synthesis is dependent upon plant-exuded tryptophan. Analyses of the more than 4000 ORFs of *Bacillus subtilis* revealed that *yqkF* are growth regulatory gene related to auxin which may manipulate hormonal processes in plants (Andrews and Harris 2000). To control the ethylene production, increased amount of ACC is hydrolysed by an ACC deaminase and ACC deaminase-producing rhizobacteria upregulate genes involved in cell division and proliferation but down-regulate stress genes thus reducing plant stress and induce root elongation and proliferation in plants, largely by lowering ethylene levels (Glick et al. 1998; Penrose and Glick 2003) which bring about ISR by fortifying the physical and mechanical strength of the cell wall (Knoester et al. 1999). Ahn et al. 2002 – by showing augmented, rapid transcript accumulation of defense-related genes including PR-1a, phenylalanine ammonia-lyase (PAL), and 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) following inoculation with PGPRs – concluded that PGPRs by a typical phenomenon of potentiation using the jasmonate pathway of defence provide ISR to the plants. Phenotypically ISR in

plants is similar to pathogen-induced systemic acquired resistance (SAR) known defense-related genes, i.e. the SA-responsive genes PR-1, PR-2, and PR-5, the ethylene-inducible gene (Karen et al. 2005).

Plant-microbe coexistence is strongly influenced by abiotic stress conditions induced by drought, high salt and temperature. Stress is an unhealthy condition and prolonged stress weakens the immune system, opening the door for a variety of ailments that an otherwise healthy plant could overcome. The major consequences of drought stress is the loss of water from the protoplasm and leads to the concentration of ions in the protoplasm, higher concentration of which are toxic to plants. However, a lack of water results in the overproduction of free radicals, leading to the damage of cellular membranes and ultimately loss of solutes from the cell and organelles (Apel and Hirt 2004). Damage to DNA under these conditions severely hinders the ability of the plant to recover, as DNA stores the genetic information that is ultimately used for the synthesis of new proteins (Fig. 4.1).

Drought is one of the most serious world-wide problems for agriculture. Fourtenths of the world's agricultural land lies in arid or semi-arid regions and many variables play a part in reaching drought conditions; these include lack of natural rainfall, soil type, air temperature, humidity, wind conditions, sun exposure and also the plant type (root depth). Plants respond to these conditions with an array of biochemical and physiological adaptations and being sessile they develop tolerance mechanisms to cope with the detrimental effects of environmental stress. They evolved some of the genes coding for an antioxidant enzyme to protect DNA against free radicals, cell membrane stabilizing enzymes and a protein which is thought to stabilize osmotic imbalances by actively transporting solutes across the cell membranes, thereby minimizing water loss during drought.

Timmusk and Wagner (1999) reported that the *Arabidopsis thaliana* plants inoculated with *Paenibacillus polymyxa* plants were more resistant to drought stress and revealed that ERD 15 and RAB 18 is a drought responsive genes and gets differentially expressed in case of *P. polymyxa* treated plants in drought and its mechanism of action of the plant growth promotion was studied by Timmusk et al. (2003). Using plasmid-borne *gfp* gene tagging of *P. polymyxa,* Timmusk et al. (2005) concluded that it colonises on root and form biofilm on plant root tips and enters intercellular spaces but does not spread throughout the plant.

Adaptation to environmental stresses is dependent upon the activation of cascades of molecular networks involved in stress perception, signal transduction, activation and regulation of specific stress tolerant genes. During stress conditions dehydration responsive transcription factor after binding with the cis acting elements promotes both, the stress inducible genes codes for osmoprotectants, scavengers or stress proteins such as cold responsive proteins (COR) or late embryogenesis abundant (LEA) with an undefined mechanism of action (Vinocur and Altman 2005) and regulatory proteins such as transcription factors or components of signal cascades and regulate the expression of a set of genes involved in stress. Both categories of genes have been shown to impart tolerance when overexpressed in plants. Significant improvement of stress tolerance in case of *A. thaliana* has been noticed by the interaction of a single transcription factor, which activates

