# 8 Secondary Metabolites of the Plant Growth Promoting Model Rhizobacterium *Bacillus velezensis FZB42* Are Involved in Direct Suppression of Plant Pathogens and in Stimulation of Plant-Induced Systemic Resistance

Rainer Borriss, Huijun Wu, and Xuewen Gao

## 8.1 Introduction

Biocontrol effects exerted by antagonistic acting bacilli are due to different mechanisms; besides direct antibiosis and competition by secretion of a spectrum of secondary metabolites in the rhizosphere, the beneficial action on the host-plant microbiome (Erlacher et al. 2014) and stimulation of plant-induced systemic resistance (ISR) (Dornboos et al. 2012) are of similar importance. ISR is induced by a range of secondary metabolites, which are called "elicitors." Different signaling pathways, such as jasmonic acid (JA), ethylene (ET), and salicylic acid (SA), are activated to trigger plant resistance. Keeping this in mind, the focus of this review is directed to the characterization of antimicrobial compounds synthesized by the biocontrol bacterium FZB42 and their beneficial action on plant health.

The group of plant-associated, endospore-forming rhizobacteria, previously known as *Bacillus amyloliquefaciens* subsp. *plantarum* (Borriss et al. 2011) and nowadays reclassified as being *B. velezensis* (Dunlap et al. 2016), are able to enhance yield of crop plants (plant growth promotion function) and to suppress plant pathogens (biocontrol activity) (Borriss 2011). Representatives of this group

R. Borriss (✉)
Institut für Biologie, Humboldt Universität, Berlin, Germany
e-mail: rainer.borriss@rz.hu-berlin.de; h0135djo@cms.hu-berlin.de

H. Wu · X. Gao
Department of Plant Pathology, College of Plant Protection, Nanjing Agricultural University, Nanjing, People's Republic of China

Key Laboratory of Integrated Management of Crop Diseases and Pests, Ministry of Education, Nanjing, People's Republic of China



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of bacteria are increasingly applied in sustainable agriculture in order to replace, at least in part, chemical pesticides and fertilizers. Taxonomically they belong to a group we have recently designated as "*B. amyloliquefaciens* operational group" (Fan et al. 2017). Besides *B. velezensis*, also *B. amyloliquefaciens*, known for its ability to produce extracellular enzymes with industrial importance (amylases, glucanases, and proteases), and *B. siamensis*, mainly occurring in Asian food, are members of this operational group, which is distinct from *B. subtilis*. FZB42 (=BGSC 10A6, DSM23117), the prototype of Gram-positive bacteria with phytostimulatory and biocontrol action, has been genome sequenced in 2007 (Chen et al. 2007) and is subject of intensive research. Since its isolation from beet rhizosphere (Krebs et al. 1998), more than 200 articles dealing with FZB42 have been published (http://amylowiki.top/reference.php).

## 8.2 Special Features of the FZB42 Genome

The 3918-kb FZB42 genome, containing an estimated 3695 protein-coding sequences (CDS), lacks extended phage insertions, which occur ubiquitously in the related *Bacillus subtilis* 168 genome, which is recently considered as being also a plant-associated bacterium (Wipat and Harwood 1999; Borriss et al. 2018). Many genes, essential for a plant-associated lifestyle, are shared between *B. subtilis* 168 and FZB42 as well. Spectacular examples are YfmS, a chemotaxis sensory transducer recognizing a still unknown substrate, is involved in the colonization of *Arabidopsis thaliana* roots (Allard-Massicotte et al. 2017) and BlrA (formerly YtvA), a blue light receptor related to plant phototropins (Borriss et al. 2018).

FZB42 secretes different hydrolases, enabling them to use external cellulosic and hemicellulosic substrates present in plant cell walls. Microbe-associated hydrolytic enzymes digesting plant cell wall structures, resulting in free oligosaccharides, have been shown to act as elicitors of plant defense (Ebel and Scheel 1997). Some genes encoding for extracellular hydrolases, such as *amyE* (α-amylase), *eglS* (endo-1,4-ß-glucanase), and *xynA* (xylanase), were found in the plant-associated representatives of the "*B. amyloliquefaciens* operational group" but not in their soil-associated counterparts (Borriss et al. 2011; Zhang et al. 2016). Similarly, an operon with *xylA*, involved in xylose degradation (EC 5.3.1.5); *xynP*, encoding an oligosaccharide transporter; *xynB*, encoding 1,4-β-xylan xylosidase (EC 3.2.1.37); and *xylR*, encoding the xylose operon repressor, are present in *B. subtilis* 168 and *B. amyloliquefaciens* FZB42 but missing in the *B. amyloliquefaciens* DSM7$^{T}$ genome (Rückert et al. 2011).

Three unique genes encoding enzymes involved in hexuronate degradation were found in *B. velezensis*: *kdgK1*, (2-dehydro-3-deoxygluconokinase EC:2.7.1.45), *kdgA* (2-dehydro-3-deoxyphosphogluconate aldolase, EC:4.3.1.1.16), and LacI-like transcription regulator *kdgR*. The three genes are part of a six-gene *kdgKAR* operon and located within a cluster of ten genes flanked by two rho-independent transcription terminators. Inside of the ten-gene cluster, three independent transcription units exist: besides the six-gene *kdgKAR* operon, a probably monocistronic *exuT* gene

with sugar phosphate transporter function and a three-gene *yndGHJ* operon with unknown function (He et al. 2012). Besides *yjmD*, a gene with putative galactitol-1-phosphate dehydrogenase function and, also present in *B. subtilis*, two genes encoding enzymes involved in D-mannonate metabolism are part of the six-gene transcription unit: the mannonate dehydratase UxuA, EC 4.2.1.8, and *uxuB* encodes mannonate oxidoreductase (EC 1.1.1.131). In addition, a second operon containing the genes *uxaC*, *uxaB*, and *uxaA* encoding enzymes for degrading and isomerizing of different hexuronates to D-altronate and D-fructuronate occurs remote from the ten-gene cluster. Since 6-phosphogluconate dehydratase converting 6-phosphogluconate to KDPG is lacking in *B. velezensis*, we assume that D-mannonate oxidoreductase, UxuB, catalyzes the NAD-dependent interconversion of D-mannonate and D-fructuronate. YjmE/UxuA dehydrates then mannonate to 2-keto-3-deoxygluconate, KDG, which is phosphorylated to 2-keto-3-deoxy-6-phosphogluconate, KDPG, by KDG kinase. This metabolic route is part of a derivative pathway of aldohexuronates in *E. coli* K12 in which UxuA, KdgK, and KdgA are involved (Portalier et al. 1980). Thus, the complete biochemical pathway from galacturonate to KDG is present in *B. velezensis* (He et al. 2012), but no gene encoding D-glucuronate isomerase was detected, suggesting that *B. velezensis* is not able to metabolize D-glucuronate. *B. subtilis yjmD*, *yjmE (uxuA)*, *yjmF (uxuB)*, and *yjmG (exuT)* displayed high similarity (75–83%) to the corresponding genes in the *B. velezensis* ten-gene cluster.

