Chapter 12
Genomic Insights and Comparative Genomics of *Bacillus* Species Having Diverse Mechanisms of Biocontrol Against Fungal Phytopathogens

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12.1 Introduction

Emerging developments in next-generation sequencing (NGS) technologies allow exploration of whole genome sequence of bacteria and other organisms (Schuster 2008). Systemic analysis of whole genome data and identification of genes that contribute to the plant growth promotion and biocontrol activity has aided in the understanding of the molecular mechanism of many bacterial species (MacLean et al. 2009). NGS have been employed to study genomes of several PGPRs such as *Pseudomonas* spp. and *Bacillus* spp. (Song et al. 2012; Duan et al. 2013). Previously, insufficient knowledge underlying the mechanisms of interaction between plants and *Bacilli* (Qiao et al. 2014) with the lack of genetic data had hindered their application in agriculture and biotechnology, although certain progress had been made in the last decade (Borriss et al. 2011). However, we assume that competence of rhizosphere and function of the biocontrol in *Bacilli* are partly caused by nonribosomally produced cyclic lipopeptides acting against phytopathogenic microorganisms (Stein et al. 1996). Over the past few years, the draft or complete genome sequencing of bacteria isolated from the rhizosphere has been carried out providing the genetic attributes involved in plant growth promotion and biocontrol. The genomic overview of the *Bacillus* isolates had provided genetic mechanisms underlying plant growth promotion and antagonism against pathogens. The data allow us to use bioinformatics to mine the genome for potential secondary metabolites effective against plant pathogenic fungi for developing agro-biotechnological agents with predictable features. Also, it is necessary to reveal and elucidate the genetic mechanisms involved in plant-associated lifestyle and whole biocontrol process achieved by PGPR (Palazzini et al. 2016). Genome mining has been used to predict

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uncharacterized genes clusters, although few known secondary metabolites have been identified; hundreds of secondary metabolites are yet to be identified accurately. The sequencing of a number of prokaryotic genomes had allowed comparing the genomes, and, accordingly, evolution of the clusters was explored using comparative genomics across all known core and accessory genomes of *Bacillus* strains (Belbahri et al. 2017). Core genome is the shared genetic material among nearly all the strains of the species. It contains the housekeeping genes and is interspersed with accessory genomic parts. An accessory genome is assumed to be present in some strains while absent in the rest of the species strains (Ozer et al. 2014).

Phytopathogenic fungi are microbial agents that cause major biotic stress which contribute significantly to the global loss in yield of crop plants (Ribera and Zuniga 2012). The fungi of the divisions *Ascomycetes*, *Basidiomycetes*, or *Oomycetes* are most prevalent as plant pathogens. The main fungal diseases of plants comprise mildew, powdery mildew, rusts, coals, guts and deformation, necrosis, cankers, leaf wilts, vascular, root rots, flower rots, fruit rots, etc. For instance, *Phytophthora* spp. cause black pod, frosty pod, and witches' broom in cacao that leads to yield loss. *Cladosporium herbarum* cause passion fruit rust; *Mycosphaerella fijiensis* cause black Sigatoka of banana which results in reduction of yield and quality of fruit (Wood and Lass 2001; Phillips-Mora et al. 2006; Cuervo-Parra et al. 2011). Phytopathogenic *Fusarium* fungi cause several diseases of grain cereals. *F. graminearum*, *F. culmorum*, *F. poae*, *F. avenaceum*, and *Microdochium nivale* are common pathogens of wheat and barley (Parry et al. 1995; Sutton 1982; Miedaner 1997; Tekauz et al. 2000; Brennan et al. 2003; Leslie et al. 1996), and *F. graminearum*, *F. moniliforme*, and *F. subglutinans* on maize (Sutton 1982; Leslie et al. 1996; Velluti et al. 2000; Torres et al. 2001) cause significant yield losses worldwide (Popovski and Celar 2013). The phytopathogenic plant disease *Fusarium* head blight (FHB) (head scab) is one of the most destructive diseases for cereals, and it is caused by a group of *Fusarium* species including *F. graminearum* and *F. culmorum* (Nasraoui et al. 2007). *Fusarium* foot rot and *Fusarium* root rot on cereals are also caused by soilborne fungi during wet seasons (Scherm et al. 2013).

Earlier, farmers have become more dependent on agrochemicals as a consistent method of crop production as agricultural production has been intensified (Santos et al. 2012). However, wide usage of chemicals has negative effect on the environment as well as human health (Tournas 2005). The plant-associated nonpathogenic microorganisms have the potential to increase soil fertility and plant health, and it has been used to replace chemical fertilizers. Due to development of resistance, various chemicals had become ineffective to control diseases, and therefore, biological control through the use of beneficial natural microflora of soil popularly known as plant growth-promoting rhizobacteria (PGPR) offers the opportunity for sustainable agricultural systems protecting the plants from pathogens while simultaneously lowering chemical residues (Vessey 2003). Biocontrol agents are able to protect plants from phytopathogenic organism infection (Bloemberg and Lugtenberg 2001). It is environmentally safe, and it employs natural antagonists of pests and pathogens (Cook 1993). Therefore, the use of biological control is considered as a safer and more sustainable strategy for profitable agricultural production.

