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

Rainer Borriss

3.1 Introduction

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

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

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

R. Borriss (✉)
Bacterial Genetics/Institute of Biology, Humboldt University Berlin, Chausseestr. 117, 10155 Berlin, Germany

ABiTEP GmbH, Glienicker Weg 185, 12489 Berlin, Germany
e-mail: rainer.borriss@rz.hu-berlin.de; rborriss@abitep.de

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

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

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

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

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

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

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

Box 3.1 Chemopesticides and Biopesticides

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

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

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

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

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

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

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

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

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

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

3.2 Bacilli as Biofungicides and Biofertilizers: Advantages and Disadvantages

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

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

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

According to Ehlers (2006)

Table 3.4 Importance of biopesticides in North America

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

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

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

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

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

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

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

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

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

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

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

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

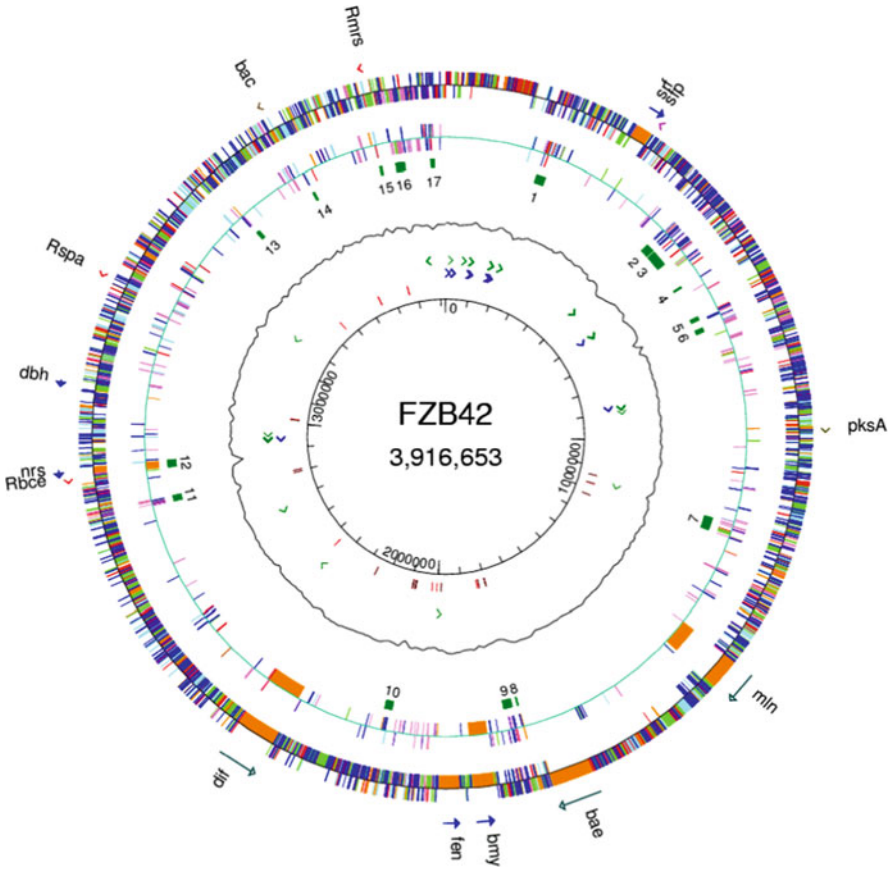

Fig. 3.1 *The genome of B. amyloliquefaciens FZB42*. The likely origin of replication was defined by similarities to the corresponding regions in *B. subtilis* and *B. licheniformis*. The first nucleotide pair of the *B. amyloliquefaciens* genome was defined congruent with *B. subtilis* as the A:T nucleotide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