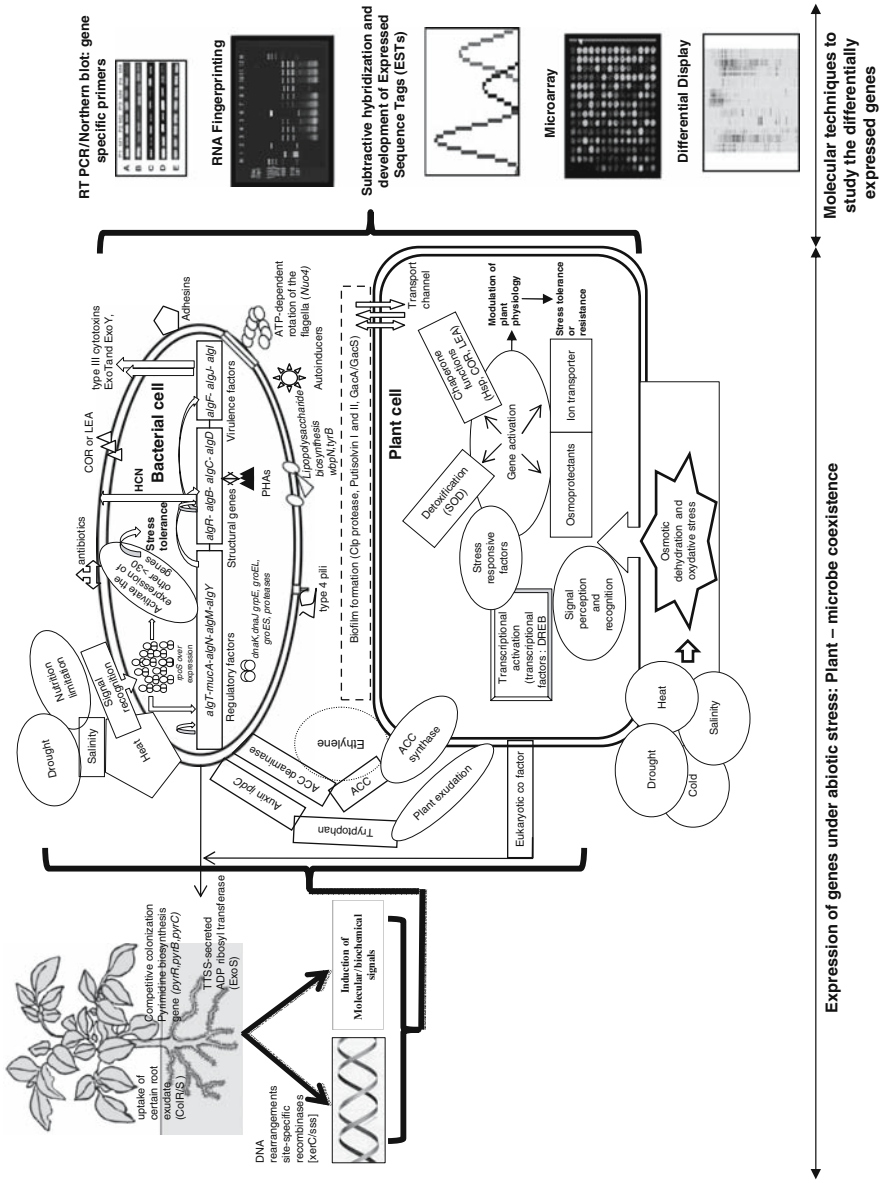

Fig. 4.1 Abiotic stress inducible gene expression in rhizobacteria

expression of downstream genes involved in drought- and salt-stress (Sakuma et al. 2006). Huang and Liu (2006) reported a novel cDNA encoding DRE-binding transcription factor, designated GhDBP3 from *Gossypium hirsutum*, showed enhanced expression by drought, NaCl, low temperature and ABA treatment. During different environmental stresses, basic cellular processes such as DNA replication, repair, recombination, transcription, ribosome biogenesis and translation initiation play essential role and all these functions are genetically controlled by helicases of DEAD-box protein superfamily. Thus, helicases might playing an important role in regulating plant growth and development under stress conditions by regulating some stress-induced pathways (Vashisht and Tuteja 2006). Kwak et al. (2007) reported that *A. thaliana* genome contains eight genes of high mobility group B (HMGB) proteins, and gets upregulated by cold salt and drought stress and downregulated by drought or salt stress. Various strategies have been used to produce transgenic plants with increased tolerance to dehydration stress. These include the overproduction of enzymes responsible for biosynthesis of osmolytes, late-embryogenesis-abundant proteins and detoxification enzymes. However, in case of crop improvement regulatory gene would have potential to play a broader role in stress tolerance and still a careful appraisal for the selection of the genes is expected (Chinnusamy et al. 2004; Sreenivasulu et al. 2007).

4.3 Genomics and Proteomics of Plant–Microbe Coexistence

The ability of microbes to exploit water resources that are less available to plants helps in buffering the soil microbial community from stress as during stressed physiological conditions these rhizomicrobes enter a state of reduced metabolic activity and go for different morphological changes (Kulkarni and Nautiyal 1999). At the onset of starvation, a regulatory response leads to the enhanced expression of particular metabolic genes and almost all virulence gene of functions such as plant cell-wall-degrading enzymes, phytotoxins, ice nucleation activity, exopolysaccharide production, and the type III protein secretion machinery of plant pathogenic bacteria exhibit increased transcription at temperatures well below the respective growth optima (Angela et al. 2001).

Several genes have been reported to be up- or down-regulated in response to different stresses in PGPR (Fig. 4.1). These genes might generate products either directly involved in protection against environmental stress or that play a role in stress regulation. Polyols such as glycerol, mannitol, sorbitol, sucrose and quaternary ammonium compounds such as glycine betaine, proline, betaine, β-alanine betaine, choline *O*-sulphate and the tertiary sulphonium compound dimethylsulphoniopropionate are effective osmoprotectants widely distributed in bacteria, marine algae and many plant families (Rathinasabapathi 2000). Lee et al. (2005) has isolated a novel strain *P. stutzeri* CJ38, that enabled direct transformation of maltose to trehalose and by the introduction of the yeast trehalose-6-phosphate synthase (TPS1) gene in tomato showed improved tolerance under drought, salt and

oxidative stress suggested that carbohydrate alterations produced by trehalose biosynthesis is linked to the stress response and increased tolerance of abiotic stress, without decreasing productivity, under both stress and nonstress conditions through trehalose biosynthesis (Cortina and Culiáñez-Macià 2005).

Under certain stress conditions local changes in the superhelicity of DNA also induce or repress genes both at the level of transcription and translation (Dorman 1991). Transcriptional profiling of *P. aeruginosa* grown under steady-state hyperosmotic stress conditions showed an up-regulation of 116 and 81 genes at least threefold in cells grown in the presence of 0.3 M NaCl and 0.7 M sucrose, respectively. However, 66 genes showed a change in expression of at least threefold in response to both stressors 40 of which are associated with virulence factors, encoding proteins of a type III secretion system (TTSS), the type III cytotoxins ExoT and ExoY, and two ancillary chaperones (Aspedon et al. 2006) (Fig. 4.1).

Heat shock response, a universally conserved stress response is governed by the positive transcriptional control of the σ32 (*rpoH*) polypeptide. Functional genomic study of stress tolerance in *P. putida* by the global mRNA expression studies in 392 regulated genes showed that 36 genes were differentially expressed more than twofold and 32 genes of 23 operons are indispensable in response to abiotic conditions (Reva et al. 2006).