Bacterial endophytes benefit plants by imparting biotic and abiotic stress to hosts, colonizing the same niche similar to that of phytopathogens (Rai et al. 2007). Some bacterial endophytes have been shown to produce anti-pest compounds to protect plants from various pathogens as well as to promote growth of the host plants (Gheler et al. 2013). *Bacillus* species comprise a physiologically versatile group of bacteria isolated from diverse habitats. *Bacillus*-based biocontrol agents have proved to be effective against a broad range of plant pathogens. Mostly, *Bacillus* species used as biocontrol agents are mobile with peritrichous flagella (Driks 2004). The species has versatile metabolic activities, and it has been regarded as a safe biological agent (Kim et al. 2003). Various *Bacillus* species were shown to have antifungal activity against phytopathogenic fungi that mark them as a good biocontrol agent (Li et al. 2014). The main mechanisms by which biocontrol agents suppress pathogens include antibiosis, competition, plant growth promotion, and induction of systemic resistance. Antibiotic production also plays a key role in biocontrol activities (Szczech and Shoda 2006). *Bacillus* strains are usually isolated as biological control agents or plant growth promoters, due to their capacities to produce a wide range of antifungal compounds, including volatiles, enzymes, lipopeptides, and several small peptides (Moyne et al. 2004; Romero et al. 2007).

Many genome sequences of PGPR isolates from the genus *Bacillus* have been published. To extend the understanding of the potential antifungal capacities, 286 genome assemblies possessing the biocontrol properties have been reported within the species of *B. velenzensis*, *B. subtilis*, *B. amyloliquefaciens*, *B. methylotrophicus*, and *B. thuringiensis*. Forty-seven strains within *B. velenzensis* have been sequenced, and 20 of them have been assembled completely (Chen et al. 2007; Cai et al. 2013; Yang et al. 2014).

This chapter highlights the genomic analysis and comparative analysis of genomes of different biocontrol *Bacillus* spp., with the aim to study the genetic traits and secondary metabolites production to reveal the potential of *Bacillus* spp. for biocontrol of phytopathogens and plant growth promotion.

12.2 Biological Control of Phytopathogens

Various *Bacillus* species including *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. cereus*, *B. mycoides*, *B. amyloliquefaciens*, *B. amyloliquefaciens* subsp. *plantarum*, *B. oryzicola*, and *B. thuringiensis* have been reported to control fungal diseases of plants because of their ability to produce antibiotics and/or suppress growth of several fungal pathogens such as *Rhizoctonia* spp., *Fusarium* spp., *Sclerotinia* spp., *Gaeumannomyces* spp., *Nectria* spp., *Pythium* spp., *Phytophthora* spp., and *Verticillium* spp. (Romero et al. 2004; Leclere et al. 2005; Kotan et al. 2009; Zhang et al. 2009). Enzymatic and endosporic products of *B. subtilis* were found to be active against many fungal pathogens (Denner and Gillanders 1996). Several strains of *B. amyloliquefaciens*, a close relative of *B. subtilis*, have been reported to be effective in control of plant pathogens (Yu et al. 2002). Generally, the mode of

action of *Bacillus* is (i) antibiosis (ii) production of extracellular enzymes (iii) competition to inhabit an ecological niche by utilizing root exudates and thus affecting growth of pathogens (Suarez-López 2010; Doornbos et al. 2012). *B. subtilis* strains have been reported to have an antagonistic effect against *Fusarium* spp. (Baysal et al. 2013).

12.3 Genomes of *Bacillus*, Which Are Antagonist of Fungal Phytopathogens

The strain *B. subtilis* RC 218 isolated from wheat anthers was suggested as a potential antagonist of *Fusarium graminearum*. It was demonstrated to reduce disease and the associated mycotoxin (deoxynivalenol) accumulation under field conditions. The genome of *B. subtilis* RC 218 was observed to encode five non-ribosomal peptide synthetase clusters – surfactin, iturin A, fengycin, the siderophore bacillibactin, and the antibacterial bacilysin along with three gene clusters for polyketide synthetase (PKS) – bacillaene, difficidin, and macrolactin (Palazzini et al. 2016).

Biocontrol strain *B. subtilis* XF-1 isolated from the rhizosphere soil of cabbage infected by *Plasmodium brassicae* had been proposed to show suppression effect on 21 fungal pathogens belonging to oomycetes, ascomycetes, basidiomycetes, and deuteromycetes (Xiong et al. 2009). The genome of XF-1 contains gene clusters for antifungal lipopeptides, i.e., surfactin and fengycin, and the siderophore bacillibactin (Guo et al. 2015).

In agriculture, several *B. velezensis* have been used as plant growth promoters and antagonist of plant pathogens (Yao et al. 2006; Chen et al. 2007; Dunlap et al. 2015). *B. velezensis* had been used as biocontrol agent as it has the ability to suppress pathogenic fungi. It was reclassified as a synonym of *B. amyloliquefaciens* subsp. *plantarum*, *B. methylotrophicus*, and *B. oryzicola* (Dunlap et al. 2015). A number of *B. velezensis* species had been described to produce lipopeptides, which have antifungal properties synthesized by non-ribosomal peptide synthetases. The whole genome sequence of endophytic biocontrol *B. velezensis* strain CC09, isolated from healthy leaves of *Cinnamomum camphora*, was reported to harbor 13 clusters for secondary metabolite production that showed a broad spectrum of antifungal activities against numerous phytopathogens such as *Glomerella glycines*, *Rhizoctonia solani*, *Alternaria alternata*, and *Blumeria graminis*. The metabolites detected are surfactin, bacillaene, macrolactin, bacitracin, difficidin, iturins, plipstatins (fengycins), bacillibactin, and bacilysin. The strain showed inhibition of growth of fungi including *Geotrichum candidum*, *Aspergillus niger*, *Phytophthora capsici*, and *Mycosphaerella pomi*, when the enzyme activity assay was performed in presence of iturin A (0.66 mg/mg) (Yang et al. 2014; Cai et al. 2017). The whole genome of *B. velezensis* M75, which is an isolate from cotton waste used for mushroom cultivation in Korea, have been reported to have antagonistic activity against fungal plant pathogens. The genome comprises operons encoding various

non-ribosomal peptide synthetases and polyketide synthases that are responsible for the biosynthesis of secondary metabolites (Kim et al. 2017).