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

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

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

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

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

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

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

3.5 Plant–Bacteria Interactions

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

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

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

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

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

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

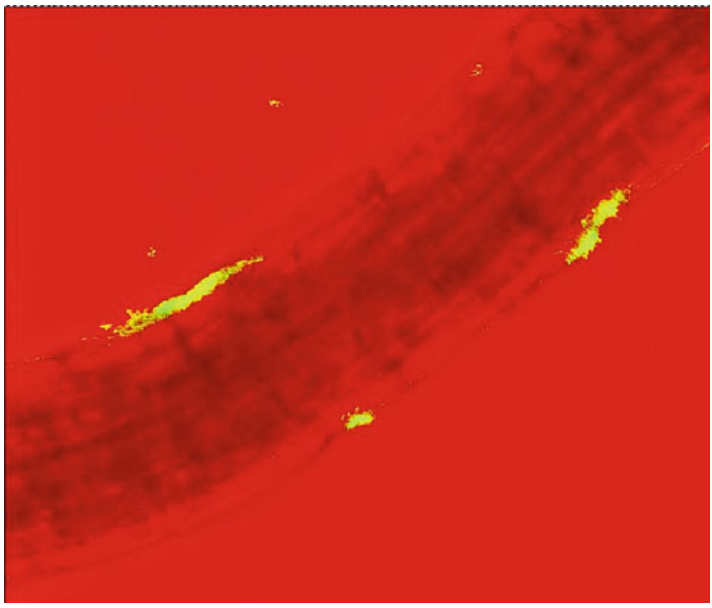

Fig. 3.2 *Colonization of plant roots by GFP-labeled FZB42*. GFP+-labeled strain BF02mut, a derivative of FZB42 (B. Fan, unpublished), forms microcolonies, appearing as *yellow* spots onto the *red* surface of the Arabidopsis primary root, grown in a gnotobiotic system. The confocal scanning laser microscopy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

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

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

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

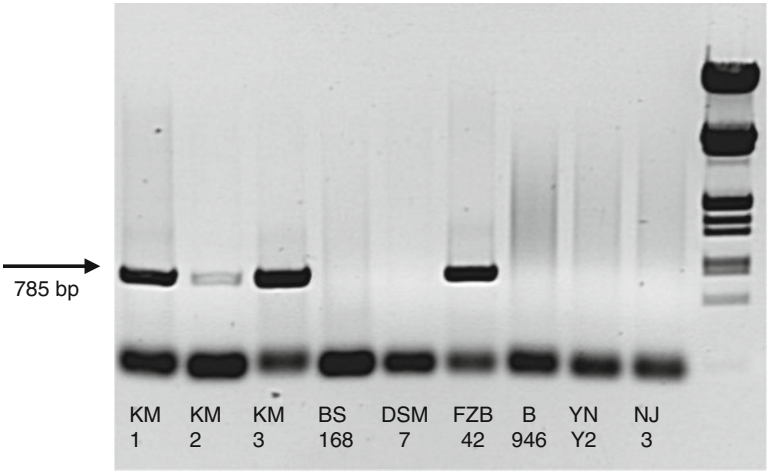

Fig. 3.3 *Detection of FZB42, reisolated from environmental samples*. Five months after its inoculation to Snapdragon plants (Antirhinum major) grown in Chenggong County, Kunming, Yunnan province, China, soil samples were taken and used for reisolation of FZB42. Colonies with the typical morphology of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