Laville et al. (1992) demonstrated that *P. fluorescens* CHA0 colonizes plant roots, produces several secondary metabolites in stationary growth phase, and suppresses a number of plant diseases, including *Thielaviopsis basicola*-induced black root rot of tobacco. Mutations in a *P. fluorescens* gene named *gacA* (for global antibiotic and cyanide control) pleiotropically block the production of the secondary metabolites 2,4-diacetylphloroglucinol (Phl), HCN, and pyoluteorin. The *gacA* mutants of strain CHA0 drastically reduced ability to suppress black root rot under gnotobiotic conditions, supporting the previous observations that the antibiotic Phl and HCN individually contribute to the suppression of black root rot. The *gacA* gene is directly followed by a *uvrC* gene. Double *gacA-uvrC* mutations render *P. fluorescens* sensitive to UV irradiation. The *gacA-uvrC* cluster is homologous to the orf-2 (= *uvrY*)-*uvrC* operon of *E. coli*. The *gacA* gene specifies a trans-active 24-kDa protein. Sequence data indicate that the GacA protein is a response regulator in the FixJ/DegU family of two-component regulatory systems. Expression of the *gacA* gene itself was increased in stationary phase. Thus Laville et al. (1992) proposed that GacA, perhaps activated by conditions of restricted growth, functions as a global regulator of secondary metabolism in *P. fluorescens*. Later Schnider et al. (1995) reported that CHA0 produces a variety of secondary metabolites, in particular the antibiotics pyoluteorin and 2,4-diacetylphloroglucinol, and protects various plants from diseases caused by soilborne pathogenic fungi. The *rpoD* gene encoding the housekeeping sigma factor sigma 70 of *P. fluorescens* was sequenced. The deduced RpoD protein showed 83% identity with RpoD of *P. aeruginosa* and 67% identity with RpoD of *E. coli*. Attempts to inactivate the single chromosomal *rpo*D gene of strain CHA0 were unsuccessful, indicating an essential role of this gene. When *rpoD* was carried by an IncP vector in strain CHA0, the production of both antibiotics was increased severalfold and, in parallel,

protection of cucumber against disease caused by *P. ultimum* was improved, in comparison with strain CHA0.

At the onset of any stressed conditions bacteria ensure its survival in changing environment by its regulatory mechanisms. These regulatory mechanisms which make essential contributions to bacterial survival under stressed conditions are the alternative sigma factors – RpoS (σ^S) and RpoE (σ^{22}). In response to starvation for carbon, nitrogen, phosphate and amino acids cells leads to enter into stationary phase and the global regulator sigma S (*rpoS*) level induced and resulting in a partial reduction of the growth rate. Additional inducing conditions for *rpoS* are hyperosmolarity, high or low temperature, acidic pH and high cell density and probably all generate a common intracellular signal. Initially it seemed that a reduction or cessation of growth might be a signal. Using an *rpoS* mutant of a cosmopolitan strain *P. aeruginosa,* Jorgenson et al. (1999) proved that during exposure to different stressors such as hydrogen peroxide, high temperature, hyperosmolarity, low pH and ethanol, disruption of the *rpo*S gene resulted in a two- to threefold increase in the rate of kill of stationary-phase cells, thus alternative sigma factor encoded by the *rpoS* gene is a general stress response regulator playing important role for survival under stressful conditions by controlling the expression of genes which confer increased resistance to various stresses. A knockout mutant study during exposure to different stressors (hydrogen peroxide, high temperature, hyperosmolarity, low pH and ethanol) has proven that *rpoS* along with some other factors regulate the desiccation tolerance in *P. aeruginosa* (Jorgensen et al. 1999). By using *rpoS-lacZ* fusions studies it has been reported that *rpoS* transcription induced in late exponential phase and reach a peak upon entry into stationary phase (Benchamas et al. 2003) and during osmotic upshock *rpoS* mRNA expression remains steady, however the protein levels increase (Henge-Aronis 1996). Hulsmann et al. (2003) also reported that *rpoS* in *V. vulnificus* is important for adaptation to environmental changes and may have a role in virulence. The expression of *rpoS*, which encodes an RNA polymerase subunit results in a 30-fold induction at the onset of stressors/stationary phase and regulate the expression of more than 30 genes simultaneously (Fig. 4.1). Kazmierczak et al. (2005) reported that sigma factors provide promoter recognition specificity to the polymerase and contribute to DNA strand separation and then they dissociate from RNA polymerase core enzyme following transcription initiation of many genes as the regulon of a single sigma factor can be comprised of hundreds of genes, sigma factors provide effective mechanisms for simultaneously regulating large numbers of prokaryotic genes (Weber et al. 2005). Arden et al. (2006) – by microarray analysis of osmotically stressed *P. aeruginosa* – have also showed differential gene expression.

Expression of *rpoS* also regulates the production of several exoproducts to alter their environment for the survival in adverse conditions and to impart a role in virulence (Roberson and Firestone 1992; Sledjeski et al. 2001). Bacteria in many environments including soil generally live in colonies within a matrix largely composed of extracellular polysaccharides (EPS). During desiccation, maintenance of functional cell envelop is also a prerequisite for matric stress adaptation as it is exposed to the external environment and is host to various essential metabolic and

structural features thus EPS envelope may protect bacteria from drying and fluctuations in water potential. Pseudomonads used to produce alginate as a main constituent of EPS towards desiccation and osmotic tolerance and which is controlled by the sigma factor AlgU (AlgT) (Keel et al. 2001). Induction of alginate biosynthesis genes and the production of alginate only under water-limited conditions, indicate that EPS production is a fitness trait for survival in low-water-content habitats. Alternatively, dehydration leads to improper folding of the transporters, which in turn activates expression of genes for their biosynthesis. Twenty-six genes homologues of *P. putida* KT2400 genome sequence have been identified for bacterial growth and survival in water-limited environments; these are involved in protein fate, nutrient or solute acquisition, energy generation, motility, alginate biosynthesis or cell envelope structure (Nelson et al. 2002).