B. velezensis strain LM2303 was reported to be effective in inhibiting the growth of *F. graminearum*, *F. culmorum*, *A. flavus*, *F. moniliforme*, *Coniothyrium olivaceum*, *Rhizomorpha roth*, and *Alternaria tenuissima* (Chen et al. 2017). The genome of LM2303 was proposed to detect a total of 29 biosynthetic gene clusters (BGCs), out of which 13 (18% of the whole genome) were identified including 4 NRPSs, 3 PKSs, and 2 PKS-NRPS hybrid synthetases. The three annotated BGCs encoding antifungal metabolites include fengycin, iturin, and surfactin. Iturin was found to be encoded by PKS-NRPS hybrid cluster with a length of 37.3 kb in LM2303 (Chen et al. 2018). Also, *srf* gene cluster spans for 26.2 kb in LM2303. The four *srf* genes (*srfAA-AD*) were found to be 78% similar with the strain FZB42. Further, the *sfp* gene was detected together with the regulatory gene *yczE*. The synthetase genes for the metabolites were shown to be 100% similar with *B. velenzensis* strains (Chen et al. 2018).

B. amyloliquefaciens FZB42 isolated from the rhizosphere of *Beta vulgaris* is a representative of a group of endophytic *Bacillus* strains. The strain had been proposed to consider this specific group of rhizobacteria as an ectomorph, which was distinct from the *B. amyloliquefaciens* type strain F (Reva et al. 2004). The strain was developed as biocontrol agent (BCA) to control diseases of tomato, cucumber, cotton, tobacco, and lettuce (Grosch et al. 1999; Yao et al. 2006, Wang et al. 2009a; Chowdhury et al. 2013, 2015), and it showed extraordinary colonizing ability on *Lemna minor*, *Arabidopsis thaliana*, *Zea mays*, *Lycopersicon esculentum*, and *Lactuca sativa* (Fan et al. 2012). The genome of *B. amyloliquefaciens* FZB42 was reported to dedicate approximately 9% (340 kb) of its genetic capacity to the synthesis of antimicrobial metabolites. It has several gene clusters directing to the synthesis of a huge range of secondary metabolites such as bacillaene, bacillibactin, bacillomycin D, bacilysin, difficidin, fengycin, macrolactin, and surfactin (Chen et al. 2009).

The plant-associated *B. amyloliquefaciens* GA1 isolated from strawberry had been observed to exhibit inhibitory activity toward the growth of fungal and oomycete plant pathogens. Incidence of post-harvest infection caused by *Botrytis cinerea*, which is the causative agent of gray mold disease in apple, was reported to be reduced by the strain GA1 (Toure et al. 2004). From the genome analysis, it has been investigated that the partial ORFs of eight gene clusters directing the synthesis of non-ribosomal peptide and polyketide synthetase have been identified in GA1. Fourteen gene clusters were observed to direct the synthesis of cyclic lipopeptides surfactin, iturin A, fengycin, and iron-siderophore bacillibactin. Of these, 11 gene clusters had a high amino acid identity with *srf* (80–96%) or *fen* (41–92%) operon that directs the synthesis of surfactin and fengycin, respectively, in *B. amyloliquefaciens* FZB42 (Chen et al. 2007).

The antagonist *B. amyloliquefaciens* AS 43.3 isolated from a wheat head was reported to reduce the incidence of *Fusarium* head blight (FHB) which is caused by *Gibberella zeae* (*Fusarium graminearum*) (Khan et al. 2001; Schisler et al. 2002). The genome sequence of the strain AS 43.3 was reported to encode nine biosynthetic

clusters associated with biocontrol activity including three lipopeptides (surfactin, iturin, and fengycin) and a siderophore (bacillibactin) (Dunlap et al. 2013).

The genome of *B. amyloliquefaciens* subsp. *plantarum* CAU B946, an isolate from rice rhizosphere, was described to commit about 8.5% (nine gene clusters) of the whole genome to be involved in non-ribosomal synthesis of antimicrobial compounds and siderophores. The strain has been reported to show great potential as a biofungicide for the control of various plant disease like tobacco black shank, rice sheath blight, cotton *Fusarium* wilt, cotton *Verticillium* wilt, and wheat scab (Blom et al. 2012; Hao et al. 2012).

The complete genome sequence of *B. amyloliquefaciens* subsp. *plantarum* YAU B9601-Y2 strain isolated from wheat rhizosphere had been reported to produce NRPS and PKS (9 gene clusters, 8.5% of the whole genome) such as bacillomycin D, fengycin, and difficidin. The strain was suggested to suppress a broad spectrum of pathogenic fungi, promote growth and rooting of crops and vegetables, and develop drought resistance of wheat, corn, and broad bean, and it was also found to reduce the number of nematodes in tomato and tobacco rhizosphere. Presence of diverse antifungal activities in these strains was unique to their genomic features (Hao et al. 2012).

