

Environmental and Microbial Biotechnology

Manoj Kumar  
Vivek Kumar  
Ram Prasad *Editors*

# Phyto- Microbiome in Stress Regulation

 Springer

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# **Environmental and Microbial Biotechnology**

## **Series Editor**

Ram Prasad, Department of Botany, Mahatma Gandhi Central University,  
Motihari, Bihar, India

Innovative and novel advances in microbial biotechnology are providing great understandings in to the machineries of nature, presenting fascinating prospects to apply principles of biology to different arenas of science. Sustainable elucidations are emerging to address the concerns on improving crop productivity through microbes, depleting natural resources, environmental pollution, microbial degradation of pollutants, nanomaterials, nanotoxicity & safety issues, safety of food & agricultural products etc. Simultaneously, there is an increasing demand for natural bio-products of therapeutic and industrial significance (in the areas of healthcare, environmental remediation, microbial biotechnology). Growing awareness and an increased attention on environmental issues such as climate change, energy use, and loss of non-renewable resources have carried out a superior quality for research that provides potential solutions to these problems. Emerging microbiome approaches potentially can significantly increase agriculture productivity & human healthcare and henceforth can contribute to meet several sustainable development goals.

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Editors

# Phyto-Microbiome in Stress Regulation

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ISSN 2662-1681

ISSN 2662-169X (electronic)

Environmental and Microbial Biotechnology

ISBN 978-981-15-2575-9

ISBN 978-981-15-2576-6 (eBook)

<https://doi.org/10.1007/978-981-15-2576-6>

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The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

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## Preface

This book presents state-of-the-art research on the many facets of the plant microbiome, including survivability aspects of plants based on ecology, physiology and genomics, as well as molecular mechanisms of plant-microbe interactions. Topics are well thought out which include the significance of microbial assisted plant growth, induced systemic resistance, tolerance to abiotic stress and biological control of plant pathogens.

The respective contributions show how microbes help plants to cope with abiotic stresses and represent significant progress towards understanding the complex regulatory networks critical to host-microbe interaction and plant adaptation in extreme environments. New insights into the mechanisms of microbial actions in inducing plant stress tolerance open new doors for improving the efficacy of microbial strategies and could produce new ways of economically increasing crop yields without harming the environment. As such, this book offers an essential resource for students and researchers with an interest in plant-microbe interaction, as well as several possibilities for employing the plant microbiome for the enhancement of crop productivity under future climate change scenarios.

Phyto-stimulation and bio-control by plant-associated microbes set a revolutionary paradigm in modern findings towards farming culture in developing countries. At the same time, genetically modified (GM) crops harbouring few significant strains, i.e. *Bacillus thuringiensis* (BT) gene(s) to combat biotic stress caused by insect pests, endorse the contemporary theme of bridging research for the agriculture sector. The book captures the cumulative efforts of contributors in a review format, which can be a meaningful addition for modern researchers.

It not only provides details on existing challenges but also offers deeper insights into the possibility of solving problems, compiled as follows, but not limited to:

- Applications of plant-microbe interactions in contaminated agro-ecosystem management.
- Development of future bio-formulations for sustainable environment.
- Heavy metals, hydrocarbons, radioactive materials and bioremediation of dyes.
- Microbes and soil and water toxicants management.
- Role of plants, genetically modified organisms (GMOs) and earthworms in bioremediation or any topic on bioremediation.

Emphasis is given on the most neglected part of scientific exploration that is rare micro-flora of earth where scientists are understanding the mechanism behind survivals; (b) targeted that the efficient plant microbiome and their benefits in agro-ecosystem, water bodies, human health and targeting global community in plant, soil, water and rare microbe synergism field; (c) highly specialized book on biotic and abiotic stresses and its management through microbial technological inputs, will help the academicians, researchers, environmentalists, industrialists, agriculturists and graduate students and (d) experts across the globe expressed the practiced knowledge in the shape of chapters which deal with zest of specific research on microbiome mediated stress tolerance.

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## About the Editors



**Manoj Kumar** is working as Associate Professor and Head of in the Department of Life Sciences, Central University of Jharkhand, Ranchi, India. He is a scientist with sanguine behavior who is adoring about research and development, with a commitment to lifelong learning. Dr. Kumar has pursued his PhD in Plant Biotechnology and continuing his career in multidisciplinary research in Plant Developmental Biology, Plant-Microbe Interaction and Forest Molecular Genetics. He is a referee for many journals, including Phytoremediation, Journal of Soil Sediments etc. He has guided several research scholars at doctoral and masters level, also he is leading a multidisciplinary research group comprised of diverse research background in biological sciences.



**Vivek Kumar** is an agricultural microbiologist having 20 Years of experiences in teaching, research and guidance, with a pledge to enduring knowledge. Dr. Kumar is working as Associate Professor Department of Microbiology, Himalayan School of Biosciences, Swami Rama Himalayan University, India.

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# Phytohormone and Biocontrol by the Plant-Associated *Bacillus amyloliquefaciens* FZB42: An Update

Rainer Borriss

## Abstract

*Bacillus amyloliquefaciens* FZB42, the type strain for representatives of the plant-associated subspecies *plantarum*, stimulates plant growth and suppresses soil-borne plant pathogens. The strain has been sequenced in 2007. The *B. amyloliquefaciens* FZB42 genome reveals an unexpected potential to produce secondary metabolites. In total, 11 gene clusters representing nearly 10% of the genome are devoted to synthesizing antimicrobial metabolites and/or to confer immunity against them. Ability to synthesize nonribosomally, the antibacterial polyketides macrolactin and difficidin and the antifungal lipopeptide bacillomycin D is a unique feature of the subspecies *plantarum*. However, according to latest research, most of the secondary metabolites are not expressed in plant rhizosphere suggesting that the anti-biome expressed during the plant-associated state of PGPR Bacilli does not reflect the vast genetic arsenal devoted to the formation of secondary metabolites. There is now strong evidence that plant-associated Bacilli trigger pathways of induced systemic resistance, which protect plants against attacks of pathogenic microbes, viruses, and nematodes.