P. aeruginosa is capable of synthesising polyhydroxyalkanoic acids (PHAs) and rhamnolipids, both of which are composed of 3-hydroxydecanoic acids connected by ester bonds and synthesising the biofilm matrix polymer alginate. Alginate is a biopolymer of D-glucouronic acid and mannouronic acid and is responsible for adherence, barrier to phagocytosis and neutralizes the oxygen radical. At normal and stress conditions comparison of mRNA concentrations shows difference in the expression of *algD*, the key gene leading to overproduction of alginate. After growth on 3% ethanol but not after heat-shock, an increase in *algD* mRNA levels and a corresponding decrease in *mucB* (a regulatory gene) mRNA levels were detected (Edwards and Saunders 2001). An isogenic knock-out mutants study on *P. aeruginosa* PAO1 and the alginate-overproducing *P. aeruginosa* FRD1 suggested that PHA biosynthesis and alginate biosynthesis are in competition with respect to a common precursor and the PAO1 PHA-negative mutant form a stable biofilm with large, distinct and differentiated microcolonies characteristic of alginate-overproducing strains of *P. aeruginosa* (Pham et al. 2004).

Alginate gene expression is transcriptionally controlled by a gene cluster at 68 min on the chromosome: *algT* (*algU*)-*mucA*-*mucB* (*algN*)-*mucC* (*algM*)-*mucD* (*algY*). The *algT* gene encodes a 22-kDa alternative sigma factor (σ22) that autoregulates its own as well as the promoters of *algR*, *algB*, and *algD* and two AlgR-binding sites, RB1 and RB2, located far upstream from the *algD* mRNA start site, are essential for the high-level activity of *algD* (Mohr et al. 1992; Mathee et al. 1997). The *rpoS* mutant impaired in transcription of *algD* showed that GacA play a role in controlling alginate production and gene expression during the stationary phase in *A. vinelandii* (Castanjeda et al. 2001). Whitchurch et al. (2002b) also stated that AlgR govern the fimbrial biogenesis, twitching motility, and biofilm formation in *P. aeruginosa*. The presence of *O*-acetyl groups plays an important role in the ability of the polymer to act as a virulence factor, and the *algF*, *algJ*, and *algI* genes are essential for the addition of *O*-acetyl groups to alginate. Random fusion study with *phoA* (alkaline phosphatase [AP] gene) to *algF*, *algJ*, and *algI* showed alkaline phosphatase activity, indicating that both AlgF and AlgJ were exported to the periplasm and AlgI, is an integral membrane protein with seven transmembrane domains and they form a complex (AlgI-AlgJ-AlgF) in the membrane which acts as a reaction center for *O*-acetylation of alginate (Franklin and Ohman 2002) (Fig. 4.1).

In *P. aeruginosa*, the response regulator AlgR is required for transcription of *algC* and *algD*, which encode key enzymes in the alginate biosynthetic pathway. In *P. syringae* FF5, however, *algR* is not required for the activation of *algD*. Interestingly, *algR* mutants of *P. syringae* remain nonmucoid, indicating an undefined role for this response regulator in alginate biosynthesis. AlgC promoter has two potential binding sites for AlgR and σ54, the alternative sigma factor encoded by *rpo*N thus AlgR has a positive role in the activation of *algC* in *P. syringae* and contributes to both virulence, epiphytic fitness and systemic movement (Alejandro et al. 2004). Expression analysis revealed that, during mid-logarithmic growth, AlgR activated the expression of 58 genes while it repressed the expression of 37 others, while during stationary phase, it activated expression of 45 genes and repression of 14 genes. Thus the new roles for AlgR are that it can repress gene transcription, can activate *fimTU-pilVWXY1Y2E* operon, regulate HCN production, and controls the transcription of the putative *cbb*3-type cytochrome PA1557 (Lizewski et al. 2004) (Fig. 4.1).

Previous work has shown that functional genomics, in particular DNA microarrays and proteomics, provides a powerful tool to study global gene expression in bacterial biofilms (Sauer and Camper 2001). Successful colonisation of rhizomicrobes includes weakening or destruction of target and competing organisms, efficient uptake of low concentrations of nutrients and competition with other cells of the same or unrelated species for nutrients and the accumulation of extracellular metabolic products, which have a regulatory role on bacterial colonisation. During these interactions microbes entering the rhizospheric environment utilize minor, non-nutrient components of root exudates as signals to guide their movement towards the root surface and elicit changes in gene expression appropriate to the complex beneficial associations and maintain themselves in a competitive manner on the root system (Kiely et al. 2006). The exact composition of the exudates is determined by many factors, including species and nutritional status of the plant, soil structure, micronutrient status and plant developmental stage and the exudation from plant root is also stimulated by microorganisms and these compounds which are mainly exuded by the plant roots promote the bacterial populations from 3- to 100-fold (Bais et al. 2006). Depending on the exact nature of the compound in the root exudates, they may play a role in activation of microbial genes responsible for recognition and initiation of symbiotic association, act as an antimicrobial plant defense, activate or disrupt key microbial genes responsible for biofilm formation, or they may simply act as an easy source of moisture, nutrients, and energy. Rhizodeposition of the roots are playing important role in plant growth promotion was confirmed by the addition of mucilage to the soil which increased the microbial C up to 23% and the number of cultivable bacteria was enhanced by 450%. Catabolic (Biolog GN2) and 16 S–23 S intergenic spacer fingerprints exhibited significant differences between control and mucilage treatments indicates that mucilage can affect both the metabolic and genetic structure of the bacterial community (Benizri et al. 2007).

Through transposon mutagenesis, Roberts et al. (2007) reported that mutants showing insertion at *aceF* [encodes the dihydrolipoamide acetyltransferase subunit of

the pyruvate dehydrogenase complex (PDHC, enzyme responsible for the conversion of pyruvate to acetyl-CoA)] are impaired of rhizosphere colonisation and maintenance of *Enterobacter cloacae* population density in diverse crop plants.