The genome of *B. amyloliquefaciens* subsp. *plantarum* UCMB5113 isolated from soil has been described to colonize plant roots and stimulate growth of the plants and limit the growth of several fungal pathogens on oilseed rape such as *Alternaria brassicae*, *Botrytis cinerea*, *Leptosphaeria maculans*, and *Verticillium longisporum* (Danielsson et al. 2007; Sarosh et al. 2009). The genome harbors gene clusters for the synthesis of lipopeptides including surfactin (*srf*), bacillomycin D (*bmy*), and fengycin (*fen*). The organization of gene clusters in UCMB5113 was found to be similar with genomic segments in the strain FZB42 (Niazi et al. 2014).

12.4 Comparative Genomes of *Bacillus* Which Are Antagonist of Fungal Phytopathogens

The genome sequencing allowed us to accurately determine the taxonomy of the strain using a phylogenomic approach. Comparative genome studies of four strains, *B. amyloliquefaciens* FZB42, *B. subtilis* 168, *B. licheniformis*, and *B. pumilus*, suggested that they belong to a closely related taxonomic unit called *B. subtilis* group (Fritze 2004). The four members of *B. subtilis* group consisted of 2139 genes sharing more than 50% identity to each other by core genome analysis. The majority of the genes of FZB42 were found to be conserved in *B. subtilis* (3271), whereas *B. amyloliquefaciens* and *B. pumilus* share 2378 genes in common. A significant portion of the FZB42 genome was found to be conserved in *B. cereus* ZK (2342), *B. anthracis* (2338), *B. thuringiensis* (2335), *B. clausii* (2162), *B. halodurans* (2105), and *Geobacillus kaustophilus* (1995) (Chen et al. 2009).

The strain GA1 which was initially identified as *B. subtilis* was proposed to assign as *B. amyloliquefaciens* which resulted from phylogenetic tree of *recA* and *recN* genes. The two genes, encode DNA repair and recombination proteins, respectively, and had been suggested to exhibit in resolving closely related taxa (Zeigler 2003). The protein-coding sequence detected in the genome of the strain GA1 was investigated to be highly similar with *B. amyloliquefaciens* FZB42 (Arguelles-Arias et al. 2009). Five *dhb* gene fragments of GAI directing the synthesis of the siderophore bacillibactin was described to be similar with in *B. amyloliquefaciens* FZB42, located between *CDS304* and *yuilV* ORFs. The *comS* gene in GA1, which encodes a competence signal molecule, was described to be embedded within *srfAB* that is similar to *B. amyloliquefaciens* FZB42 and *B. subtilis* 168. This proposed that an interspecies horizontal gene transfer might follow between *B. subtilis* and *B. amyloliquefaciens* (Arguelles-Arias et al. 2009).

Borriss et al. (2011) proposed that *B. amyloliquefaciens* comprises two subspecies: the plant-associated *B. amyloliquefaciens* subsp. *plantarum* and the non-plant-associated *B. amyloliquefaciens* subsp. *amyloliquefaciens* based on phylogenic analysis, physiological characteristics, and production of antibiotics. A comparative genome analysis of *B. amyloliquefaciens* and *B. plantarum* subsp. strains showed a high level of genomic homogeneity sharing 72–84% coding genes among the genomes which specified that these two groups have recently diverged. In addition, two gene clusters encoding difficidin and macrolactin were not found in the genome sequence of *B. amyloliquefaciens.* Genes which are unique to plant-associated strains differentiate biocontrol strains from non-plant-associated species (Niazi et al. 2014).

Dunlap et al. (2015) stated that it was difficult to differentiate *B. amyloliquefaciens*, *B. subtilis*, and *B. velezensi*s on the basis of 16S rRNA sequence similarity, morphological characteristics, and physiological and chemical reactions. Several heterotypic synonyms such as *B. subtilis*, *B. methylotrophicus*, *B. amyloliquefaciens* subsp. *plantarum*, and *B. oryzicola* have been regrouped as *B. velezensis* based on the genome sequencing, comparative genomics analysis, and DNA-DNA relatedness calculations (Dunlap et al. 2015). Earlier reports regrouped *B. subtilis* RC 218 and *B. amyloliquefaciens* IT45, Y2, and LFB112 into *B. velezensis* (Palazzini et al. 2016). Wang et al. (2008) described *B. velezensis* as a heterotypic synonym of *B. amyloliquefaciens*. The unique characteristics of *B. velezensis* consist of plant growth promotion, antifungal metabolite production, and competent colonization on plants (Madhaiyan et al. 2010; Borriss et al. 2011; Dunlap et al. 2015).

The core genome of 35 strains of *B. velezensis*, *B. amyloliquefaciens*, *Bacillus* sp. Pc3, and *B. subtilis* 168 was reported to share 257,259 bp in length including 2574 CDSs. Phylogenomic tree constructed from these core genome sequences grouped the strain CC09 as *B. velezensis* which was previously identified as *B. amyloliquefaciens* (Cai et al. 2017).

Based on a comparative genomic analysis of *Bacillus* strains, three clusters are conserved in strains of *B. velezensis*, *B. amyloliquefaciens*, and *B. subtilis* 168, and nine clusters were conserved in *B. velezensis* and *B. amyloliquefaciens* and two in all strains of *B. velezensis*. Until now, there are few reports on comparative genome

data of *B. velezensis* strains regarding the production of secondary metabolites. Earlier reports suggested two clusters encoding NRPS and NRPS-Trans ATPKS, respectively, were found only in some strains of *B. velezensis* that might lead to the synthesis of new bioactive compounds (Ongena and Jacques 2008; Chen et al. 2009; Arrebola et al. 2010; Alvarez et al. 2012).