## Keywords

*Bacillus amyloliquefaciens* · Macrolactin · Antimicrobial-metabolites · *plantarum*

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© Springer Nature Singapore Pte Ltd. 2020

M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, [https://doi.org/10.1007/978-981-15-2576-6\\_1](https://doi.org/10.1007/978-981-15-2576-6_1)

## 1.1 Introduction

Environmental-friendly biotechnological approaches, such as the use of microbial biopesticides, offer alternatives to chemical control of plant diseases and pests. Among these alternatives, the use of bioformulations, which are manufactured from plant growth-promoting rhizobacteria (PGPR) with biocontrol activity (BC) (Lugtenberg et al. 2013), is steadily increasing. At present, due to the long-term shelf life of their endospores, bacilli are the most widely used bacteria on the biopesticide market. Their use in agriculture has been previously reviewed (Borriss 2011). An update of *Bacillus*-based bioformulations, currently available for the farmer interested on sustainable agriculture, is presented in Table 1.1.

Plant rhizosphere is a highly competitive environment in which bacteria are abundantly present due to availability of nutrients actively secreted by the plant root and mucilage. Some of these bacteria which are living within or in the vicinity of plant roots and supporting plant growth are generally referred to as PGPR (Kloepper et al. 1980). In many cases, the plant growth-promoting activity is linked with their ability to suppress soil-borne plant pathogens (bacteria and microfungi), occurring in the competing microflora. Different mechanisms are discussed in this context. Besides production of antimicrobial (“antibiotics”) and nematicidal compounds, also stimulation of plant-induced systemic resistance (ISR, Doornbos et al. 2012), and a beneficial effect on the composition of the host-plant microbiome might contribute to their suppressive effect (Erlacher et al. 2014). In other PGPR, termed “biofertilizer”, plant growth promotion by hormone-like compounds and increased accessibility of nutrients 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, hemicellulases), phytohormones (auxin, cytokinin, and gibberellins), and volatile growth stimulants (such as acetoin and 2,3 butanediol) (Borriss 2011).

*Bacillus amyloliquefaciens* FZB42 is the type strain for a group of plant-associated *Bacillus* spp. classified as *B. amyloliquefaciens* subsp. *plantarum* (Borriss et al. 2011). Its 3918-kb genome, containing an estimated 3693 protein-coding sequences, lacks extended phage insertions, which occur ubiquitously in the closely related but nonplant-associated *Bacillus subtilis* 168 genome. Further analysis revealed that FZB42 is a bacterium with impressive capacity to produce metabolites with antimicrobial activity (Chen et al. 2007). Its antifungal activity is due to nonribosomal synthesis of the cyclic lipopeptides bacillomycin D and fengycin (Koumoutsi et al. 2004), whilst its antibacterial activity is mainly due to nonribosomally synthesized polyketides (Chen et al. 2006), bacilysin (Chen et al. 2009a), and ribosomally synthesized bacteriocins (Scholz et al. 2011, 2014). Recent proteome and transcriptome studies revealed that plant root exudates stimulate expression of genes involved in root colonization and plant–bacteria interactions (Borriss 2015a, b; Fan et al. 2012a, b, 2015; Kierul et al. 2015). Its plant colonizing ability was demonstrated with a GFP-labeled FZB42 strain on maize and *Arabidopsis* using confocal laser scanning microscopy (Fan et al. 2011). Beneficial effects on plant growth and disease suppression were documented for *B. amyloliquefaciens*

**Table 1.1** Examples for commercial use of *Bacillus*-based bioformulations in agriculture

Trade name	<i>Bacillus</i> strain	Known properties	Company
Kodiak™	<i>Bacillus subtilis</i> GB03	EPA-registered (71065–2) biological and seed treatment fungicide	Bayer Crop Science, former Gustafsson LLC
Companion	<i>Bacillus subtilis</i> GB03	EPA-registered (71065–2) biofungicide, prevent and control plant diseases. It produces a broad-spectrum Iturin antibiotic that disrupts the cell-wall formation of pathogens, and it triggers an advantageous induced systemic resistance (ISR) in plants, whereby a plant's natural immune system is activated to fight plant diseases	Growth Products Ltd., White Plains, NY 10603
Yield Shield	<i>Bacillus pumilus</i> GB34 (=INR7)	EPA-registered biofungicide (264–985), suppression of root diseases caused by <i>Rhizoctonia</i> and <i>Fusarium</i>	Bayer Crop Science, previously Gustafsson
BioYield™	<i>B. amyloliquefaciens</i> GB99 + <i>Bacillus subtilis</i> GB122	Combination of strong ISR activity (GB99) with phytostimulaton (GB122)	Bayer Crop Science, previously Gustafsson
Subtilex®, INTEGRAL®	<i>Bacillus subtilis</i> MBI600	EPA-registered (71840–8.) biofungicide, provides protection against soil-borne pathogens such as <i>Rhizoctonia solani</i> , <i>Pythium</i> spp. and <i>Fusarium</i> spp. to help prevent damping-off and other root diseases	Becker Underwood, Saskatoon, Canada acquired by BASF
VAULT®	<i>Bacillus subtilis</i> MBI600	Produced by “BioStacked®” technology, enhancing growth of soy beans and pea nuts	Becker Underwood, Saskatoon, Canada
	<i>Bacillus pumilus</i> BU F-33	EPA-registered (71840-RG, –RE, 2013) plant growth stimulator, induced systemic resistance	Becker Underwood, Saskatoon, Canada
SERENADE Max	<i>Bacillus subtilis</i> QST713	EPA-registered (69592–11) biofungicide, Annex 1 listing of the EU agrochemical registration directive (91/414)	Bayer Crop Science, previously AgraQuest
SERENADE SOIL <sup>(R)</sup>	<i>Bacillus subtilis</i> QST713	EPA-registered (69592-EI, 2012) biofungicide for food crops	Bayer Crop Science, previously AgraQuest

(continued)