After a recent literature search, we found 576 genes involved in plant-bacteria interaction (http://amylowiki.top/interaction.php).

## 8.3 Structure of Gene Clusters Involved in Synthesis of Secondary Metabolites in FZB42

The FZB42 genome reveals a huge potential to produce secondary metabolites, including the polyketides bacillaene, macrolactin, and difficidin (Chen et al. 2006; Schneider et al. 2007) and the lipopeptides surfactin, bacillomycin D, and fengycin (Koumoutsi et al. 2004). In total, the FZB42 genome harbors 13 gene clusters involved in non-ribosomal and ribosomal synthesis of secondary metabolites with putative antimicrobial action. In two of them, in the nrs gene cluster and in the type III polyketide gene cluster, their products are not identified till now (Table 8.1). Similar to *B. subtilis* $168^T$, the genome of the non-plant-associated soil bacterium *B. amyloliquefaciens* $DSM7^T$ harbors a significantly lower number of gene clusters involved in non-ribosomal synthesis of secondary metabolites than strain $FZB42^T$ (Table 8.1). Polyketides and lipopeptides comprise two families of natural products biosynthesized in a similar fashion by multimodular enzymes acting in assembly line arrays. The monomeric building blocks are organic acids or amino acids, respectively (Walsh 2004). Synthesis of lipopeptides and polyketides is depending on Sfp, a PPTase that transfers 4′-phosphopantetheine from coenzyme A to the carrier proteins of nascent peptide or polyketide chains. In *B. subtilis*-type strain $168^T$, there is a frame shift mutation within the *sfp* gene hindering non-ribosomal

**Table 8.1** Presence of genes and gene clusters encoding for secondary metabolites in *B. velezensis* FZB42, *B. amyloliquefaciens* DSM7$^T$, and *B. subtilis* 168$^T$

| Gene cluster | Size | metabolite | FZB42 NC_009725.1 | FZB42 genome | DSM7 NC_014551.1 | BS168 NC_000964.3 | MIBiG accession |
|---|---|---|---|---|---|---|---|
| Sfp-dependent non-ribosomal synthesis of lipopeptides (NRPS) | | | | | | | |
| *srfABCD, aat,ycxC, ycxD,sfp,yczE* | 29.1 kb | Surfactin | 342,618–374,584 | Core genome | 333,123–362,173 | 376,967–408,887 | BGC0000433 |
| *bmyCBAD* | 39.7 kb | Bacillomycin D | 1,871,171–1,908,422 | Core genome | 1,968,514–2008850[a] | – | BGC0001090 |
| *fenABCDE* | 48.1 kb | Fengycin | 1,921,411–1,969,477 | G15: 1939781–1,967,431 | 2,017,516–2040900[b] | 1,949,681–2,002,351 | BGC0001095 |
| *nrsABCDEF* | 15.0 kb | Orphan | 2,885,927–2,868,410 | G22: 2868278–2,887,889 | – | – | – |
| Sfp-dependent non-ribosomal synthesis of Bacteriocin-Nrps | | | | | | | |
| *dhbABCDEF* | 27.2 kb | Bacillibactin | 3,019,044–3,038,453 | Core genome | 3,053,649–3,066,379 | 3,278,324–3,297,919 | BGC0001185 |
| Sfp-dependent non-ribosomal synthesis of polyketides (Transatpks-Nrps type I) | | | | | | | |
| *mlnABCDEFGHI* | 52.2 kb | Macrolactin | 1,391,841–1,444,003 | G13: 1402380–1,445,564 | – | – | BGC0000181 |
| *baeBCDE,acpK,baeGHIJLMNRS* | 71.1 kb | Bacillaene | 1,700,344–1,772,787 | Core genome | 1,785,330–1,856,436 | 1,782,712–1,859,783 | BGC0001089 |
| *dfnAYXBCDEFGHIJKLM* | 69.5 kb | Difficidin | 2,276,742–2,346,266 | G19: 2276734–2,347,685 | – | – | BGC0000176 |
| Type III polyketide synthesis | | | | | | | |
| *bpsAB* | 1.6 kb | Triketide pyrone | 2,122,078–2,123,684 | Core genome | 2,189,857–2,191,463 | 2,316,446–2,318,053 | – |
| Sfp-independent non-ribosomal synthesis | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *bacABCDE,ywfG* | 7.3 kb | Bacilysin | 3,593,876–3,601,174 | Core genome | 3,654,159–3,660,055 | 3,867,492–3,874,150 | BGC0001184 |
| Ribosomal synthesis of modified peptides (RiPP) | | | | | | | |
| *pznFKGHIAJCDBEL* | 9.96 kb | Plantazolicin | 726,469–736,360 | GI 6: 724191–740,699 | – | – | BGC0000569 |
| *acnBACDEF* | 4.2 kb | Amylocyclicin | 3,044,505–3,048,679 | Core genome | 3,076,887–3,081,038 | – | BGC0000616 |
| *lci* | 0.3 kb | Antibacterial peptide | 310,858–311,142 | Core genome | 1,296,288–1,296,563 | – | – |
| Immunity, but no synthesis genes | | | | | | | |
| *mrsK2R2FGE* (partial) | 4.82 kb | Mersacidin | 3,769,734–3,774,552 | Core genome | – | – | BGC0000527 |
| *bceBASR* (partial) | 4.49 kb | Bacitracin | 2,856,835–2,861,322 | Core genome | – | – | BGC0000310 |
| *spaKREF*(partial) | 4.29 kb | Subtilin | 3,210,423–3,214,712 | Core genome | – | – | BGC0000559 |

Genomic islands (GIs) in FZB42 were identified by SeqWord and M-GCAT (Rückert et al. 2011). The MIBiG accession numbers (Medema et al. 2015) are indicated

[a]DSM7$^T$ contains the gene cluster for synthesis of iturin A (BGC0001098), which is closely related to *bacillomycin* D

[b]The gene cluster for non-ribosomal synthesis of *fengycin* is only present in part in the genome of DSM7$^T$

synthesis of surfactin, fengycin, and bacillaene in this domesticated laboratory strain (Borriss et al. 2018). Around 8.5% of the whole genomic capacity of FZB42 is devoted to non-ribosomal synthesis of these both families of secondary metabolites (Chen et al. 2009b) (Fig. 8.1).