A comparative analysis of four *B. velezensis* genomes M75, FZB42, CAU B946, and YAU B9601 had shown that the genome sequence of M75 strain (4,007,450 bp) was found to be larger than other genome of FZB42 strain (3,918,589 bp) and smaller than other *B. velezensis* strains (4,242,774–4,019,861 bp) (Kim et al. 2017). In another study, He et al. (2012) suggested that the genomes of *B. amyloliquefaciens* B9601 and the closely related type strain have 3426 CDS in common. The M75 strain was observed to have 145 unique genes that were not found in other *B. velezensis* strains. The strain M75 possesses 22–23 extra genes associated with secondary metabolites biosynthesis as compared to the other *B. velezensis* strains. It has been investigated that M75 strains has the highest number of genes (87 genes and 2.6% of the whole genome) associated with the secondary metabolite biosynthesis, transport, and catabolism. The core genome analysis of four strains M75, CAU B946, FZB42, and YAU B9601 consisted of 3414 CDSs with average identity of 95%, and pan genome consisted of 4558 CDSs. All the four strains have been suggested to have strong antagonistic activities toward plant pathogens as the operons that encode the biosynthetic enzymes for the secondary metabolites were found to be highly homologous. However, the operon encoding biosynthetic enzymes in the four *B. velezensis* strains showed a difference in gene clusters (Kim et al. 2017).

The whole genome of the strain LM2303 has been reported to harbor the largest number of gene clusters associated with secondary metabolite biosynthesis, transport, and catabolism (119 genes, 3.08% of the whole genome) when compared with three biocontrol strains FZB42, M75, and CAU B946, which specify that LM2303 has higher potential as biocontrol agent than other *B. velezensis* strains. Using phylogenomic analysis of single-copy core genes, *Bacillus* strain LM2303 was described as a member of *B. velezensis* clade as it was found to cluster closely with *B. velezensis* strains CAU B946 and M75 (Chen et al. 2018).

Another comparative analysis of eight genomes of *B. amyloliquefaciens* was reported to identify 3,316,600 bp core genome and 5,529,004 bp pan genomes for the species. It was observed that the alignment of four biocontrol genomes were similar, with *B. amyloliquefaciens* AS 43.3 most similar to *B. amyloliquefaciens* FZB42. The strain AS 43.3 comprised 263,690 bp genes unique to the biocontrol strains. The nine metabolite clusters present in the strains were investigated to be found in the same relative physical location in the genome with same gene order (Dunlap et al. 2013). The core genome of biocontrol strains identified three large synthetic cluster (macrolactin, difficidin, and fengycin) that are specific for plant-associated *B. amyloliquefaciens* subsp. *plantarum*. Although a part of fengycin cluster (*fenDE*) was found in strain *B. amyloliquefaciens* DSM-7, it is not functional due to lack of other needed enzymes (*fenABC*) (Borriss et al. 2011).

Genome comparison of *B. amyloliquefaciens* subsp. *amyloliquefaciens* $DSM7_T$, UCMB5113, FZB42 identified 112 unique coding sequence in the genome

UCMB5113. The core genome of *Bacillus* species comprised of 2391 orthologs. The genome of *B. subtilis* 168, *B. amyloliquefaciens* FZB42, and *B. amyloliquefaciens* UCMB5113 was found to share 3421 orthologs, whereas UCMB5113 and $DSM7_T$ were found to share 3345 orthologs (Niazi et al. 2014). The comparative genomes of *Bacillus* strains with the gene clusters encoding antifungal metabolites are given in Table 12.1.

The core genomes of all representatives of *B. amyloliquefaciens* subsp. *plantarum* was detected to contain 3347 CDS, on the other hand, representatives of the two *B. amyloliquefaciens* subsp. "*plantarum*" and "*amyloliquefaciens*" contained 3153 CDS. Also, the core genome analysis of *B. amyloliquefaciens* B9601 with other representatives of *B. amyloliquefaciens*, *B. atrophaeus*, and *B. subtilis* was found to contain 2459 CDS. The strain B9601-Y2 was identified to harbor 81 unique genes. Moreover, it revealed that 130 genes were found in the representatives of *B. amyloliquefaciens* subsp. *plantarum*; however, it was absent in *B. amyloliquefaciens* subsp. *amyloliquefaciens* (He et al. 2012).

Furthermore, phylogenomic analysis of the sequenced genome allows us to check taxonomic validity of the isolates and define the extent of interspecies genome variability within the strains. Phylogenomic analysis based on the core genomes (1906 genes) of *B. methylotrophicus*, *B. amyloliquefaciens* subsp. *plantarum*, and several closely related strain showed that *B. methylotrophicus* KACC 130105^T and *B. amyloliquefaciens* subsp. *plantarum* $FZB42^T$ were closely related and they were found to share 95% of the same genes. This approach confirmed that the strain *B. amyloliquefaciens* subsp. *plantarum* $FZB42^T$ was not clustered with other *B. amyloliquefaciens* and proposed that the subspecies *B. amyloliquefaciens* subsp. *plantarum* should be regrouped as *B. methylotrophicus* (Dunlap et al. 2015).

12.5 Plant-Microbe Interaction

The microorganisms that exist within the tissues of plants without producing negative effects to the host are termed as endophytes. They produce anti-pest compounds to protect plants from several pathogens and insects (Wilson 1995). Plant-associated bacteria are described to play an important role in stimulating plant growth and protecting them from phytopathogens with the production of secondary metabolites. Efficient colonization on plant roots is essential for the biocontrol strains for disease suppression and for plant growth promotion (Timmusk et al. 2005).