**Table 1.1** (continued)

Trade name	<i>Bacillus</i> strain	Known properties	Company
SERENADE Optimum®	<i>Bacillus subtilis</i> QST713	EPA-registered (2013) biofungicide/bactericide for prevention. It works by stopping spore germination, disrupting cell membrane, and inhibiting attachment of the pathogen to leaves. For use in leafy and fruiting vegetables, strawberries and potatoes. Active against fungal (Botrytis, Sclerotinia), and bacterial pathogens (Xanthomonas and Erwinia)	Bayer Crop Science, previously AgraQuest
CEASE®	<i>Bacillus subtilis</i> QST713	Aqueous suspension biofungicide, recommended for leafy and fruiting vegetables, herbs and spices, and ornamentals	BioWorks, Inc., Victor, New York, USA
SONATA®	<i>Bacillus pumilus</i> QST2808	EPA-registered (69592–13) biofungicide, powdery mildew control	Bayer Crop Science, previously AgraQuest Inc.
RhizoVital®	<i>Bacillus amyloliquefaciens</i> FZB42	Biofertilizer, plant growth promoting activity, provides protection against various soil-borne diseases, stimulation of ISR	ABiTEP GmbH, Berlin
RhizoPlus®	<i>Bacillus subtilis</i>	Plant growth-promoting rhizobacterium and biocontrol agent. It can be used for potatoes, corn, vegetables, fruits, and also turf	ABiTEP GmbH, Berlin
Taegro®	<i>Bacillus subtilis</i> FZB24	EPA-registered biofungicide. FZB24 has been originally isolated by FZB Berlin, the forerunner of ABiTEP GmbH. Registration as a biofungicide for the United States was performed by Taegro Inc. and then sold to Novozymes without agreement with ABiTEP GmbH where the product is still offered	Syngenta, Basel, previously Novozyme, Davis, California and Earth Biosciences
POMEX	<i>Bacillus subtilis</i> CMB26	Microbial fungicide, control and inhibition germination effect on powdery mildew, <i>Cladosporium fulvum</i> and <i>Botrytis cinerea</i>	NIN Co. Ltd.,
	<i>Bacillus subtilis</i> CX9060	EPA-registered 71840-RG-RE (2012) fungicide, bactericide for food crops, turf and ornamentals	Certis Columbia, MD USA

(continued)

**Table 1.1** (continued)

Trade name	<i>Bacillus</i> strain	Known properties	Company
Easy Start® TE-Max	<i>Bacillus subtilis</i> E4-CDX	Rhizosphere bacterium that competes with harmful pathogens for space around the roots of the grass plant. Once established, this unique strain physically protects the roots and inhibits the advance of soil-borne fungi	COMPO Expert GmbH, Münster, Germany
Double Nickel 55™	<i>B. amyloliquefaciens</i> D747	EPA-registered (70051-RNI, 2011) a broad spectrum preventive biofungicide for control or suppression of fungal and bacterial plant diseases (Powdery mildew, <i>Sclerotinia</i> , <i>Botrytis</i> , <i>Alternaria</i> , bacterial leaf spot, bacterial spot and speck, Fire blight, <i>Xanthomonas</i> , <i>Monilinia</i> )	Certis Columbia, MD USA
Amylo-X®	<i>B. amyloliquefaciens</i> D747	Annex 1 listing of the EU agrochemical registration directive. Launched in Italy by Intrachem Bio Italia SpA for control of <i>Botrytis</i> and other fungal diseases of grapes, strawberries, and vegetables, and bacterial diseases such as fire blight in pome fruit and PSA in kiwi fruit	Certis Columbia, MD USA/ Intrachem Bio Italia SpA
BmJ WG	<i>Bacillus mycoides</i> BmJ	It works entirely as a microbial SAR activator with no direct effect on the plant pathogen itself. Under development	Certis Columbia, MD USA
	<i>Bacillus pumilus</i> GHA 181	EPA-registered fungicide (2012), food crops, seeds, ground cover, and ornamentals	Premier Horticulture
BioNem	<i>Bacillus firmus</i> GB-126	EPA-registered (2008), suppressing plant pathogenic nematodes, <i>Bacillus firmus</i> creates a living barrier that prevents nematodes from reaching the roots	AgroGreen, Israel acquired by Bayer Crop Science

*Note:* The U.S. governmental EPA registration does not depend on successful field trials; it is only necessary to demonstrate that no negative effects are connected with the use of the biofungicide. The table is taken from Borriss (2015b)

FZB42 on tomato, cucumber, cotton, tobacco, and lettuce, for example (Grosch et al. 1999; Idriss et al. 2004; Yao et al. 2006; Guel et al. 2008; Wang et al. 2009; Chowdhury et al. 2013). Two review articles published in open access journals in 2015 (Chowdhury et al. 2015b; Wu et al. 2015b) cover the aspects stressed in this contribution in more detail and are recommended for further reading.



## 1.2 Root Colonization by FZB42 and Its Impact on the Host Plant Microbiome

The ability of FZB42 to colonize the rhizoplane is a precondition for plant growth promotion. Using a GFP-tagged derivative (Fan et al. 2011, 2012a, b), the fate of bacterial root colonization was recently studied. It ruled out that the bacterium behaves distinctly in colonizing root surfaces of different plants. In contrast to maize, FZB42 colonized preferentially root tips when colonizing *Arabidopsis thaliana* (Dietel et al. 2013). On duckweed, *Lemna minor*, FZB42 accumulated preferably along the grooves between epidermal cells of roots and in the concave spaces on ventral sides of fronds. In vitro studies performed with maize seedlings revealed that the segment within 2–8 cm distant from the basal site of the primary root was a most colonized region by FZB42. On the contrary, few bacterial cells could be observed within the range of 2 cm of root tip. In general, the green fluorescent FZB42 cells were decreasingly observed from the upper part of a root down to the root tip. Scanning electron microscopy confirmed the presence of FZB42 on root hairs, where the bacterial cells were usually associated with a wealth of presumed root exudates (Fan et al. 2012a, b). In lettuce, *Lactuca sativa*, seedlings, bacterial colonization occurred mainly on primary roots and root hairs, as well as on root tips and adjacent border cells. Occurrence of labeled bacteria decreased towards the root tips of the lateral roots, and no colonization of the finer roots could be observed (Chowdhury et al. 2015a).