Many bacteria have evolved sensory transduction systems that utilize diffusible signals or "pheromones" to sense and respond to their biotic environment, including their own population density (Kaiser and Losick 1993; Salmond et al. 1995; Swift et al. 1996; Gray 1997). A two component sensor kinase [ColR/S] is involved in the competitive root colonisation ability of *P. fluorescens* WCS365 in response to an environmental stimulus and maintains the cells internal pH, enhance the uptake of certain root exudate due to the proton motive force generated by the NADH dehydrogenases (Dekkers et al. 1998c) and also involved in chemotaxis towards exudate compounds to initiate a high growth rate in the rhizosphere (Dekkers et al. 1998a). Lambda integrase family of site-specific recombinases [xerC/sss] also seems to be essential for colonisation. In certain rhizomicrobes efficient root colonisation is linked to their ability to secrete a site-specific recombinase (Dekkers et al. 1998b).

Margarita et al. (2002) provided evidence that shows that the lack of NADH dehydrogenase I, an enzyme of the aerobic respiratory chain encoded by the *nuo* operon, is responsible for the impaired root-colonisation ability of PCL1201. The complete sequence of the *nuo* operon (ranging from *nuoA* to *nuoN*) of *P. fluorescens* WCS365 was identified, including the promoter region and a transcriptional terminator consensus sequence downstream of *nuoN*. It was shown biochemically that PCL1201 is lacking NADH dehydrogenase I activity. Since it was assumed that low-oxygen conditions were present in the rhizosphere, the activity of the *nuo* and the *ndh* promoters at different oxygen tensions were analysed. The results showed that both promoters are up-regulated by low concentrations of oxygen and that their levels of expression vary during growth. By using *lacZ* as a marker, it was shown that both the *nuo* operon and the *ndh* gene are expressed in the tomato rhizosphere. In contrast to the *nuo* mutant PCL1201, an *ndh* mutant of WCS365 appeared not to be impaired in competitive root tip colonisation.

Martínez-Granero et al. (2006) has demonstrated that in the biocontrol agent *P. fluorescens* F113, phenotypic variation is mediated by the activity of two site-specific recombinases, Sss and XerD. By overexpressing the genes encoding either of the recombinases, large numbers of variants (mutants) after selection either by prolonged laboratory cultivation or by rhizosphere passage were generated. All the isolated variants were more motile than the wild-type strain and appear to contain mutations in the *gacA* and/or *gacS* gene. By disrupting these genes and complementation analysis, it was observed that the Gac system regulates swimming motility by a repression pathway. Variants isolated after selection by prolonged cultivation formed a single population with a swimming motility that was equal to the motility of *gac* mutants, being 150% more motile than the wild type. The motility phenotype of these variants was complemented by the cloned *gac* genes. Variants isolated after rhizosphere selection belonged to two different populations: one identical to the population isolated after prolonged cultivation and the other comprising variants that besides a *gac* mutation harbored additional mutations

conferring higher motility. Results showed that *gac* mutations are selected both in the stationary phase and during rhizosphere colonisation. The enhanced motility phenotype is in turn selected during rhizosphere colonisation. Several of these highly motile variants were more competitive than the wild-type strain, displacing it from the root tip within two weeks.

Certain well-conserved genes in fluorescent *Pseudomonas* spp. are involved in pathogenic interactions between the bacteria and evolutionarily diverse hosts including plants, insects and vertebrate animals. One such gene, *dsbA*, encodes a periplasmic disulfide-bond-forming enzyme implicated in the biogenesis of exported proteins and cell surface structures. Role of *dsbA* in *P. fluorescens* Q8r1-96, a biological control strain that produces the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) and is known for its exceptional ability to colonise the roots of wheat and pea was elucidated (Mavrodi et al. 2006a). The deduced DsbA protein from Q8r1-96 is similar to other predicted thiol:disulfide interchange proteins and contains a conserved DsbA catalytic site, a pattern associated with the thioredoxin family active site, and a signal peptide and cleavage site. A *dsbA* mutant of Q8r1-96 exhibited decreased motility and fluorescence, and altered colony morphology; however, it produced more 2,4-DAPG and total phloroglucinol-related compounds and was more inhibitory in vitro to the fungal root pathogen *Gaeumannomyces graminis* var. *tritici* than was the parental strain. When introduced separately into a natural soil, Q8r1-96 and the *dsbA* mutant did not differ in their ability to colonise the rhizosphere of wheat in greenhouse experiments lasting 12 weeks. However, when the two strains were co-inoculated, the parental strain consistently out-competed the *dsbA* mutant. It was concluded that *dsbA* does not contribute to the exceptional rhizosphere competence of Q8r1-96, although the *dsbA* mutation reduces competitiveness when the mutant competes with the parental strain in the same niche in the rhizosphere. The results also suggest that exoenzymes and multimeric cell surface structures are unlikely to have a critical role in root colonisation by this strain (Mavrodi et al. 2006a). Recently, interaction of *Pseudomonas* spp. with various hosts were investigated to determine their contributions to the unusual colonisation properties of strain Q8r1-96 (Mavrodi et al. 2006b). Mutants were characterized to determine their 2,4-DAPG production, motility, fluorescence, colony morphology, exoprotease and hydrogen cyanide (HCN) production, carbon and nitrogen utilization, and ability to colonise the rhizosphere of wheat grown in natural soil. The data suggested novel functions for two genes, *ptsP* and *orfT*, that previously were linked with pathogenesis in *P. aeruginosa*. The *ptsP* and *orfT* mutants of Q8r1-96 did not have nonspecific growth defects in vitro, and the effects of the mutations became apparent only when the mutants were tested in the rhizosphere, either individually or in competition with the parental strain. Both genes fulfill the criteria for "true" rhizosphere colonisation determinants, and is the first report to provide evidence that *ptsP* is involved in rhizosphere colonisation by fluorescent pseudomonads.