The process for colonization in the plant roots relies on the passive movement in the water fluxes or by surface motility and capable of biofilm formation by bacterial cells (Guo et al. 2015; Zeriouh et al. 2014; Arguelles-Arias et al. 2009). Establishment of plant-microbe interaction is the next efficient step for colonization by non-specific binding or by forming of bacterial biofilm formed by various extracellular matrix (Guo et al. 2015). In many *Bacillus* strains, the expression of genes for flagellar movement is important to confirm cell motility (*fli*D, *fli*P, *flg*M) and chemotaxis (*che*C, *che*D, *che*V) in response to root exudates (Fredrick and Helmann 1994).

Table 12.1 Comparative genomes of *Bacillus* strains with gene clusters encoding antifungal metabolites

Features	*B. velezensis*			*B. amyloliquefaciens*			*B. amyloliquefaciens subsp. plantarum*		*B. subtilis*
Strains	CC09	M75	LM2303	FZB42	As 43.3	GA1	YAU B9601	UCMB5113	RC218
Genome size (mb)	4,167,153	4,007,450	3,989,393	3,918,589	3,961,291	461.5 kbp	4,242,774	3,889,530	3,873,821
GC content (%)	46.1	46.6	46.68	46.5	46.6	46.6	45.85	46.71	46.5
CDS	4141	3803	3866	3695	3919	358	3991	3106	3603
Surfactin	*srfABCD*	*srfABCD*	–	*srfABCD*	*srfABCD*	*srfABCD*	*srfABCD*	*srfABCD*	*srfABCD*
Iturin	*ituDABC*	–	*ituDABC*	–	*ituDABC*	*ituDABC*	*ituDABC*	–	*ituDABC*
Bacilomycin	–	–	–	*bmyCBAD*	–	–	*bmyD*	*bmyCBAD*	–
Fengycin	*fenABCDE*	*fenABCDE*	*fenABCDE*	*fenABCDE*	*fenABCDE*	*fenABCDE*	*fenABCDE*	*fenABCDE*	*fenABCDE*
Bacillibactin	*dhbABCDEF*	*dhbACEBF*	–	*dhbABCDEF*	*besA, dhbACEBF*	*dhbABCDEF*	*dhbABCDEF*	*dhbACEBF*	*besA, dhbACEBF*
References	Cai et al. (2017)	Kim et al. (2017)	Chen et al. (2018)	Chen et al. (2009)	Dunlap et al. (2013)	Arguelles-Arias et al. (2009)	Hao et al. (2012)	Niazi et al. (2014)	Palazzini et al. (2016)

The presence of gene clusters (*flgBCDEGKLMN*, *flhABFOP*) and *swrABC* have been investigated in the genomes of different *B. amyloliquefaciens* (Ghelardi et al. 2012). *B. subtilis* XF-1 has flagellar biosynthesis operon (*fli*/*che*) and two stator elements, *mot*AB and *mot*PS (Werhane et al. 2004). These gene clusters which are responsible for cell envelope and cellular processes motility and chemotaxis have been identified in different genomes of *Bacillus* strains (Niazi et al. 2014). *Bacillus velezensis* LM2303 has gene clusters for flagellar assembly (*flg* and *fli* cluster) and bacterial chemotaxis (*che* cluster). LM2303 has operons encoding Tas*A* protein for biofilm (*yqxM-sipW-tasA*), regulator gene (*sinR* and *sinI*), and exopolysaccharide (*eps* cluster) for colonization (Chen et al. 2018). Presence of gene clusters (*flg*, *flh*, *fli*) for production of functional flagellar components along with *swrA* gene for regulation of expression of flagellar genes in *B. amyloliquefaciens* subsp. *plantarum* UCMB5113 made it to exhibit swarming motility. Also operons *epsA-O* and *yqxM-sipW-tasA* for making up biofilm were found in strain UCMB5113 (Niazi et al. 2014). Homology-based searches and annotation of genes have been conducted in *Bacillus* genomes encoding exopolysaccharide using *B. subtilis epsA-O* operon genes *tapA*, *tasA*, *sipW*, *pgsB*, and *bslA* (Vlamakis et al. 2013). Moreover, a positive correlation between surfactin production and biofilm formation was observed, insufficiency in production of surfactin directed to a defect in biofilm formation and ultimate reduction of suppression of disease (Zeriouh et al. 2014). Further, surfactin was described to be able to inhibit formation of biofilm by pathogenic bacteria, thus providing colonization of biocontrol strains with antagonistic advantage (Chen et al. 2009). In the biocontrol system, certain interactions of plant, pathogen, and biocontrol strains can be generated by biocontrol strains, for example, direct antagonism toward pathogens, plant growth promotion, and ISR. Biocontrol strains promote plant growth by making it easier for plants to get nutrients or by producing plant growth-promoting hormones (Niazi et al. 2014). There are many *Bacillus* species that have been reported as endophytic bacteria in higher plants which promote plant growth and secrete antimicrobial compounds (Wang et al. 2009b; White et al. 2014). Surfactin and iturin favor plant root colonization by stimulating cell spreading, swarming, and biofilm formation (Kinsinger et al. 2003; Hofemeister et al. 2004). Also, iturins and fengycin exhibit strong activity against fungus and are inhibitory for the growth of various plant pathogens (Maget-Dana et al. 1992).