The rhizosphere competence of FZB42 was recently studied using a combination of field and greenhouse trials. FZB42 is able to effectively colonize the rhizosphere (7.45 to 6.61 Log<sub>10</sub> CFU g<sup>-1</sup> root dry mass) within the growth period of lettuce in the field. Our results demonstrated that FZB42 is able to effectively reduce the disease severity of bottom rot caused by soil-borne pathogen *Rhizoctonia solani* on lettuce (Chowdhury et al. 2013).

From a practical point of view, it is interesting to note that the application mode of the biocontrol agent is a key factor for efficacy of FZB42. An effective suppression of *R. solani* was found only after two times application of FZB42, before and after transplanting. For the settlement of the inoculated strain in the rhizosphere in a sufficient high number, it might be important that the microflora in the rhizosphere of young plants is not yet stabilized (Berendsen et al. 2012).

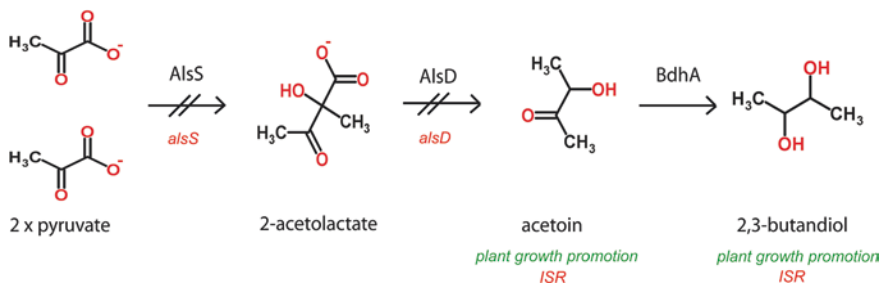
As revealed by T-RFLP, application of FZB42, independent of its mode of application, did not shift the composition of rhizosphere bacterial community in a measurable extent—as also shown for *B. amyloliquefaciens* BNM122 on soybean (Correa et al. 2009). By contrast, inoculation with the pathogen did change the rhizosphere microbial community structure. In complementing that study, the effect of FZB42 and the pathogen *R. solani* on the microbial community of lettuce was more deeply analyzed by 454-amplicon sequencing focusing on the presence of gamma-proteobacteria (Erlacher et al. 2014). Clear differences between plants infected by *R. solani* compared to noninoculated healthy plants were found, corroborating the results obtained by T-RFLP. A significant increase in gamma-proteobacterial diversity was detected in samples inoculated with the pathogen. However, together with

FZB42, this increase was less distinct, suggesting a selective compensation of the impact of a pathogen on the indigenous plant-associated microbiome by FZB42. The number of DNA fragments corresponding to FZB42 in samples taken in vicinity of plant roots was steadily decreasing. After five weeks, only 55% of the initial number of FZB42 DNA was traceable (Kröber et al. 2014).

### 1.3 Plant Growth Promotion

Although the ability of FZB42 to support growth of potatoes, maize, cotton, tobacco, leafy and fruiting vegetables, and ornamentals is well documented (Bochow et al. 2001; Yao et al. 2006; Guel et al. 2008; Burkett-Cadena et al. 2008; Chowdhury et al. 2013), the molecular reasons for the “biofertilizer” effect of beneficial plant-associated Bacilli are still not completely understood. However, we know that several factors are involved in the complex interplay between root-colonizing bacteria and plant:

1. Ability to colonize and to persist at plant roots (see previous section). Their ability to suppress soil-borne pathogens might positively affect the indigenous microbiome of the rhizosphere.
2. Stimulation of plant growth by tryptophan-dependent synthesis of indole-3-acetic acid. Inactivation of genes involved in tryptophan biosynthesis and in a putative tryptophan-dependent IAA biosynthesis pathway led to reduction of both IAA concentration and plant growth-promoting activity in the respective mutant strains (Idris et al. 2007).
3. Volatiles, as 2,3-butanediol and 3-hydroxy-2-butanone (acetoin), released by *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a were reported as enhancing plant growth (Ryu et al. 2003). To synthesize 2,3-butanediol, pyruvate is converted to acetolactate by the acetolactate synthase (AlsS), which is subsequently converted to acetoin by the acetolactate decarboxylase (AlsD) (Fig. 1.1). FZB42 mutant strains, deficient in synthesis of volatiles due to mutations interrupting the *alsD* and *alsS* genes, were found impaired in plant growth promotion (Borriss 2011).



**Fig. 1.1** Anaerobic and aerobic formation of 2,3-butanediol via acetoin involves acetolactate synthase and decarboxylase encoded by the *alsSD* operon. The *alsS* insertion mutation abolishes synthesis of 2,3-butanediol (Renna et al. 1993; Cruz Ramos et al. 2000). The figure is taken from Chowdhury et al. (2015b)

4. Enhancement of nutrient availability by phytase-producing bacteria. Soil phosphorous is an important macronutrient for plants. Improved phosphorous nutrition is achievable by “mobilization” of phosphorous fixed as insoluble organic phosphate in phytate (myo-inositol-hexakisphosphate) by soil bacteria (Singh and Satyanarayana 2011). The extracellular 3(1)-phytase of the plant growth-promoting *B. amyloliquefaciens* FZB45 hydrolyzed phytate to D/L-Ins(1,2,4,5,6)P5 in vitro. A phytase-negative mutant strain, whose *phyA* gene was disrupted, did not stimulate plant growth under phosphate limitation (Idriss et al. 2002). Further experiments under field conditions revealed that FZB45 can only stimulate plant growth when phytate is present in soils, which are poor in soluble phosphate, suggesting that phytase acts only under certain conditions as a plant growth stimulator (Ramírez and Kloepper 2010).