### 8.3.1 Type I and Type III Polyketides

Polyketides are an important class of secondary metabolites, which are synthesized through decarboxylative condensation of carboxylic acids by polyketide synthases (PKSs). PKSs are a giant assembly of multifunctional polypeptides, each consisting

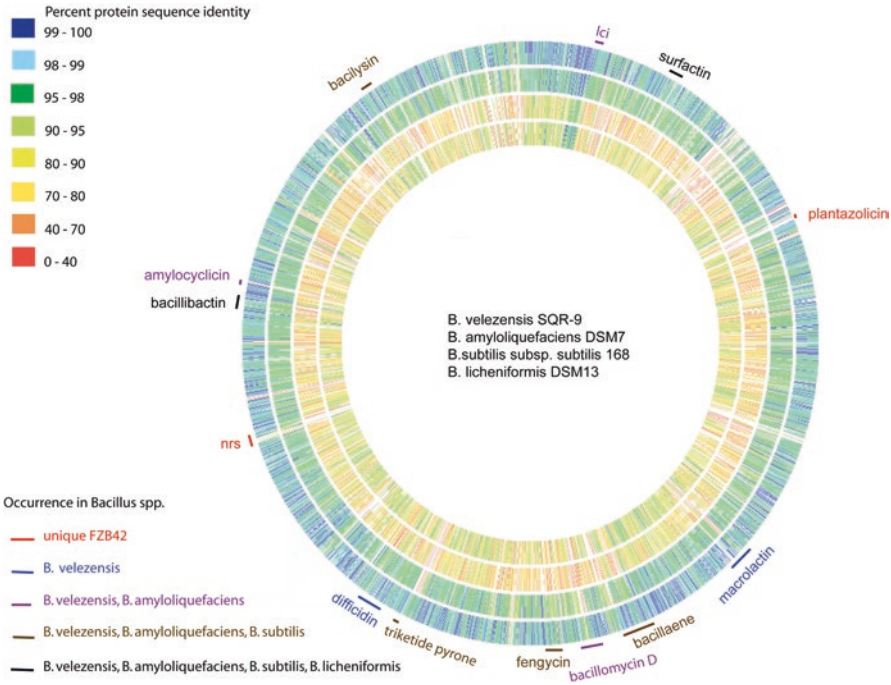


**Fig. 8.1** Genome comparison of FZB42 with *B. velezensis*, *B. amyloliqufaciens*, *B. subtilis*, and *B. licheniformis*. The whole genomes of *B. velezensis* SQR-9 (outside circle), *B. amyloliquefaciens* DSM7$^T$ (2nd circle), *Bacillus subtilis* 168$^T$ (3th circle), and *B. licheniformis* DSM13$^T$ (inner circle) were aligned with FZB42$^T$ using the RAST server (Aziz et al. 2008). The color code indicates % similarity of single gene products. Thirteen sites (genes or gene clusters) involved in synthesis of antimicrobial compounds were identified within the genome of FZB42 (compare also Table 8.1). The gene clusters responsible for non-ribosomal synthesis of the polyketides macrolactin and difficidin are unique in *B. velezensis*. The gene cluster for synthesis of bacillomycin D/iturin A and amylocyclicin and the gene for synthesis of the antimicrobial peptide Lci occur also *in B. amyloliquefaciens*. The gene clusters for non-ribosomal synthesis of bacillaene, fengycin, and the hypothetical tripeptide pyrone occur *in B. velezensis*, *B. amyloliquefaciens*, and *B. subtilis*. (The figure has been redrawn after Fig. 1 in Chowdhury et al. 2015b).

of a series of catalytic domains. Essential domains for chain elongation are ketosynthase (KS), acyl transferase (AT), and acyl carrier protein (ACP). In bacilli, e.g., FZB42, a special class of PKSs that lack the cognate AT domain and require a discrete AT enzyme acting iteratively *in trans* (trans-AT), was detected (Shen 2003). Unfortunately, structural instability of these polyketides excluded until now their use as antibacterial agents.

Besides type I PKS, also genes encoding type III polyketide synthases are present in the genome of FZB42. By contrast to type I PKSs, the type III PKSs do catalyze the priming, extension, and cyclization reactions iteratively to form a huge array of different polyketide products (Yu et al. 2012). In *Bacillus subtilis*, gene products of bspA-bspB operon were functionally characterized and found to be involved in synthesis of triketide pyrones. The type III PKS BspA is responsible for the synthesis of alkylpyrones and BspB is a methyltransferase that acts on the alkylpyrones to yield alkylpyrone methyl ethers (Nakano et al. 2009). However, their biological role needs further elucidation. Orthologs of *bspA* and *bspB* are present in FZB42 and DSM7$^T$ (Table 8.1).

### 8.3.2 NRPS

Another important class of secondary metabolites, also non-ribosomally synthesized by giant multifunctional enzymes (peptide synthetases, NRPS), is formed by lipopeptides. Similar to PKS, three catalytic domains are involved in each elongation cycle: (1) the A-domain (adenylation domain) selects its cognate amino acid; (2) the PCP domain (peptidyl-carrier domain) is equipped with a PPan prosthetic group to which the adenylated amino acid substrate is transferred and bound as thioester; and (3) the condensation domain (C-domain) catalyzes formation of a new peptide bond (Duitman et al. 1999).

Nearly 10% of the FZB42 genome is devoted to synthesizing antimicrobial compounds by pathways either involving or not involving ribosomes. Notably, the gene clusters involved in non-ribosomal synthesis of the antifungal lipopeptide bacillomycin D and the antibacterial polyketides difficidin and macrolactin are absent in DSM7$^T$ and other representatives of *B. amyloliquefaciens* suggesting that synthesis of these secondary metabolites might be important for the plant-associated lifestyle. Instead of the bacillomycin D synthesis genes, the gene cluster for synthesis of iturin A is present within the DSM7$^T$ genome. Notably, the genes involved in synthesis of fengycin are only fragmentary present in DSM7$^T$ (Table 8.1). It has been shown experimentally that DSM7$^T$ is unable to produce fengycin (Borriss et al. 2011).