Environmental signals through quorum sensing trigger many plant-associated bacteria to compete with a diverse community of microorganisms including multicellular differentiation, fruiting body development, and sporulation by a process of

signal exchange, which enables bacterial populations to coordinate gene expression in rhizosphere competence (Mazzola et al. 1992). At the onset of effective root colonisation bacteria goes to phase variation, a regulatory process for DNA rearrangements which is orchestrated by site-specific recombinase (Dybvig 1993). Urgel et al. (2000) studied some transposon mutants defective in attachment to corn seeds and sequence analysis of these mutants showed similarity with genes of known functions such as putative surface and membrane proteins, including a calcium-binding protein, a hemolysin, a peptide transporter, and a potential multidrug efflux pump. The second attachment step requires the synthesis of bacterial cellulose fibrils that cause a tight and irreversible binding of the bacteria to the roots and overexpression of a colonisation gene (with functions in the cell envelope, chemotaxis and motility, transport, secretion, DNA metabolism and defense mechanism, regulation, energy metabolism, stress, detoxification, and protein synthesis) of a rhizobacterium of tomato plant caused an increase in the extent of colonisation. Transposon insertion in two overlapping genes with different orientations, *wbpN* and *tyrB* (aromatic amino acid amino transferase) genes, plays a role in root colonisation, whose function in lipooligosaccharide synthesis is still unknown; a regulatory protein (*pyrR)* gene and biosynthetic genes (*pyrB* and *pyrC)* of pyrimidine biosynthetic pathway were also defective mutants (Tn5*lac*) of competitive colonisation (Lugtenberg et al. 2001) and *dsbA*, encodes a periplasmic disulfide-bond-forming enzyme implicated in the biogenesis of exported proteins; cell surface structures were also reported as a gene for competitive root colonisation (Mavrodi et al. 2006a). Recently Rodriguez et al. (2007) also confirmed that cyclic glucans, capsular polysaccharide, and cellulose fibrils are involved in the phenomenon of root colonisation.

More than 80% of known bacterial species shows motility by means of flagella and flagellar motility among bacteria is found as a prerequisite for the movement towards favourable conditions and avoiding detrimental factors and it also allows the bacteria to compete with other rhizobacteria present in the environment. Weger et al. (1987) reported that the flagella are involved in the colonisation of the deeper root parts in potato. During flagellar movement bacteria has to expend too much of energy and a *nuo4* genes coding for the subunits of NADH:ubiquinone oxidoreductase, generate a proton motive force to drive ATP synthesis, active transport and ATP-dependent rotation of the flagella play an important role in root colonisation (Anraku and Gennis 1987; Moens and Vanderleyden 1996). Howie et al. (1987) reported through transposon study that the most severely impaired colonisation mutants are non-motile mutants and mutants impaired in *O*-antigen synthesis (Dekkers et al. 1998c). In addition to motility and attachment, *O*-antigenic side chain of the outer membrane lipopolysaccharides also enhances survival of *P. fluorescens* within tomato root (Duijff et al. 1997). In a steep nutrient concentration gradient environment bacterial chemotaxis is thought to play a critical role in structuring microbial communities, characterisation of the function of two chemotaxis gene clusters (*che1* and *che2*) in *R. leguminoserum* in controlling motility behaviour for effective nodulation also supporting the previous evidences (Miller et al. 2007).

Scher et al. (1988) reported that in wheat and soybean nonmotile mutants of *Pseudomonas* are not impaired in root colonisation and type 4 pili are involved in colonisation of both plants and these pili perform competitive root colonisation through twitching motility. In *P. aeruginosa*, type 4 pili mediate the initial contact between the bacteria and the epithelial cell surface by driving the locomotion through twitching motility and attaching to abiotic surfaces to form biofilms. Once rhizobacteria reach a root and are tightly bound to it they colonise the root by their ability to maintain and grow on the root system to initiate biofilm formation and cytoplasmic Clp protease protein, participates in biofilm formation (O'Toole and Kolter 1998). The matrix, which holds bacterial biofilms together, has been presumed to be derived from lysed cells and is not a important component of biofilm structure including exopolysaccharides, proteins, and DNA which bacteria produce in substantial quantities through a mechanism independent of cellular lysis and it has been confirmed by the treatment of bacterial biofilm to DNaseI (Whitchurch 2002a). The bacterial cells on the surface of the biofilm are different from the cells within the biofilm matrix. The embedded cells behavior can change the thickness of the biofilm. The surface cells are metabolically active and large. These surface cells divide and increase the thickness of the biofilm. Little oxygen is available to the embedded cells, therefore they are smaller and grow slower. The bacteria exist in a somewhat dormant state, becoming active when cells in the outer layers are killed. Recently, a (*mvaT*) gene, a negative regulator of *cupA* (chaperone-usher pathway) was shown to be required for biofilm formation on abiotic surfaces (Isabelle et al. 2004). Among Gram-positives, *B. subtilis* is a ubiquitous soil bacterium that forms biofilms in a process that is negatively controlled by the transcription factor AbrB and different AbrB-regulated genes. YoaW, a putative secreted protein, and SipW, a signal peptidase had a role in biofilm formation (Hamon et al. 2004). The lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes *rmlA*, *rmlC*, and *xanB* are necessary for biofilm formation and twitching motility (Huang et al. 2006). A transposon (Tn5*luxAB*) study of PCL1627 (biosurfactant mutant) has shown that heat shock genes (*dnaK*, *dnaJ* and *grpE*) regulate Putisolvin biosynthesis at the transcriptional level and is dependent on the GacA/GacS two-component signaling and the lipopeptides Putisolvin I and II with surfactant activity, which is induced by the quorum-sensing signals and consequently control biofilm formation by *P. putida* PCL1445 (Kitagawa et al. 1991; Dubern et al. 2006). In response to these quorum sensing signals, bacteria interact with plant tissues through adhesins including polysaccharides, surface proteins, and the recognition of plant produced lectins and their cognate carbohydrates is a common means of specificity; the whole process of biofilm development and intimate interactions is under the control of cell-cell communication between colonising bacteria. These plant-associated biofilms undergo chromosomal rearrangements and are hot spots for conjugative plasmid transfer, favored by the close proximity between cells and the constant supply of nutrients coming from the plant in the form of exudates or leachates (Danhorn and Fuqua 2007).