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

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

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

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

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

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

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

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

3.5.3 Bacterial Compounds, Beneficial for Plant Growth

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

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

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

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

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

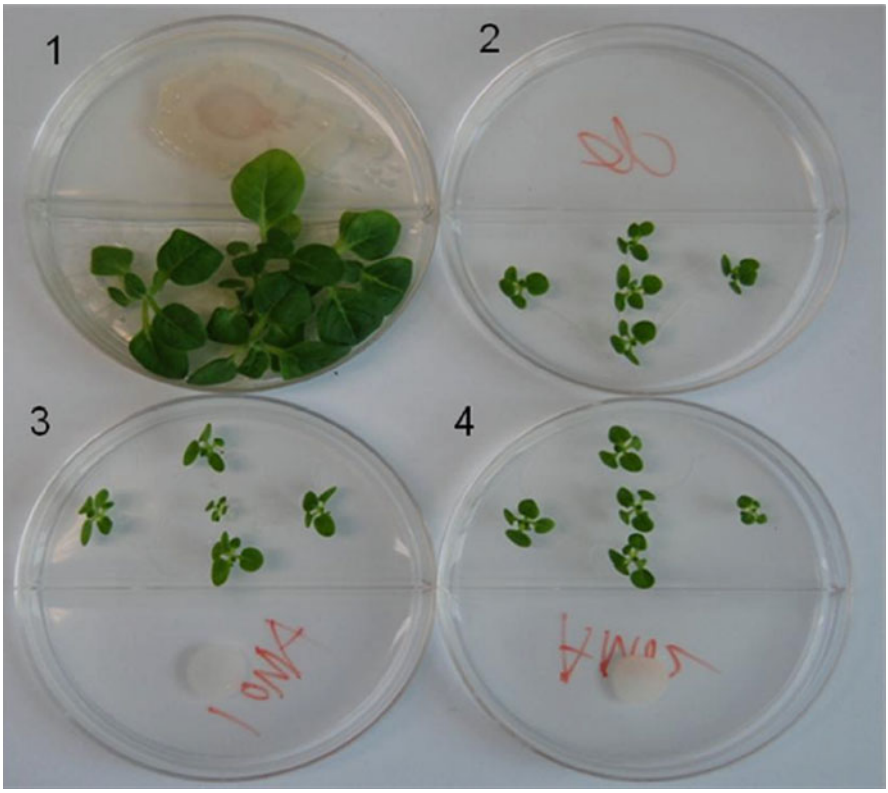

Fig. 3.4 *Influence of volatiles emitted by FZB42 on plant growth*. The picture was taken 4 weeks after inoculation of Arabidopsis with FZB42 (1), and the mutant strains, unable to produce volatiles due to defects in the *alsD* (3) and *alsS* (4) gene, respectively. The water control is indicated by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

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

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

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

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

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

3.6 Biofertilizer Function

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

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

3.6.1 Soil Aggregation

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

3.6.2 Nitrogen and Nitrite Extrusion

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

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

3.6.3 Mineral-Solubilizing Bacteria and Phytase Activity

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

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

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

3.7 Biological Control of Soil and Airborne Plant Diseases

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

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

3.7.1 Nonribosomal Lipopeptides

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

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

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

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

Box 3.3 Nonribosomal Synthesis of Lipopeptides

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

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

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

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

3.7.2 Nonribosomal Synthesized Polyketides

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

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

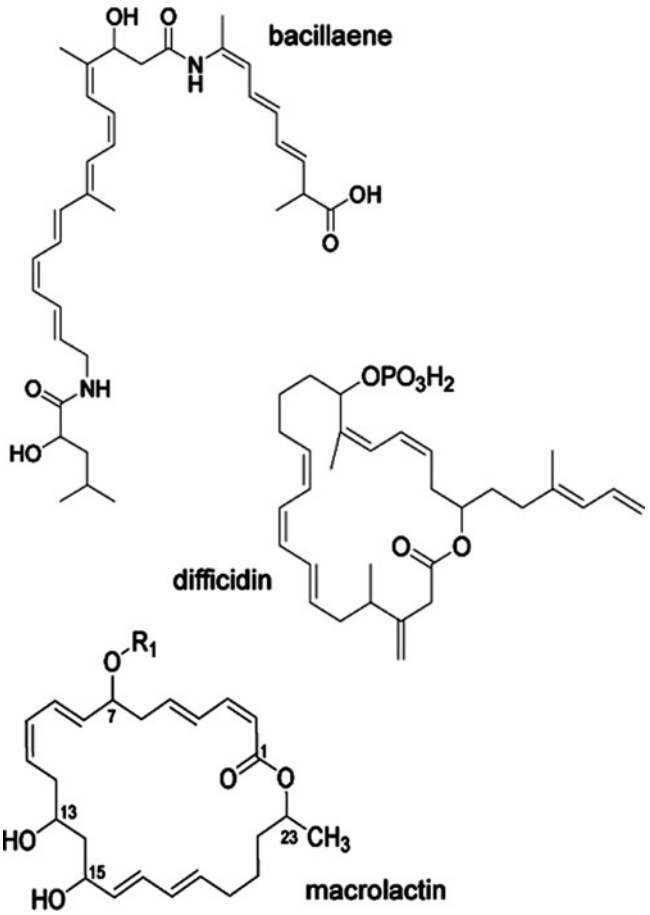

Fig. 3.5 Chemical structure of the polyketides synthesized in FZB42

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

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

3.7.3 Ribosomal Synthesized Peptides (Lantibiotics and Bacteriocins)

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

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

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

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

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

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

3.7.5 Induced Systemic Resistance

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

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

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

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

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

3.7.5.1 Comparison of FZB42 with Related Strains

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

3.8 Conclusions and Outlook

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

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

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

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

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