Five out of a total of 13 gene clusters are located within variable regions of the FZB42 chromosome (Table 8.1), suggesting that they might be acquired via horizontal gene transfer. Except the fengycin gene cluster (see above), all others (bacillomycin D, macrolactin, difficidin, plantazolicin, and the orphan *nrsA-F* gene cluster) were without counterpart in DSM7$^T$ and *B. subtilis* 168$^T$.

### 8.3.2.1 Lipopeptides

The lipopeptides of *Bacillus* are small metabolites that contain a cyclic structure formed by 7–10 amino acids (including 2–4 D-amino acids) and a beta-hydroxy fatty acid with 13–19 C atoms (Zhao et al. 2017). They can be classified into four main families: the surfactins, the iturins, the fengycins or plipastatins, and the kurstakins (Jacques 2011). Lipopeptides could act by direct antibiosis against fungi and bacteria but were also found to stimulate ISR (Ongena et al. 2007). *B. velezensis* SQR9 mutants deficient in surfactin, bacillomycin, and fengycin synthesis were found impaired in triggering induced systemic resistance in *Arabidopsis* plantlets against plant pathogens *P. syringae* pv. tomato (Pst DC3000) and *Botrytis cinerea* (Wu et al. 2018).

#### Surfactin

Surfactin is a heptapeptide with an LLDLLDL chiral sequence linked by a ß-hydroxy fatty acid consisting of 13–15 carbon atoms to form a cyclic lactone ring structure. Surfactin is surface active (biotenside) and acts hemolytic, antiviral, and antibacterial by altering membrane integrity (Peypoux et al. 1999). The biological role of surfactin is thought as supporting colonization of surfaces and acquisition of nutrients through their surface-wetting and detergent properties. Similar to *B. subtilis* (Kovacs et al. 2017), FZB42 is capable of sliding on surfaces, dependent on the presence of surfactin. Mutants of *B. amyloliquefaciens*, blocked in surfactin biosynthesis, were shown to be impaired in biofilm formation (Chen et al. 2007).

Besides direct antagonism of phytopathogens, surfactin could also interact with plant cells as determinant for turning on an immune response through the stimulation of the induced systemic resistance pathway (Chowdhury et al. 2015a, b). 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. Syntheses of iturin and fengycin were also detected but found delayed until the end of the aggressive phase of colonization (Nihimborere 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). Mutant strains of FZB42, devoid in synthesis of surfactin (CH1, CH5), were found impaired in triggering of JA/ET-dependent ISR in lettuce plants, when challenged with plant pathogen *R. solani* (Chowdhury et al. 2015a). The lower expression of the JA/ET-inducible plant defensin factor (PDF1.2) in mutant strain CH5 (Δsfp) compared to CH1 (Δsrf) suggests that secondary metabolites other than surfactin might be involved in triggering plant response.

Gray leaf spot disease caused by *Magnaporthe oryzae* is a serious disease in perennial ryegrass (*Lolium perenne*). A mutant strain of FZB42 (AK3) only able to produce surfactin but no other lipopeptides (Bacillomycin D, fengycin) was shown to induce systemic resistance (ISR). A similar effect as in live cells was obtained in root-drench application of solid-phase extraction (SPE)-enriched surfactin.

Treatment led to reduced disease incidence and severity on perennial ryegrass. ISR defense response was characterized by enhanced hydrogen peroxide ($H_2O_2$), elevated cell wall/apoplastic peroxidase activity, and deposition of callose and phenolic/polyphenolic compounds underneath the fungal appressoria in naïve leaves. Moreover, a hypersensitive response (HR)-type reaction and enhanced expression of *LpPrx* (Prx, peroxidase), *LpOXO4* (OXO, oxalate oxidase), *LpPAL* (PAL, phenylalanine ammonia lyase), *LpLOXa* (LOX, lipoxygenase), *LpTHb* (putative defensin), and *LpDEFa* (DEFa, putative defensin) in perennial ryegrass were associated with SPE-enriched surfactin and live AK3 cell treatments, acting as a second layer of defense when preinvasive defense responses failed (Rahman et al. 2015). Surprisingly there are *B. velezensis* strains descibed which could positively affect plant growth and health although they were found impaired in synthesis of surfactin (He et al. 2012).

### Bacillomycin D

Members of the iturin family are iturins A, C, D, and E; bacillomycins D, F, and L; bacillopeptin; and mycosubtilin. They contain one ß-amino fatty acid and seven α-amino acids (Chen et al. 2009b). The peptide moiety of the iturin lipopeptides contains a tyrosine in the D-configuration at the second amino acid position and two additional D-amino acids at positions 3 and 6. While the majority of *B. velezensis* strains were found to contain a gene cluster encoding bacillomycin D, strain CAU B946 was found to synthesize iturin A which is reflected by its *ituA* operon located at the same site as the bmyD gene cluster in FZB42 (Blom et al. 2012). The same is true for the type strain of *B. amyloliquefaciens* DSM7$^T$ (Borriss et al. 2011). Transcription of the bacillomycin D gene cluster is directly controlled by global regulator DegU. A transmembrane protein of unknown function, YczE, is also necessary for synthesis of bacillomycin D (Koumoutsi et al. 2007).

The members of the iturin family exhibit strong fungicidal activity, and bacillomycin D has been identified as the main antifungal activity directed against fungal plant pathogens in *B. velezensis* strains FZB42 and C06. Mycelium growth and spore germination are suppressed in *Fusarium oxysporum, Rhizoctonia solani*, and *Monilinia fructicola* (Koumoutsi et al. 2004; Chowdhury et al. 2013). Purified iturin A suppressed the *Fusarium* yellows at tatsoi by soil amendment at relatively low concentration (0.47 mg/L soil) (Yokota and Hayakawa 2015). Recently, bacillomycin D was proven to show strong fungicidal activity against *Fusarium graminearum*. Bacillomycin D caused morphological changes in the plasma 60 membrane and cell wall of *F. graminearum*, induced accumulation of reactive oxygen species, and ultimately caused cell death in *F. graminearum*. Interestingly, when challenged by bacillomycin D, deoxynivalenol production, gene expression, mitogen-activated protein kinases phosphorylation, and pathogenicity of *F. graminearum* were significantly altered. Similar as in other cyclic lipopeptides, bacillomycin triggers ISR against plant pathogens (Wu et al. 2018).