In the comparison of the protein or transcription profiles of *Pseudomonas* spp. employing comparative proteomics, Sauer and Camper (2001) detected

45 differentially expressed proteins in biofilms of *P. putida* ATCC 39168 following 6 h of attachment, indicating that this strain undergoes a global change in gene expression after surface-adherence. Furthermore, the expression rate of 16 proteins was changed when planktonic cells were grown in a medium supplemented with 3-oxo-C12-HSL. Only one protein, the periplasmatic putrescine binding protein PotF, was found to be downregulated in biofilm cells as well as in the presence of AHL signal-molecules. On the basis of this result it was suggested that QS does not play an important role in the initial attachment process. Sauer and Camper (2001) studied global changes in the expression profile of intracellular proteins during the initial stage of biofilm formation of an AHL-negative *P. putida* strain. At present there is an ongoing debate on whether biofilm differentiation into mature biofilms necessarily requires a defined genetic programme or merely constitutes the sum of cellular adaptations and growth cycles influenced by the nutrient diffusion conditions in individual communities (Kjelleberg and Molin 2002). As a consequence of the latter assumption even global methodologies employing DNA-array technology or proteome analyses will hardly be able to define a "universal biofilm regulon". Moreover, as bacterial cells are distributed in a structured biofilm in numerous microenvironments with different physiological activities, results can only be considered as an overlay of gene expression alterations of all occurring sub-populations. A dissection and consequent proteome analysis of these sub-populations would certainly improve our understanding of the interplay of quorum sensing and sessile lifestyle to regulate gene expression in *P. putida* but is technically very challenging as conventional 2-DE requires high amounts of biomass.

Differentially expressed spots were identified by matrix-assisted time of flight mass spectrometry (MALDI-TOF MS) and database search in the recently completed genome sequence of *P. putida* KT2440 (Nelson et al. 2002). Arevalo-Ferro et al. (2005) investigated the impact of QS and biofilm formation on the protein profile of surface-associated proteins of *P. putida* IsoF. This was accomplished by comparative proteome analyses of the *P. putida* wild type IsoF and the QS-deficient mutant F117 grown either in planktonic cultures or in 60 h old mature biofilms. Differentially expressed proteins were identified by peptide mass fingerprinting and database search in the completed *P. putida* KT2440 genome sequence. The sessile life style affected 129 out of 496 surface proteins, suggesting that a significant fraction of the bacterial genome is involved in biofilm physiology. In surface-attached cells 53 out of 484 protein spots were controlled by the QS system, emphasizing its importance as global regulator of gene expression in *P. putida* IsoF. Most interestingly, the impact of QS was dependent on whether cells were grown on a surface or in suspension; about 50% of the QS controlled proteins identified in planktonic cultures were found to be oppositely regulated when the cells were grown as biofilms. Of all identified surface-controlled proteins, 57% were also regulated by the *ppu* QS system. The data provide strong evidence that the set of QS-regulated proteins overlaps substantially with the set of proteins differentially expressed in sessile cells. In fact, the most striking result of comparative proteome analysis was the finding that expression of QS-regulated proteins in *P. putida* IsoF is strongly dependent on the life style of the organism.

4.4 Strategies to Enhance Plant–Microbe Coexistence

Weller's group has focused on the role of the antifungal metabolite DAPG in biological control of soil-borne pathogens by fluorescent *Pseudomonas* spp. (Thomashow and Weller 1996). Genetic studies, modeled after Koch's postulates, demonstrated unequivocally that DAPG plays a major role in the suppression of a variety of soil-borne plant pathogens by fluorescent *Pseudomonas* strains (Raaijmakers et al. 1999). They have demonstrated that genotypic diversity within a group of antagonistic microorganisms that share a common biocontrol trait has great potential for improving biological control. This approach capitalizes on existing knowledge concerning mechanisms, while exploiting the differences among strains to face the challenges of diverse soil and rhizosphere environments. By matching rhizobacterial genotypes with crops or varieties for which they have a colonisation preference, root colonisation and biocontrol can be increased without increasing the amount of inoculum (Raaijmakers and Weller 2001). The biosynthetic locus for 2,4-DAPG includes five genes, *phlACBDE. phlD*, a key gene in the biosynthesis of the antibiotic and highly conserved in nature (De La Fuente et al. 2006), is widely used for identification of 2,4-DAPG producers. Using molecular fingerprinting by BOX-PCR and *phlD*-RFLP, Weller et al. (2007) have described 22 genotypes. A distinction was made between the superior ("premier") root colonisation of strains of certain genotypes, which reach and maintain large population sizes for long periods of time, and the "average" colonisation of most rhizobacteria, whose rhizosphere populations decline within days or weeks after introduction into the soil. For example, D-genotype strains (i.e. Q8r1-96) are premier colonists of wheat and pea roots, whereas genotype B and L strains (i.e. Q2-87 and 1M1-96, respectively) are average colonists of these crops. Superior rhizosphere competence is a trait that permits a 2,4-DAPG producer to consistently protect roots against soilborne pathogens (Weller et al. 2007). Thus the factor(s) responsible for superior rhizosphere competence of 2,4-DAPG-producing *P. fluorescens* belonging to certain genotypes on some crops remain(s) elusive and variable colonisation of introduced strains remains a major impediment to the widespread use of biocontrol agents.