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## 1.4 Biocontrol

Genome analysis revealed that nearly 10% of the genome is devoted to synthesizing antimicrobial metabolites and their corresponding immunity genes (Chen et al. 2009b). FZB42 harbors 11 gene clusters involved in synthesis of antimicrobial compounds. Nine of them are involved in nonribosomal synthesis of lipopeptides and polyketides and two in conventional synthesis and modification of bacteriocin peptides. In addition, three further gene clusters contain genes mediating immunity against antimicrobial compounds produced by other related *Bacillus* strains (Table 1.2). This antibiotic arsenal makes *B. amyloliquefaciens* FZB42 and related *B. amyloliquefaciens plantarum* strains to an efficient microbial biopesticides, developed to control plant diseases (Borriss 2011).

For a long time, the plant protective activity of PGPR has been correlated with the potential to secrete a wide array of antibiotic compounds upon growth as planktonic cells in isolated cultures under laboratory conditions. We determined expression of the corresponding secondary metabolites by MALDI TOF mass spectrometry from FZB42 cultures grown in liquid Landy medium under laboratory conditions. Except the orphan *nrs* gene cluster, all expected bioactive compounds were synthesized in reasonable amounts, but the iron siderophore bacillibactin was detected only under iron-deprived conditions. In recent years, it became doubtful that synthesis of metabolites by the planktonic cells grown under laboratory conditions does correspond to their capability to produce those compounds also when grown in biofilm-related structures on the surface of plant tissues.

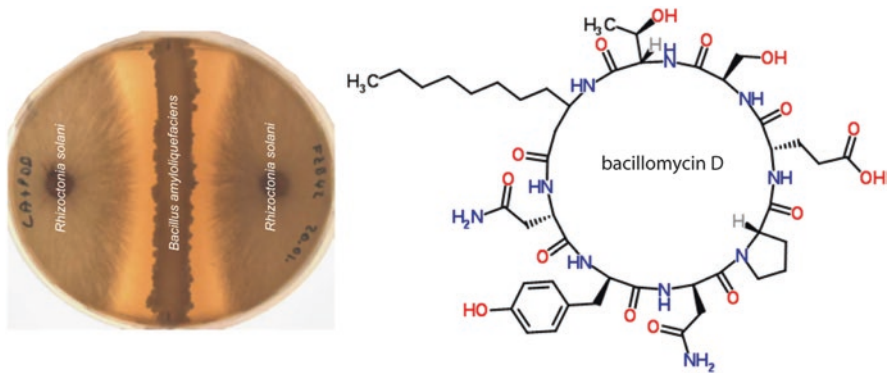
### 1.4.1 Lipopeptides, Bacillibactin, and Antifungal Activity

Five gene cluster involved in nonribosomal synthesis of cyclic lipopeptides and the iron-siderophore bacillibactin were identified in the genome of FZB42 (Table 1.2). Three of the respective gene clusters were assigned for synthesis of surfactin,

**Table 1.2** Genes and gene cluster encoding for secondary metabolites and immunity against bacteriocin in FZB42

Gene cluster	From	To	Size	Metabolite	Effect against	Reference
<b>Sfp-dependent nonribosomal synthesis of lipopeptides</b>						
<i>stfABCD, aat, 334, yct, Cysx2, sfp, yczE</i>	342,618	368,776	32.0 kb	Surfactin	Virus	Koumoutsi et al. (2004)
<i>bmyCBAD</i>	1,871,172	1,908,422	39.7 kb	Bacillomycin D	Fungi	Koumoutsi et al. (2004)
<i>fenABCDE</i>	1,931,328	1,968,997	38.2 kb	Fengycin	Fungi	Koumoutsi et al. (2004)
<i>mrsABCDEF</i>	2,868,410	2,885,927	15.0 kb	Orphan NRP1	Unknown, siderophore?	Chen et al. (2007)
<i>dthABCDEF</i>	3,021,250	3,032,970	12.8 kb	Bacillibactin	Iron deficiency, siderophore	Chen et al. (2007)
<b>Sfp-independent nonribosomal synthesis of polyketides</b>						
<i>mlnABCDEFghi</i>	1,391,841	1,445,094	53.9 kb	Macrolactin	Bacteria	Schneider et al. (2007)
<i>baeBCDE, acpK, baeGHIJLMNRS</i>	1,700,345	1,701,022	74.3 kb	Bacillaene	Bacteria	Chen et al. (2006)
<i>dfrAYXBCDEFGHIJKLM</i>	2,276,743	2,346,266	71.1 kb	Difficidin	Bacteria	Chen et al. (2006)
<b>Sfp-independent nonribosomal synthesis</b>						
<i>bacABCDE, ywfg</i>	3,593,877	3,599,784	6.9 kb	Bacilysin	Bacteria	Chen et al. (2009a, b, c)
<b>Ribosomal synthesis of modified peptides (bacteriocins)</b>						
<i>pznFKGHIAJCDBEL</i>	726,469	736,360	9.96 kb	Plantazolicin	Gram-positive bacteria	Scholz et al. (2011)
<i>acnBACDEF</i>	3,044,506	3,048,678	4.49 kb	Amylocyclicin	Closely related bacteria	Scholz et al. (2014)
<b>Immunity, but no synthesis genes</b>						
<i>mrsK2R2FGE</i>	3,769,734	3,774,552	4.82 kb	Mersacidin	Resistance against Y2	He et al. (2012)
<i>bceBASR</i>	2,856,835	2,861,322	4.49 kb	Bacitracin	Resistance against <i>B. cereus</i>	Unpubl. Results
<i>spaKREF</i>	3,210,423	3,214,712	4.29 kb	Subtilin	Resistance against <i>B. subtilis</i>	Unpubl. Results

The table is taken from Chowdhury et al. (2015b) with modifications



**Fig. 1.2** Effect of FZB42 on *Rhizoctonia solani*. A clear inhibition zone indicating growth suppression of the fungal pathogen is visible on agar plates simultaneously inoculated with both microbes. Bacillomycin D was detected as the only prominent compound by MALDI TOF mass spectrometry of samples taken from the surface of the agar plate within the inhibition zone. The figure is taken from Chowdhury et al. (2015b) with slight modifications

fengycin, and bacillomycin D. Bacillomycin D was identified as being the most powerful antifungal metabolite produced by FZB42 (Fig. 1.2).