**Fengycin**

Fengycin (synonymous to plipastatin) is a cyclic lipo-decapeptide containing a ß-hydroxy fatty acid with a side change of 16–19 carbon atoms. Four D-amino acids and one non-proteinogenic ornithine residue have been identified in the peptide portion of fengycin. Fengycin is active against filamentous fungi and is known for inhibiting phospholipase $A_2$. Similar to bacillomycin D, toxicity against pathogenic fungi relies mainly on their membrane permeabilization properties. Due to its high productivity in synthesizing fengycin, biocontrol exerted by strain C06 relies rather on fengycin than on bacillomycin D (Liu et al. 2011). Fengycin is known for triggering induced systemic resistance in *B. velezensis* (Wu et al. 2018).

## 8.3.3 Type I Polyketides

### 8.3.3.1 Bacillaene

The *pks* genes encode the enzymatic mega-complex that synthesizes bacillaene (Chen et al. 2006; Straight et al. 2007). The majority of *pks* genes appear to be organized as a giant operon (>74 kb from *pksC-pksR*). Bacillaene is, due to its molecular structure, a highly unstable inhibitor of prokaryotic protein synthesis and does have no effects on eukaryotic organisms (Patel et al. 1995). NMR studies of partially purified extracts from *B. subtilis* revealed bacillaene as an open-chain, unsaturated enamine acid with an extended polyene system (Butcher et al. 2007). Features of bacillaene synthesis, the archetype of trans-AT PKS, were uncovered, and bacillaene B bearing a glucosyl moiety was identified as the final product of the *bae* pathway (Moldenhauer et al. 2007, 2010).

Regulation of bacillaene synthesis has been extensively investigated in *B. subtilis*. A deletion of the *pks* operon in *B. subtilis* was found to induce prodiginine production by *Streptomyces coelicolor* (Straight et al. 2007). Expression of the *pks* genes in liquid culture requires the master regulator of development, Spo0A, through its repression of AbrB and the stationary phase regulator, CodY, which regulates metabolism in response to nutrient status and can bind to multiple sites in the bacillaene operon (Belitzky and Sonenshine 2013). Deletions of *degU*, *comA*, and *scoC* had moderate effects, disrupting the timing and level of *pks* gene expression (Vargas-Bautista et al. 2014). Interestingly, the polyketide bacillaene, produced in *B. subtilis* NCIB3610, functions as a significant defense protecting *Bacillus* cells from predation by *Myxococcus xanthus* (Müller et al. 2014).

### 8.3.3.2 Difficidin

Difficidin and oxydifficidin were identified as products of the *dfn* gene cluster in FZB42$^T$ (Chen et al. 2006). Difficidin has been shown to inhibit protein biosynthesis (Zweerink and Edison 1987), but the exact molecular target remains unknown. The polyketides are highly unsaturated 22-membered macrocyclic polyene lactone phosphate esters (Wilson et al. 1987) and are by far the most effective antibacterial compounds produced by FZB42$^T$. Difficidin is the most effective antibacterial compound produced by FZB42$^T$. Notably, difficidin is efficient in suppressing plant

pathogenic bacterium *Erwinia amylovora,* which causes fire blight disease at orchard trees (Chen et al. 2009a). In addition, difficidin produced by FZB42 was efficient in suppressing rice pathogens *Xanthomonas oryzae.* Together with bacilysin (see below), difficidin caused downregulated expression of genes involved in *Xanthomonas* virulence, cell division, and protein and cell wall synthesis (Wu et al. 2015). Analyses using fluorescence, scanning electron, and transmission electron microscopy revealed difficidin and bacilysin caused changes in the cell wall and structure of *Xanthomonas*. Biological control experiments on rice plants demonstrated the ability of difficidin and bacilysin to suppress economically damaging rice diseases such as bacterial blight and bacterial leaf streak.

#### 8.3.3.3 Macrolactin

Macrolactins are the biosynthesis product of the *mln* gene cluster in FZB42$^T$ and were characterized as an inhibitor of peptide deformylase (Yoo et al. 2006). Macrolactins, originally detected in an unclassified deep-sea bacterium, contain three separate diene structure elements in a 24-membered lactone ring (Gustafson et al. 1989). 7-O-malonyl macrolactin induces disruptions of cell division, thereby inhibiting the growth of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* (Romero-Tabarez et al. 2006). In the culture fluid of FZB42$^T$, four macrolactins were identified – macrolactins A and D as well as 7-O-malonyl and 7-O-succinyl macrolactin (Schneider et al. 2007). By contrast to other polyketides, macrolactin triggers ISR in *Arabidopsis* plantlets against *P. syringae* pv. tomato (Pst DC3000) and *Botrytis cinerea* (Wu et al. 2018).

### 8.3.4 Bacilysin

Like difficidin, the dipeptide bacilysin was found as also being involved in suppression of *Erwinia amylovora*. Bacilysin [L-alanyl-[2,3-epoxycyclohexanone-4]-L-alanine] contains L-alanine residue at the N-terminus and non-proteinogenic L-anticapsin, at the C-terminus. The peptide bond with L-alanine proceeds with a non-ribosomal mode catalyzed by amino acid ligase DhbE. Bacilysin is active in a wide range of bacteria and against the yeast, *Candida albicans,* due to the anticapsin moiety, which becomes released after uptake into susceptible cells and blocks glucosamine synthetase, an essential enzyme of cell wall biosynthesis. 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,* the causative agent of fire blight at orchard trees (Chen et al. 2009b). More recent experiments demonstrated that bacilysin is efficient in suppressing *Microcystis aeruginosa*, the main causative agent of cyanobacterial bloom in lakes (Wu et al. 2014a), and *Xanthomonas oryzae*, the causative agent of bacterial rice blight and bacterial leaf streak on rice (Wu et al. 2015).