12.6 Protection Against Phytopathogens

Antagonism or mycotrophic ability of any organism includes special strategies to expand the pathogens and pests. It could be through a direct mycotrophic interaction or indirectly by inducing a defense response in plants to combat against the attackers. The effectiveness of biological control agents are determined by its diversity of mechanisms through which biological control agents are able to attack antagonistically on plant pathogens and benefit plants by suppressing disease (Van-Loon et al. 1998; Pozo and Azcón-Aguilar 2007; Jamalizadeh et al. 2011). About 2428

antimicrobial peptides identified from various organisms, 756 peptides have several degrees of antifungal properties (Microbiology UDoPa 2016). The mechanisms by which fungi lead to death include blockage, distraction, and holes formation in the cell wall and cell membranes of the fungi induced by antifungal metabolites produced by biocontrol organisms. Furthermore, some peptides disintegrate fungal intracellular organs such as nucleic acid and mitochondria (Zhao et al. 2013).

Chen et al. (2018) proposed that *B. velezensis* LM2303 can control fungal disease in wheat by four different mechanisms: (i) direct antagonistic action against *F. graminearum* and other pathogens mediated by *Bacillus* lipopeptides and antibacterial metabolites, (ii) stimulation of ISR in wheat by surfactin and volatiles, and (iii) plant growth promotion by producing plant growth hormones, and (iv) competition for space and nutrients through efficient colonization and persistence. A similar mechanism was found in *Bacillus* species which produce other metabolites like chitinases and other cell wall-degrading enzymes and volatile compounds that kill the fungus, and they also provoke plant resistance mechanisms (Pelletier and Sygusch 1990). Chitosanase and proteases have been reported to play an important role in dissolving and penetrating the cell walls of *Rhizoctonia solani* (Mcquilken and Gemmell 2004). Besides β-1,3-glucanase synthesized by *Paenibacillus*, *B. cepacia* has the capability to control the growth of *F. oxysporum*, *Rhizoctonia solai*, *S. rolfsii*, and *Pythium ultimum* cell walls.

Siderophore production had also been suggested to be one of the mechanisms of biocontrol actions of various rhizobacteria (Loper and Buyer 1991). Siderophores are ferric iron chelators that enable bacterial cells to accumulate and acquire iron in environments where bioavailability of iron is limited. Biocontrol antagonists have been recognized for competing for essential micronutrients like iron in the rhizosphere whose availability is dependent on soil. Concentration of iron in ferric form in aerated soil may be as low as 10^{-8} mol/L which is insufficient for growth and development of microorganisms. Presence of microorganisms which are able to produce siderophore in the rhizosphere adds plant health by completing iron and making it less available to phytopathogens (Kloepper et al. 2004). A functional gene cluster (*dhb*) had shown to direct the synthesis of catecholate iron-siderophore bacillibactin. It is the cyclic trimeric lactone of the 2, 3-dihydroxybenzoyl-Gly-Thr (Chen et al. 2007). Synthesis of bacillibactin is dependent on a functional Ppant transferase (s*fp*). Purified bacillibactin from *B. subtilis* AH18 has exhibited suppression against *Phytophthora capsici* which demonstrates the role of siderophore as biocontrol systems (Woo and Kim 2008). It had been proposed that bacillibactin was not produced in *B. subtilis* 168, although the respective gene cluster was present (May et al. 2001). Also, the gene cluster (*dhbACEBF)* in *B. amyloliquefaciens* FZB42 was examined to be 87–93% similar with *B. amyloliquefaciens* GA1 (Chen et al. 2009).

Studies on mechanisms of biocontrol by PGPR have revealed that various PGPR strains have the ability to protect plants from pathogenic organisms by colonizing the roots. The resulting subsequent resistance due to an inducing agent when infected by a pathogen is called induced systemic resistance (ISR) (Hammerschmidt and Kuc 1995). Concentration of antifungal lipopeptides determined in plants have been reported to be low, and other antimicrobial activity were not detected so far in

surrounding area of plant roots colonized by PGPR bacilli. Therefore, ISR is assumed as one of the main factors for suppressing phytopathogens by PGPR bacilli. It depends on the capability of some strains to actuate defense systems in the host plants, thereby reducing the susceptibility of host to successive infection in plant tissues, without provoking any symptoms themselves (Stein 2005). ISR acts through two signaling pathways, i.e., salicylic acid (SA) and jasmonic acid (JA) pathway. ISR is induced when the plant is challenged by pathogenic organisms, and it stimulated physical and mechanical strength of the cell wall and physiological and biochemical reaction of the host and thus invades pathogen on its ability to build a defense chemicals against them by the host (Benhamou et al. 1996). It has been investigated that PGPR induces structural modification of the cell wall in response to pathogenic attack (M'Piga et al. 1997). *B. pumilus* strain SE 34 used in seed treatment have shown to induce strengthening of cell wall in tomato against *F. oxysporum* f.sp. *radicislycopersici* (Benhamou et al. 1998).

It depends on the capability of some strains to actuate defense systems in the host plants, thereby reducing the susceptibility of host to successive infection in plant tissues, without provoking any symptoms themselves (Stein 2005). Various strains of *Bacillus* like *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* have been reported to produce induced systemic disease resistance which caused reduction of disease incidence of host (Ryu et al. 2003). Figure 12.1 shows the different mechanisms of *Bacillus* species in controlling plant disease. Five *Bacillus* species have been evaluated to suppress bacterial wilt