The heptapeptide moiety of bacillomycinD, belonging to the iturin family of cyclic lipopeptides (LP), is attached to a  $\beta$ -amino fatty acid chain of variable length ( $C_{14}$ – $C_{17}$ ). The peptide moiety of the heptapeptide surfactin is linked to a  $\beta$ -hydroxyl fatty acid ( $C_{12}$ – $C_{16}$ ), whilst the fengycin decapeptides are linked to a  $\beta$ -hydroxyl fatty acid chain ( $C_{14}$ – $C_{18}$ ). Their synthesis is performed by multimodular peptide synthetases and depends on a functional phospho-pantetheinyl transferase (Sfp), which transfers 4'-phosphopantetheine from coenzyme A to the carrier proteins during nonribosomal synthesis.

Within last few years, Ongena and coworkers performed pioneering work for elucidating antibiotic production *in planta* using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging (MALDI MSI). They investigated antibiotic production in a gnotobiotic system in which the plantlet and the associated *B. amyloliquefaciens* S499, a close relative of FZB42, were growing on a gelified medium covering the MALDI target plate. Under these conditions, S499 grows as biofilm on the surface of the plant roots, allowing exact assays of secondary metabolites in the vicinity of root surface. Surfactins were detected in the root environment in much higher relative amounts, which are representing more than 90% of the whole LP production, and their synthesis is rapidly progressing during early biofilm formation. By contrast, synthesis of iturin and fengycin was delayed until the end of the aggressive phase of colonization (Nihorimbere et al. 2012; Debois et al. 2014). Earlier experiments performed with FZB42 colonizing duckweed (*Lemna minor*) plantlets corroborated that surfactin is the most prominent compound which could be detected by MALDI TOF MS in the plant-bacteria system (Idris et al. 2007). Using a gnotobiotic quartz sand system consisting of lettuce plants, the beneficial bacterium FZB42, and the pathogen *R. solani*, it was demonstrated by using alternative techniques (e.g., Fourier Transform Ionen-Cyclotron Massenspectrometry) that

lipopeptides were detectable in the order surfactin > bacillomycinD > fengycin at the plant–bacteria interface (Chowdhury et al. 2015a).

An early surfactin secretion could be of biological relevance since this lipopeptide, although less fungitoxic than iturins and fengycins, is essential for moving on tissues (Kinsinger et al. 2003) and for matrix formation in biofilms (Hofemeister et al. 2004; Lopez et al. 2009a, b). Considering the relative low amounts of the fungitoxic iturins and fengycins in vicinity of plant roots, it might be concluded that their biocontrol effect is possibly less important than expected. The same is true for the iron siderophore bacillibactin, which could not be detected under the conditions of the artificial plant–bacteria associations applied in these studies.

#### 1.4.1.1 Polyketides, Bacilysin, and Bacteriocins Direct Antibacterial Activity

The polyketides, nonribosomally synthesized by FZB42 (Chen et al. 2006; Schneider et al. 2007), have been extensively reviewed previously (Chen et al. 2009b, 2009c; Borriss 2013). The three gene clusters encoding the modularly organized polyketide synthases (PKS) for synthesis bacillaene, macrolactin, and difficidin cover nearly 200 kb and are the largest ones, which are occurring in the FZB42 genome (Table 1.2). Difficidin is the most effective antibacterial compound produced by FZB42<sup>T</sup>, but also macrolactin and bacillaene possess antibacterial activity. Difficidin is efficient in suppressing plant pathogenic bacterium *Erwinia amylovora*, which causes fire blight disease in orchard trees (Chen et al. 2009a).

Another product of nonribosomal synthesis, the dipeptide bacilysin consisting of anticapsin and alanine moieties, was found to be also involved in suppression of *Erwinia amylovora*. By contrast to the lipopeptides and polyketides mentioned above, bacilysin synthesis is not dependent on the Sfp PP-transferase. A mutant strain CH3, with a disruption of the *sfp* gene and unable to produce any polyketide or lipopeptide, was still able to synthesize bacilysin and to suppress *E. amylovora* (Chen et al. 2009a). Recent experiments, performed by the group of Xuewen Gao, Nanjing Agriculture University, demonstrated that bacilysin, despite difficidin, is efficient in suppressing *Microcystis aeruginosa*: the main causative agent of cyanobacterial bloom in lakes and rivers (Wu et al. 2015a). However, corroborating these results in field trials has to be done. Until now, polyketides and bacilysin have not been detected in plants colonized by *B. amyloliquefaciens* (Debois et al. 2014).

Antimicrobial peptides, ribosomally synthesized as linear precursor peptides, remained unknown in *B. amyloliquefaciens plantarum* for a long time with one remarkable exception: mersacidin, a B-type lantibiotic, was detected in *Bacillus* sp. HIL Y85 (Chatterjee et al. 1992). The strain HIL Y85 was later classified as being *B. amyloliquefaciens plantarum* (Herzner et al. 2011). Nowadays, mersacidin production was also detected in *B. amyloliquefaciens* B9601-Y2 (He et al. 2012). Genes involved in mersacidin self-protection reside also in the genome of FZB42. Transfer of mersacidin biosynthesis genes from HIL Y85 resulted in efficient mersacidin production by the surrogate strain constructed from the FZB42 host (Herzner et al. 2011).



elucidated, unveiling a hitherto unusual number of thiazoles and oxazoles formed from a linear 14mer precursor peptide (Kalyon et al. 2011).

By transposon mutagenesis of the FZB42 mutant strain RS06, we identified a hitherto unknown gene cluster involved in synthesis and post-translational processing of a novel circular bacteriocin, named amylocyclicin (Fig. 1.3). It ruled out that amylocyclicin inhibits growth of bacterial strains closely related to FZB42, suggesting that this bacteriocin might have a function in competing with other *Bacillus* strains attracted to the plant rhizosphere (Scholz et al. 2014).