The study of Wu et al. (2014a) is of special interest, since they described carefully the molecular effects exerted by FZB42 on cyanobacteria, especially on

*Microcystis aeruginosa*, the causative agent of harmful algal blooms in lakes and rivers. The authors could show that the suppressing effect was due to bacilysin. In a mutant strain disrupted in the *bacB* bacilysin synthesis gene, the suppressing effect on *Microcystis* growth was found abolished, but this was restored when bacilysin synthesis was complemented. Bacilysin caused apparent changes in the algal cell wall and cell organelle membranes, and this resulted in cell lysis. Bacilysin addition led to downregulating of genes involved in peptidoglycan synthesis, photosynthesis, microcystin synthesis, and cell division in *M. aeruginosa*.

In order to enhance bacilysin synthesis in FZB42, a genetic approach using the powerful Cre-Lox system was applied. Replacement of the native bacilysin promoter by constitutive promoters P*repB* and P*spac* was achieved. These strains contained two antibiotic resistance genes, and markerless strains were constructed by deleting the chloramphenicol resistance cassette and promoter region bordered by two *lox* sites (*lox*71 and *lox*66) using Cre recombinase expressed from the temperature-sensitive vector pLOSS-cre. The vector-encoded spectinomycin resistance gene was removed by high-temperature (50 °C) treatment. The engineered strains produced up to 173.4% and 320.1% more bacilysin than wild type, respectively. Bacilysin overproduction was accompanied by enhancement of the antagonistic activities against *Staphylococcus aureus* (an indicator of bacilysin) and C*lavibacter michiganense* subsp. *sepedonicum* (the causative agent of potato ring rot). Both the size and degree of ring rot-associated necrotic tubers were decreased compared with the wild-type strain, which confirmed the protective effects and biocontrol potential of these genetically engineered strains (Wu et al. 2014b).

### 8.3.5 Bacteriocins

Besides the secondary metabolites (lipopeptides and polyketides), which are synthesized independently from ribosomes, bacteriocins are ribosomally synthesized and present a class of posttranslationally modified peptide antibiotics (Schnell et al. 1988). Together with peptides without antibiotic activity, they are generally termed RiPPs (ribosomally synthesized and posttranslationally modified peptides). RiPP precursor peptides are usually bipartite, being composed of an *N*-terminal leader and *C*-terminal core regions. RiPP precursor peptides can undergo extensive enzymatic tailoring, yielding structurally and functionally diverse products, and their biosynthetic logic makes them attractive bioengineering targets (Burkhart et al. 2015). According to our current knowledge about their biosynthesis, more than 20 distinct compound classes can be distinguished (Arnison et al. 2013). In recent years, two RiPPs with antibacterial activity (bacteriocins) were identified in FZB42 (Scholz et al. 2011, 2014).

#### 8.3.5.1 Plantazolicin

Plantazolicin (PZN) was predicted by bioinformatics to be an excreted metabolite from FZB42 (Lee et al. 2008). An antibacterial substance still produced by FZB42 mutant, deficient in the Sfp-dependent synthesis of lipopeptides and polyketides

and in the Sfp-independent bacilysin synthesis, was identified as being the searched compound together with the gene cluster responsible for its biosynthesis. This cluster encodes a small precursor peptide that is posttranslationally modified to contain thiazole and oxazole heterocycles. These rings are derived from Cys and Ser/Thr residues through the action of a trimeric "BCD" synthetase complex, which consists of a cyclodehydratase (C), a dehydrogenase (B), and a docking Protein (D) (Scholz et al. 2011). Cyclodehydration was shown to precede dehydrogenation in vivo as hypothesized from earlier work on microcin B17 and azol(in)e-containing cyanobactins (Molohon et al. 2011). PZN A and B structures have been resolved unveiling a hitherto unusual number of thiazoles and oxazoles formed from a linear 14mer precursor peptide (Kalyon et al. 2011). PZN A has striking antimicrobial selectivity for *Bacillus anthracis (Sterne)*, the causative agent of anthrax (Molohon et al. 2011), and is efficient against plant pathogenic nematodes (Liu et al. 2013), while precursor molecule PZNB is inactive (Kalyon et al. 2011).

Biosynthetic *pzn* genes are located in a variable part of the genome within a genomic island, together with unique genes involved in the restriction and modification of DNA. They are transcribed into two polycistronic mRNAs (*pznFKGHI* and *pznJCDBEL*) and a monocistronic mRNA for *pznA* as revealed by reverse transcriptase PCR (RT-PCR) (Scholz et al. 2011).

Recently, PZN was described as a selective small molecule antibiotic toward *B. anthracis*. Its mode of action was first examined by gene expression profiling, which yielded an expression signature distinct from broader-spectrum antibiotics. It ruled out that the bacterial membrane is the most probable target of PZN. Remarkably, PZN localizes to the cell envelope in a species-selective manner and is associated with rapid and potent membrane depolarization. Thereby PZN interacts synergistically with the negatively charged phospholipid, cardiolipin (CL), suggesting that PZN causes transient weaknesses specifically in the *B. anthracis* cell membrane (Molohon et al. 2016).

#### 8.3.5.2 Amylocyclicin

The head-to-tail cyclized bacteriocin amylocyclicin was firstly described in *B. amyloliquefaciens* FZB42 (Scholz et al. 2014). Circular bacteriocins are non-lanthionine-containing bacteriocins with broad-spectrum antimicrobial activity, including against common food-borne pathogens, such as *Clostridium* and *Listeria* spp. The positively charged patches on the surface of the structures are thought to be the driving force behind the initial attraction to and subsequent insertion into the negatively charged phospholipid layer of the target cell membrane (van Belkum et al. 2011). Transposon mutagenesis and subsequent site-specific mutagenesis combined with matrix-assisted laser desorption time of flight mass spectroscopy revealed that a cluster of six genes covering 4490 bp was responsible for the production, posttranslational maturation including cleavage and cyclization, and export of the highly hydrophobic compound (Scholz et al. 2014). Amylocyclicin was highly efficient against Gram-positive bacteria, especially against a *sigW* mutant of *B. subtilis* (Y2) (Butcher and Helmann 2006). An orthologous gene cluster was also detected in *B. amyloliquefaciens* DSM7$^T$ (Table 8.1).