Genomic subtraction is among the best methods currently available for exploring structural differences between the genomes of closely related bacteria (Straus and Ausubel 1990; Lan and Reeves 2000), including fluorescent pseudomonads (Schmidt et al. 1998). Thomashow's group described the application of genomic suppressive subtractive hybridization (SSH) (Akopyants et al. 1998; Westbrock-Wadman et al. 1999) as one approach to identify genes that contribute to the exceptional rhizosphere competence of D-genotype strains. DNA sequences present in the superior root coloniser *P. fluorescens* Q8r1-96 but not in the less rhizosphere-competent strain Q2-87 were cloned, their sequences determined and analyzed, and their expression in the rhizosphere and distribution among 29 other 2,4-DAPG-producing strains representative of 17 different genotypes were assessed. Several subtracted fragments distributed primarily among isolates of the D genotype or expressed in the rhizosphere were identified as candidates for further analysis

(Mavrodi et al. 2002; Landa et al. 2002, 2003). The development of bacterial artificial chromosome (BAC) systems has allowed the construction of large insert-sized DNA libraries (Shizuya et al. 1992). Compared with YAC and cosmid cloning, BAC has a lower rate of chimerism and higher efficiency of cloning. It is also easier to handle and is more stably maintained. To date, BAC libraries have been constructed in many kinds of organisms, e.g. human (Kim et al. 1996), plants (Woo et al. 1994), fungi (Nishimura et al. 1998) and bacteria (Dewar et al. 1998) have become a powerful tool for genome analysis (Blomberg and Lugtenberg 2001). Because it employs large-sized DNA inserts, the BAC system offers the following significant advantages for cloning and analysis of bacterial genomes: (i) it requires only a relatively small number of clones to provide complete coverage of the bacterial genome and (ii) it facilitates cloning of clustered genes, such as those for certain metabolic processes, for secretion, or for pathogenicity (e.g. *hrp* genes). Such libraries are powerful tools for genome analysis, physical mapping, map-based cloning, and simple screening for specific genomic sequences because of their low chimeric clone formation rates and high cloning efficiency. Moreover, they are easy to handle and can be stably maintained. The physical organisation of phytobeneficial genes was investigated in the plant growth promoting rhizobacterium *A. lipoferum* 4VI by hybridization screening of a BAC library (Blaha et al. 2005).

Ryu et al. (2005) have investigated the mechanisms by which PGPR, elicit plant growth promotion from the viewpoint of signal transduction pathways within plants. Results suggest that elicitation of growth promotion by PGPR in *Arabidopsis* is associated with several different signal transduction pathways and that such signaling may be different for plants grown in vitro vs in vivo.

Recently Zuo et al. (2007) reported that an ERF transcription factor gene (GbERF2) was cloned by suppression subtraction hybridization from sea-island cotton after *Verticillium dahliae* attack. These results show that GbERF2 plays an important role in response to ethylene stress and fungal attack in cotton.

Developments in high-throughput DNA sequencing have resulted in elucidation of the whole-genome sequences. Current data (as of August 2, 2007 on BLAST with microbial genomes 912 bacterial/46 archaeal/139 eukaryotic genomes tree) can be obtained from URL NCBI data base (http://www.ncbi.nlm.nih.gov/sutils/genome table.cgi) data base and references quoted therein). *P. fluorescens* SBW25 (PfSBW25) is a Gram-negative bacterium that grows in close association with plants. In common with a broad range of functionally similar bacteria it plays an important role in the turnover of organic matter and certain isolates can promote plant growth. Despite its environmental significance, the causes of its ecological success are poorly understood. Gal et al. (2003) described the development and application of a simple promoter trapping strategy IVET to identify PfSBW25 genes showing elevated levels of expression in the sugar beet rhizosphere. A total of 25 rhizosphere-induced (*rhi*) fusions are reported with predicted roles in nutrient acquisition, stress responses, biosynthesis of phytohormones and antibiotics.

One of the promises in the genomics era is an improved ability to identify causal relationships among genotype, phenotype and the environment, and to do this on a genome wide scale. In particular, the advent and dissemination of genomics

information and technologies has resulted in the development of several powerful new approaches that allow one to simultaneously analyze both the phenotype and the genotype of thousands of mutants. Collectively, these tools improve the ability to map phenotypic landscapes and develop integrated models connecting genetic alterations and their resultant phenotypes. As a result of advances in genomics technologies, several techniques now exist that substantially improve researchers ability to identify such genes. Metabolic engineers now have the ability using DNA micro-arrays to map phenotypic landscapes of considerable genetic diversity, which should improve understanding of the relationships that exist among phenotype, genotype, and environment. It has became apparent that bacteria of a certain species living in close association with different plants either as associated rhizosphere bacteria or as plant pathogens or symbiotic organisms typically reflect this relationship in their genetic relatedness and is markedly influenced by soil management and soil features. Further studies must address the consequences of the co-operation between microbes in the rhizosphere under field conditions to assess their ecological impacts and biotechnological potential.

4.5 Concluding Remarks and Future Prospects

There are more opportunities available for microbiologists today than at any time in the history of the field. Although the microbiological advancements of the last century have been profound, a great deal of biology remains to be discovered and described through study of the microbial world. Knowledge of microbial diversity and function in soils is limited because of the taxonomic and methodological limitations associated with studying these organisms. Although methods to study diversity (numerical, taxonomic, and structural) are improving for both bacteria and fungi, there is still not a clear association between diversity and function. Even if an organism is functionally redundant in one function, chances are it is not redundant in all functions and will have different susceptibilities and tolerances to abiotic and biotic stresses. It is generally thought that a diverse population of organisms will be more resilient to stress and more capable of adapting with environmental changes. Our understanding of plant–soil interactions can be greatly refined through the development of "smart" field technology, where real-time, computer-controlled electronic diagnostic devices can be used to monitor rhizosphere and plant health. The maximization of production efficiencies will also involve the development of crop cultivars that are bred specifically to capitalize on beneficial plant–microbial associations.

The application of molecular tools is enhancing our ability to understand and manage the rhizosphere and will lead to new products with improved effectiveness. Thus, future research in rhizosphere biology will rely on the development of molecular and biotechnological approaches to increase our knowledge of rhizosphere biology and to achieve an integrated management of soil microbial populations. The new tools of recombinant DNA technology, mathematical modeling, and

computer technology combined with a continuation of the more classical approaches such as crop rotation, various tilling strategies, addition of organic amendments such as compost, mulch or manures, should quickly open up new ways to harness the power of microbes to improve soil, plant, human and the environment health.

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