#### 1.4.1.2 Nematicidal Activity Is Directed by Plantazolicin

Parasitic nematodes of plants are important plant pathogens that represent a significant financial burden on agriculture. The annual losses in agriculture resulting from this pest amounted to \$125 billion worldwide in past years (Sasser and Freckman 1987; Oka et al. 2000). Chemical insecticides of nonselective nature possessing rapid nematicidal effects are widely used as control measures against these pathogens. However, the potential negative impact on the environment and ineffectiveness after prolonged use have led to banning or restricting of the use of most chemical nematicides. Therefore, identification of safe and effective nematicides is urgently needed, and biocontrol measures have recently been given much attention as viable options (Xia et al. 2011). BioNem<sup>®</sup> prepared from *Bacillus firmus* GB-126 (Table 1.1) was proven for its efficiency in greenhouse and field trials. The numbers of nematode females, eggs, and vermiform life stages at the end of the growing season decreased in the presence of the biocontrol agent, and the cotton yields were similar to those from Aldicarb, the chemical nematicide standard; however, the molecular reason for this effect remained unknown (Castillo et al. 2013).

FZB42 has been shown to reduce nematode eggs in roots, juvenile worms in soil, and plant galls on tomato (Burkett-Cadena et al. 2008). In order to identify specific nematicide-related genes, a random transposon insertion library of FZB42 was screened for relevant genes involved in nematicidal activity and, surprisingly, a gene within the *pzn* gene cluster was identified as a pathogenic factor against nematodes. Further experiments revealed that PZN displayed a moderate nematicidal activity (Liu et al. 2013).

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## 1.5 Induced Systemic Resistance Is Triggered by Plant Growth-Promoting Bacilli

Except surfactin, concentration of antifungal lipopeptides determined *in planta* was found relatively low. Moreover, antibacterial polyketides were not detected so far in vicinity of plant roots colonized by PGPR Bacilli (Debois et al. 2014). Therefore, it is tempting to speculate that induced systemic resistance (ISR) is a main factor for suppressing plant pathogens by PGPR Bacilli. ISR occurs when the plant's defense mechanisms are stimulated and primed to resist infection by pathogens (Van Loon 1997). This activation is distinct from systemic acquired resistance (SAR) in which the response is triggered by pathogenic microorganisms associated with the aerial



portions of the plant. Selected *Bacillus* PGPR strains emit volatiles (VOCs) that can elicit plant defenses. Exposure to VOCs consisting of 2,3-butanediol and acetoin (3-hydroxy-2-butanone) from PGPR *Bacillus amyloliquefaciens* activates ISR in *Arabidopsis* seedlings (Ryu et al. 2004). *Arabidopsis thaliana* plants exposed to *Bacillus subtilis* strain FB17 results in reduced disease severity against *Pseudomonas syringae* compared to plants without FB17 treatment. Exogenous application of acetoin triggers ISR and protects plants against the pathogen in the aerial parts whilst 2,3-butanediol did not (Rudrappa et al. 2010). In this context, it is worth to mention that expression of AlsS of FZB42 involved in synthesis of acetoin (Fig. 1.1) was triggered in the presence of maize root exudate (Kierul et al. unpublished), suggesting that root exudates play a role in eliciting of acetoin biosynthesis in FZB42. It is known that some of the plant metabolites present in root exudates, such as organic acids, trigger the *alsSD* operon (Rudrappa et al. 2010). *B. amyloliquefaciens* FZB24 and FZB42 applied to tobacco roots led to a reduction of tobacco mosaic virus symptoms visible on tobacco leaves and led to decreasing amounts of virus proteins present in leaf tissues. Due to spatial distance between beneficial bacterium and the pathogen, plant ISR, stimulated by the rhizobacterium, might be responsible for this effect (Wang et al. 2009).

The induction of ISR when treated with PGPRs is mediated primarily through plant-signaling molecules such as jasmonic acid (JA), a lipoxygenase pathway product, and ethylene (ET). Salicylic acid (SA) appears to be a critical plant messenger of pathogen exposure and disease resistance in systemic acquired resistance (SAR) (Durner et al. 1997). ISR restricts pathogen multiplication and disease progression through a SA/ET and NPR1-dependent mechanism. In order to determine the signaling pathways triggered by FZB42, the expression of several marker genes in lettuce plants, exposed to FZB42 and the pathogenic fungus *Rhizoctonia solani*, was analyzed by quantitative real-time (RT)-PCR (S. Paul Chowdhury et al.: 'Systemic resistance of *Lactuca sativa* against *R. solani* and secondary metabolite production by FZB42 in an axenic model system', unpublished). In absence of the pathogen, FZB42 increased expression of PR1 (pathogenesis protein 1, SA marker gene) and PDF1.2 (defensin, JA/ET marker gene), suggesting that SA and ET pathways are involved in upregulating defense response by ISR in lettuce. A similar result was obtained previously, when *Arabidopsis* plantlets were inoculated with *Bacillus subtilis* FB17 and acetoin (Rudrappa et al. 2010). In simultaneous presence of FZB42 and the pathogen *R. solani*, PDF1.2 expression was dramatically enhanced, suggesting a synergistic activation of the JA/ET pathway, whilst the SA pathway—as indicated by a decreased expression of PR-1—was suppressed in presence of both antagonists.

It was found that the circular lipopeptides surfactin and fengycin can act as elicitors of host plant immunity and contribute to increased resistance toward further pathogenesis ingress in bean and tomato plants (Raaijmakers et al. 2010). In bean, purified fengycins and surfactins provided a significant ISR-mediated protective effect on bean plants against the fungal pathogen *Botrytis cinerea*, similar to the one induced by living cells of the producing strain *B. amyloliquefaciens* S499 (Ongena et al. 2007).

We found (Chowdhury et al. 2015a) that the dramatic increase of the defensin 1.2 gene (PDF1.2) expression in simultaneous presence of both antagonists occurred only when wild-type cells of FZB42 were applied. Mutant strains deficient in synthesis of surfactin, fengycin, or acetoin did not stimulate expression of the JA/ET pathway, suggesting that cyclic lipopeptides and acetoin contribute together to the ISR plant response triggered by FZB42.