#### 8.3.5.3 Mersacidin

Mersacidin, a representative of globular type B lantipeptides, is not synthesized in FZB42, but parts of the mersacidin gene cluster are still remnant in the chromosome (Table 8.1) allowing immunity against this compound. MIC determinations of HIL Y-85 (25 mg/l) and FZB42$^T$ (25 mg/l) demonstrated that FZB42$^T$ was at least as resistant to mersacidin as the producer strain. Interestingly, mersacidin was first detected in *Bacillus* sp. HIL Y-85 (Chatterjee et al. 1992), a strain which was shown later as closely related to FZB42 (Herzner et al. 2011). Another plant-associated *Bacillus* strain, *B. velezensis* Y2, is also able to synthesize mersacidin (He et al. 2012). It was possible to reconstitute synthesis of heterologous mersacidin in FZB42$^T$ by introducing the respective biosynthetic genes cloned from HIL Y-85 (Herzner et al. 2011).

Another representative of the type B lantibiotics, *amylolysin* from *B. velezensis* GA1, was recently described. Similar as mersacidin, it is active on an array of Gram-positive bacteria, including *Listeria* spp. and methicillin-resistant *S. aureus* by interacting with the membrane lipid II (Arguelles Arias et al. 2013).

#### 8.3.5.4 Subtilin

By contrast to mersacidin, subtilin is a representative of the type A lantipeptides. 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 but are inactive to Gram-negative bacteria. Their inactivity against Gram-negative bacteria results from their relatively large size (approximately 1800–4600 Da) which prevents them from penetrating the outer membrane of the Gram-negative cell wall (Stein 2005). Subtilin was the first lantibiotic isolated from *B. subtilis*. As in the case of mersacidin, only the immunity genes are present in FZB42, while biosynthesis and modification genes are missing. However, a corresponding gene cluster involved in synthesis of the lantibiotic-like peptide ericin was found in plant-associated *Bacillus* sp. A1/3 (Stein et al. 2002). We characterized strain A1/3 as a member of the *B. amyloliquefaciens plantarum* group (Borriss et al. 2011), nowadays *B. velezensis*, and therefore, ericin can be considered as an early example of a lantibiotic produced by plant-associated bacilli.

#### 8.3.5.5 Antimicrobial Peptide Lci

Lci was reported as an antimicrobial peptide synthesized by a *B. subtilis* strain with strong antimicrobial activity against plant pathogens, e.g., *Xanthomonas campestris* pv. *oryzae* and *Pseudomonas solanacearum* PE1. Its solution structure has a novel topology, containing a four-strand antiparallel β-sheet as the dominant secondary structure (Gong et al. 2011). The gene is not present in the *B. subtilis* 168 genome but was detected in FZB42 and *B. amyloliquefaciens* DSM7$^T$ (Table 8.1).

### 8.3.6 Volatiles

A blend of volatile organic compounds (VOCs) is released by several PGPR *Bacillus* strains, including FZB42$^T$ (Borriss 2011, Tahir et al. 2017a). These are low molecular weight, gaseous, metabolic compounds, which are emitted from bacterial cells having no physical contact to their target cells. The volatiles 3-hydroxy-2-butanone (acetoin) and 2,3 butandiol are triggering enhanced plant growth, control plant pathogens, and induce systemic resistance (Ryu et al. 2003). To synthesize 2,3-butanediol, pyruvate is firstly converted into acetolactate by acetolactate synthase (AlsS) under conditions of low pH and oxygen starvation. The next step of this alternative pathway of pyruvate catabolism, conversion of acetolactate to acetoin, is catalyzed by acetolactate decarboxylase (AlsD). The final step, from acetoin to 2,3-butandiol, is catalyzed by the *bdh*A gene product, acetoin reductase/2,3-butanediol dehydrogenase (Nicholson 2008). The FZB42$^T$ genome contains all the three genes encoding this pathway. FZB42$^T$ mutant strains, incapable of producing volatiles due to knockout mutations introduced into the *als*S and *als*D genes, are unable to support growth of *Arabidopsis* seedlings (Borriss 2011).

Besides plant growth promotion, volatiles act against plant pathogens by inducing systemic resistance in plants; in addition direct inhibitory effect of VoCs against plant pathogenic fungi was reported (Tahir et al. 2017b). Thirteen VOCs produced by FZB42 were identified using gas chromatography-mass spectrometry analysis (Table 8.2). Benzaldehyde, 1,2-benzisothiazol-3(2 H)-one, and 1,3-butadiene significantly inhibited the colony size, cell viability, and motility of *Ralstonia*

**Table 8.2** VOC profile of *Bacillus velezensis* FZB42

| Volatile compound (VOC) | Abbreviation | Inhibition[a] |
|---|---|---|
| Silanediol, dimethyl | SDD | – |
| 1,2-Benzisothiazol-3(2H)-one | 1,2-BIT | +++ |
| Benzeneacetamide | BAM | ++ |
| Oxime-, methoxy-phenyl | OMP | NT |
| (1R)-2,6,6 Trimetyhlbicyclo[3–1.1] hept-2-ene | TMB | + |
| Benzoic acid,- formyl - dimethoxy -,8,8 - dimethoxyoct - 2 - yl | BA | + |
| Benzaldehyde | BDH | +++ |
| Sulfurous acid, cyclohexyl-methyl isobutyl ester | SCE | – |
| 6-Tridecen, 2,2,4,10,12,12-hexamethyl-7-(3,5,5-trimrthylhexyl) | 6-THT | NT |
| 2-Undecanethiol, 2-methyl | 2-UT,2-M | – |
| Dodecane, 1-fluoro | DCF | ++ |
| Dodecane | DCN | ++ |
| Phenol, 2-(1,1-dimethylethyl)-6-methyl | PH | – |

According to Tahir et al. (2017a)

[a] Inhibition of *Ralstonia solanacearum*

*solanacearum*, the causative agent of bacterial wilt in a wide variety of potential host plants (Tahir et al. 2017a). Severe morphological and ultrastructural changes in cells of *R. solanacearum* were registered. Furthermore, VOCs downregulated transcription of type III (T3SS) and type IV secretion (T4SS) system, extracellular polysaccharides (*eps*), and chemotaxis-related genes (*motA, fliT*), which are major contributors to pathogenicity, resulting in decreased wilt disease. The VOCs significantly upregulated the expression of genes related to wilt resistance and pathogen defense. Transcription of tobacco resistance gene RRS1 was enhanced in the presence of VOCs. Overexpression of plant defense genes *EDS1* and *NPR1* suggests the involvement of salicylic acid (SA) pathway in induction of systemic resistance (Tahir et al. 2017a).