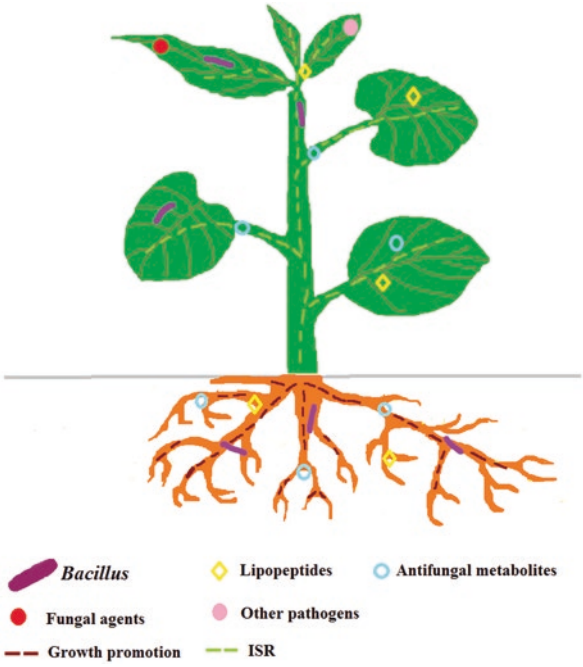

Fig. 12.1 Mode of action of *Bacillus* in rhizosphere. The figure illustrates the interaction of biocontrol *Bacillus* species (purple rod), fungal pathogens (red filled circle), and plant. The bacteria produce variety of secondary metabolites (light blue) and form a protective zone. Lipopeptides (yellow rhombus) act against fungal agents, inhibiting the growth of pathogenic fungi. Other metabolites stimulate ISR-mediated protection against the pathogen. The growth of the plant (red lines) is enhanced, by plant growth-promoting nutrients and growth regulators

caused by *R. solanacearum*. The bacterial strains *B. vallismortis* EXTN-1 have shown to reduce disease in plant which resulted from production of host plant resistance genes. The main constituents of systemic-induced resistance are phenolic compounds, genetic and structural modifications, plant resistance activators, and activation of enzymatic weapons (Park et al. 2007). PGPR strains can induce systemic resistance in plants through activation of various defense-related enzymes like chitinases, β-1,3-glucanase, peroxidase (PO), phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO) (Bharathi 2004).

Biocontrol *Bacillus* PGPR strains secrete specific metabolites that elicit plant defenses. The two well-known plant growth promoters 3-hydroxy-2-butanone (acetoin) and 2,3-butendiol act as volatile elicitors for ISR, and exposure to such volatile organic compounds results in reduced disease incidence in *Arabidopsis* (Ryu et al. 2004). Acetoin production in *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937 involves two enzyme-encoding genes *alsS* and *alsD* that encode acetolactate synthase and acetolactate decarboxylase, respectively, and *alsR* regulates these genes (Ryu et al. 2004). Rate et al. (1999) stated that some common plant metabolites like organic acids especially oxaloacetate activate the *alsSD* operon which is required for acetoin production. Acetolactate synthase (*alsS*) catalyzes the condensation of two pyruvate molecules into acetolactate, and acetolactate decarboxylase (*alsD*) converts it into acetoin. The *als* gene cluster (*alsR*, *alsS*, *alsD*) which encodes 3-hydroxy-2-butanone and the gene *bdhA* which encodes the enzyme (R-R)-butanediol dehydrogenase for catalyzing 3-hydroxy-2-butanone to 2,3-butanediol were identified in LM2303 (Chen et al. 2018). The UCMB5113 chromosome also harbors genes that ferment pyruvate to acetoin and 2,3-butanediol (Niazi et al. 2014). Two synthetic pathways (*alsSDR* and *ilvHC*) for plant growth-promoting volatile compounds were identified in the genome of XF-1. The later pathways are the parts of leucine, valine, and isoleucine biosynthesis. The XF-1 genome had observed to possess six alcohol dehydrogenase whose function is thought to catalyze the reduction of acetoin to 2,3-butanediol (Guo et al. 2015). Cassan et al. (2009) suggested that the biosynthesis systems of acetoin and 2,3-butanediol in PGPR can change from acidic products, lactate, and toxic compounds to neutral volatile compounds which are plant growth promoters to carbohydrate catabolism. This shift may be beneficial for strain colonization and plant symbiotic relationship in the rhizosphere.

12.7 Conclusion

The *Bacillus* genus includes species that have been isolated from a wide range of sources, and they are used as biocontrol agents because the spores produced by them have the ability to withstand extreme environmental conditions. The effective application of PGPR in agriculture is interesting, and appropriate information is required to understand the biological processes mediating the modes of action that enhance plant growth. They synthesize several kinds of lipopeptides with specific

activity against pathogens, and it gives a unique importance in agriculture. The mechanisms of biological control of plant diseases are diverse and complex, and the biocontrol ability of microbial strains is the outcome of complex interaction with each other. *Bacillus* biocontrol agents use a variety of mechanisms, and the most reported ones are attributed to non-ribosomally produced lipopeptides (Ongena and Jacques 2008). There are numerous reports where different species of *Bacillus* have been reported for their plant growth enhancement and biocontrol activities. The genomic data of the isolates have facilitated a better understanding of the *Bacillus* strains as biocontrol agents against phytopathogenic fungi. Nevertheless, detailed studies are needed to investigate the genetic components associated with plant pathogens' suppression. Various genomes of *Bacillus* have been reported to encode gene clusters for synthesis of various NRPS (bacillibactin, fengycin, surfactin, iturin) which regulate broad antifungal activities as well as their roles in disease control. Genes dedicated for plant colonization, motility, plant growth promotion, and ISR have been identified by genomic approach. In addition, comparative genome studies allowed us to identify a subset of genes shared in the species and unique genes of the isolate characterizing the potential metabolite for biocontrol application. An overview of comparative genomics of plant growth-promoting *Bacillus* strains may perhaps aid in studying the evolution of plant growth promotion and mechanisms of biocontrol species. These data, together with experiments performed in the laboratory, will help to clarify the potential *Bacillus* bacteria have for effective biocontrol agents to be used for plant growth and plant disease control.

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