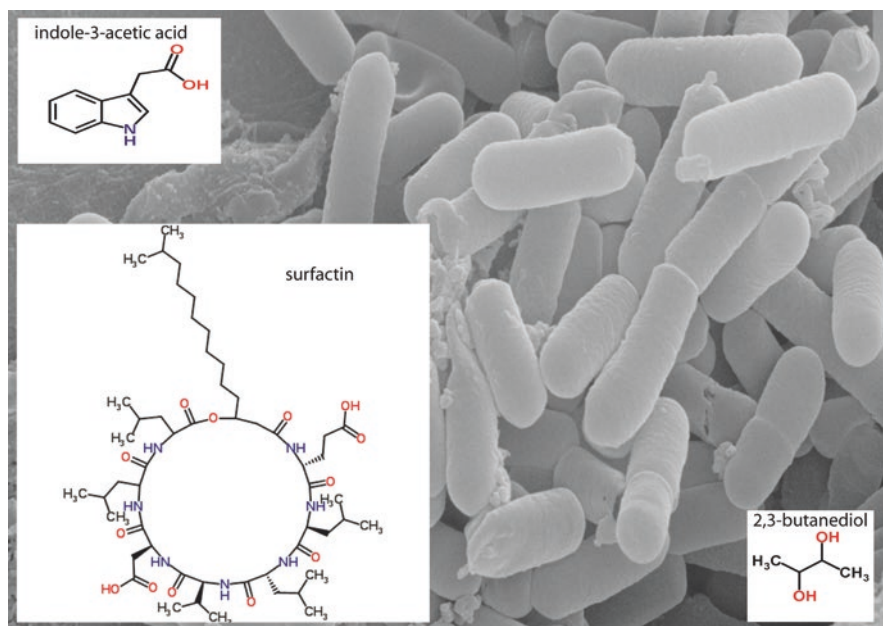
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## 1.6 Conclusion

An increasing amount of data have been accumulated in course of the last years, suggesting that the antibiome expressed during the plant-associated state of PGPR Bacilli does not necessarily reflect the vast genetic arsenal devoted to the formation of lipopeptides, polyketides, and bacteriocins, which has been elucidated, for example, in the *B. amyloliquefaciens plantarum* FZB42 genome. Obviously, there is a large discrepancy in gene expression of the planktonic cells growing in liquid laboratory cultures and cells growing as biofilms on plant tissue surfaces. Except cyclic lipopeptides no other bioactive compounds such as polyketides were detected in samples taken from the vicinity of plant roots colonized by PGPR *B. amyloliquefaciens* (Debois et al. 2014). Interestingly, surfactin has multiple biological functions in motility, biofilm formation, and cell-to-cell signaling, but is less efficient in direct suppressing of other competing microbes than other lipopeptides or polyketides; it was by far the most prominent compound occurring in the plant rhizosphere, previously being inoculated by PGPR *B. amyloliquefaciens*. For this reason, I conclude that the direct effects exerted by the array of secondary metabolites encoded by the *Bacillus* genome might not be as important and that the biocontrol effects exerted by the Gram-positive bacteria are mainly due to other more indirect effects. I assume that under field conditions, the stimulating effects on plant ISR are more important than direct biocontrol by secreted secondary metabolites. In case of Bacilli, it is very likely that ISR stimulation is a multifactorial process dependent on several compounds produced by the rhizobacteria. Candidate compounds are surfactin, and volatiles, especially acetoin and 2,3 butanediol (Fig. 1.4), since mutants of FZB42, deficient in synthesis of these compounds, were found unable to protect plants from pathogens. Moreover, high expression of defensin, indicating the JA/ET pathway in ISR, was not found when the mutant strains were applied to the plant.

These findings are important for future strategies for screening of powerful PGPR and BC strains. It is known for long time that high efficiency in suppressing fungal or bacterial pathogens do not necessarily reflect the potential of these selected strains for their performance under field conditions. Novel screening procedures have to be developed for functional tests under more appropriate conditions, either directly on plants or at least under conditions allowing biofilm formation on artificial surfaces. However, performance under field conditions remains the most important criterion.

Taken together, the beneficial effect of *Bacillus* PGPR depends, besides their rhizosphere competence, on at least three main factors:



**Fig. 1.4** Scanning electron microscopy of FZB42 cells colonizing *Arabidopsis thaliana* roots. Important compounds as surfactin, indole-3-acetic acid, and 2,3-butanediol, which are formed when growing on root surfaces (*in planta*), are indicated

1. Stimulation of plant ISR by bacterial metabolites produced in vicinity of plant roots. Volatiles, such as acetoin and 2,3 butanediol, contribute not only to ISR, but have a direct plant growth-promoting effect, whilst surfactin is important in the early stage of colonization and biofilm formation. In addition, surfactin strengthens the plant ISR response, which suppresses growth of fungal, bacterial, viral, and other plant pathogens.
2. Direct antifungal action by secondary metabolites, such as iturins (e.g., bacillo-mycin D) and fengycins, secreted into the rhizosphere. However, the suppressing effect exerted by such compounds might be relatively weak, since the amount of such compounds in vicinity of plant root was found relatively low. Until now, antibacterial compounds, such as polyketides, were not detected in this environment.
3. Application of PGPR Bacilli, as FZB42, might compensate, at least in part, undesired changes in the composition of the plant microbiome, caused by the presence of pathogens, as *R. solani*.

Without doubt, other features of PGPR, as production of plant hormones and making available fixed macro- and micro nutrients for plant nutrition, contribute also to the beneficial effect exerted by these microbes, but could not be appropriately treated in this review due to space limitation.

**Acknowledgements** Many of the recent data, reported in this review, have been obtained in close collaboration with the Helmholtz Center in Munich in frame of the PATHCONTROL project, and the laboratory of Yuewen Gao, Nanjing Agricultural University, China, in frame of a Chinese Collaborative project, financially supported by the BMBF, the German Ministry of Education and Research. I thank especially Soumitra Paul Chowdhury, Anton Hartmann, Joachim Vater, Liming Wu, Xuewen Gao, and Ben Fan (Nanjing Forestry University) for their fruitful collaboration during the last years.

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