A recent analysis performed with FZB42 volatiles revealed that signal pathways involved in plant systemic resistance were positively affected. JA response (VSP1 and PDF1.2) and SA response genes (PR1 and FMO1) were triggered either in the leaves or roots of *Arabidopsis* plantlets after incubation with the volatiles. Noteworthy, defense against nematodes were elicited by volatiles in *Arabidopsis* roots (Hao et al. 2016).

Our present knowledge about the complex network of biocontrol actions exerted by FZB42 within a tripartite model system consisting of the plant (e.g., lettuce), the pathogen (*R. solani*), and the beneficial bacterium (FZB42) is tentatively summarized in Fig. 8.2.

## 8.4 Outlook

Most of the biocontrol agents currently in use are based on living microbes. Representatives of the *B. subtilis* species complex, including *B. amyloliquefaciens*, *B. subtilis*, and *B. pumilus*, are increasingly used for commercial production of biofungicides (Borriss 2016). Most of them are stabilized liquid suspensions or dried formulations prepared from durable endospores. They are developed for seed coating, soil, or leave application. Unfortunately, it is very unlikely that concentration of *Bacillus*-synthesized CLPs (iturins and fengycins) within the plant rhizosphere reaches levels sufficient for antibiosis (Debois et al. 2014). A possibility for circumventing this problem are bioformulations consisting of both *Bacillus* spores and concentrated culture supernatants with antimicrobial metabolites. However, only a few bioformulations currently on the market, such as SERENADE$^{(R)}$ prepared from *B. subtilis* QST713 and Double Nickel 55 prepared from *B. amyloliquefaciens* D747, contain together with living spores antimicrobial compounds, such as cyclic lipopeptides (iturins, fengycin). Unfortunately, also in these products only the number of spores is declared as active ingredient of the biofungicide. In contrast to chemical fungicides, there is no indicative about metabolites and their concentration, excluding an exact treatment of pathogen-infected plant parts. I recommend indicating a fixed concentration of the active principle for suppressing the target pathogen on the label of the biocontrol product. This would allow comparison of chemical and biological pesticides (Borriss 2015). To the best of my knowledge, no bioformulations containing exclusively antimicrobial metabolites are commercially

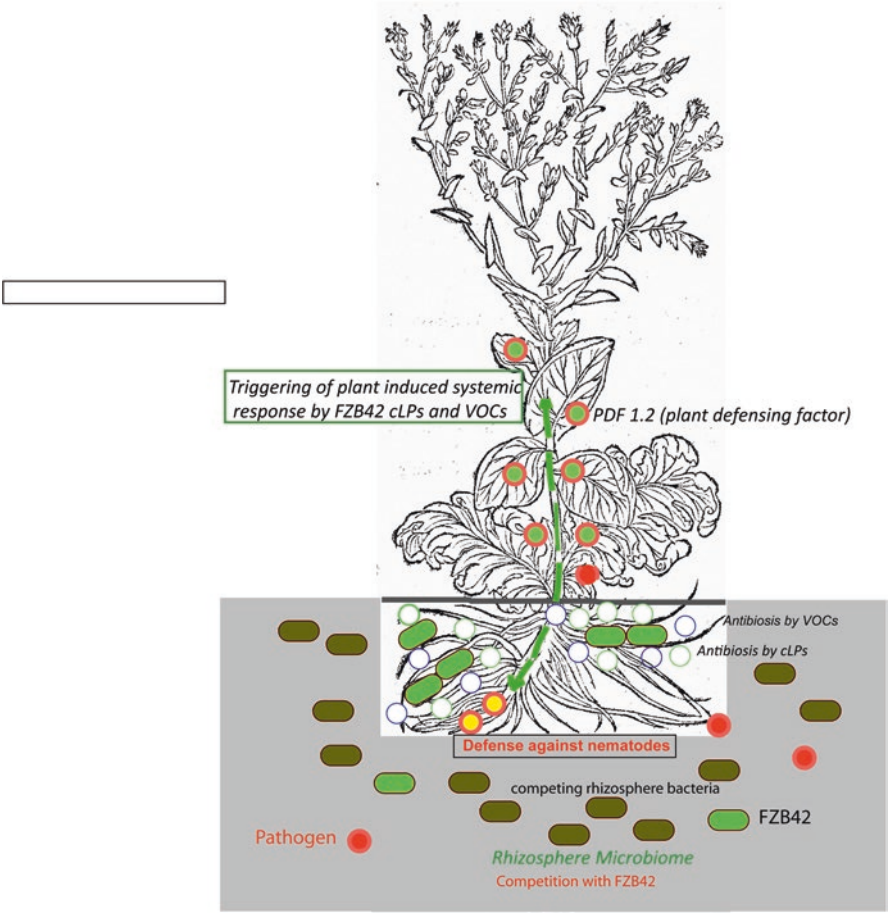


**Fig. 8.2** Biological control exerted by FZB42. The cartoon illustrates our present picture about the complex interactions between a beneficial Gram-positive bacterium (FZB42, light green), a plant pathogen (*R. solani,* symbolized by red-filled circles), and plant (lettuce, *Lactuca sativa*). FZB42 colonizes the root surface and is able to produce cyclic lipopeptides (green circles) and VOCs (blue circles). Direct antibiosis and competition for nutrients (e.g., iron) suppress growth of bacterial and fungal plant pathogens in the rhizosphere. However, these effects seem to be of minor importance, since the composition of the root microbiome is not markedly affected by inoculation with FZB42 (Erlacher et al. 2014). Due to production of *Bacillus*-signaling molecules (cLPs and VOCs) and in simultaneous presence of the pathogen, the plant defensing factor 1.2 (PDF1.2) as indicated by the green-filled red circles is dramatically enhanced and mediates defense response against plant pathogens (Chowdhury et al. 2015a). VOCs have shown to trigger defense against nematodes within plant root tissues (Hao et al. 2016). The picture of the *lettuce* plant ("*Lactuca crispa*") was taken from Bock 1552, p. 258. (Adapted after Fig. 5 in Chowdhury et al. 2015b)

available, although companies like ABiTEP performed extended large-scale trials with concentrated and stabilized *Bacillus* supernatants in order to suppress plant pathogens. Concerning biosafety issues, no representatives of the *B. subtilis* species complex and of the genus *Paenibacillus* spp. have been listed as risk group in "The

Approved List of biological agents" (2013). However, *B. cereus* and *B. anthracis* were listed in human pathogen hazard group 3, excluding their use as biocontrol agents in agriculture.

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