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Zhou, Lu

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**Discovery of Natural Products from
Bacilli and *Pseudomonas* for Biocontrol
of Plant Diseases**

Lu Zhou

2021

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university of
groningen

Discovery of Natural Products from *Bacilli* and *Pseudomonas* for Biocontrol of Plant Diseases

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Monday 8 November 2021 at 09.00 hours

by

Lu Zhou

born on 06 June 1988
in Sichuan, China

Supervisor

Prof. O.P. Kuipers

Prof. G. N. Moll

Assessment Committee

Prof. A.J.M. Driessen

Prof. J.T.M. Elzenga

Prof. Á. T. Kovács

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Chapter 1

General Introduction

Lu Zhou

*Department of Molecular Genetics, University of Groningen, Groningen, The
Netherlands*

Tomato

Tomato (*Solanum lycopersicum*) is a major agricultural plant that also serves as a model system for studying fruit development (Consortium, 2012). Tomatoes and tomato derivatives are among the most often consumed vegetables in the world. They are the most eaten non-starchy vegetable in terms of quantity and are the most important source of dietary lycopene, which is a strong antioxidant with increased bioavailability after heating and processing (Burton-Freeman and Reimers, 2011). Tomato fruit is high in calories, carotenoids, flavonoids, phenolics, mineral elements, vitamin C, and dietary fibers, among other compounds. Because of its numerous health advantages, tomato fruit has earned the distinction of being a food with positive functional characteristics (Ramesh et al., 2021). Many scientific investigations have found that including tomatoes in one's diet on a daily basis reduces the risk of some forms of cancer and hence protects one's health (Wold et al., 2004; Rao and Rao, 2007; Burton-Freeman and Reimers, 2011). However, there are several plant diseases that may easily infect tomato plants during the growing season, thereby reducing quality and output. Despite good achievements in managing tomato diseases with chemical treatments, e.g. insecticides, and fungicides, chemical residues may pose a lot of hazards to our human health and the environment (Janahiraman et al., 2016; Parray et al., 2016; Zhou et al., 2019).

Microbiome in Rhizosphere Soil

Hiltner coined the term rhizosphere, which is derived from the Greek words rhiza, which means root, and sphere, which means field of influence. The rhizosphere, as described by him, is the zone of soil directly next to legume roots that supports high levels of bacterial activity (Hiltner, 1904). Later, the word has been expanded to cover both the

volume of soil affected by the root and the root tissues colonized by microorganisms (Pinton et al., 2007). The rhizosphere soil is home to a diverse range of organisms, including bacteria, fungi, oomycetes, archaea, protozoa, and algae, as well as nematodes and arthropods. Their population per gram of soil ranges between 10^8 and 10^9 for bacteria, to 10^3 and 10^2 for nematodes (Mendes et al., 2013). The numerous metabolites produced by plant roots feed and affect microorganisms in the rhizosphere. In return, microorganisms and their metabolites interact in a variety of positive, negative, and neutral ways with plant roots. Such interactions can have an impact on plant growth and development, affect nutrient dynamics, and make plants either more or less susceptible to disease and abiotic stress (Morgan et al., 2005; Mendes et al., 2013).

Plant Growth Promoting Rhizobacteria (PGPR)

Kloepper first introduced the term Plant Growth Promoting Rhizobacteria (PGPR) to refer to bacteria that are associated with plant roots and exert beneficial effects on plant development (Kloepper et al., 1980). PGPR are naturally occurring soil microorganisms that can assist plants by promoting plant growth or decreasing disease (Xie et al., 2018). As a result, PGPR are increasingly being used as eco-friendly bio-fertilizers or biocontrol agents to stimulate plant growth and/or control plant diseases thereby reducing chemical pollution (Trinh et al., 2018). Growing interest in developing a sustainable agriculture has encouraged farmers to minimize the usage of chemical fertilizers and pesticides by increasing the application of PGPR.

Beneficial Effects of PGPR on Plant Growth

PGPR, in general, stimulate plant growth either directly by enabling resource acquisition

(e.g. nitrogen, phosphorus and critical minerals) or indirectly by lowering the inhibitory effects of different diseases on plant growth and development in the form of biocontrol agents (Glick, 2012;Ahemad and Kibret, 2014) (Figure 1).

The direct effects of PGPR include nitrogen fixation, phosphate solubilization, siderophore production, phytohormone production, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase production. Nitrogen is a critical element for the growth of all living creatures, including plants and microorganisms. The observation of nitrogen shortage in soil has resulted in the usage of significant volumes of nitrogenous fertilizers to compensate for the essential plant needs in order to attain maximum plant yield in most soils (Zhang et al., 2015). Despite the fact that nitrogen is abundant in the earth's atmosphere (approximately 78 %), this form of gaseous nitrogen [$N_2(g)$] is not readily accessible to most organisms, i.e., it is not appropriate for plant absorption unless it is first transformed to ammonia (Baas et al., 2014;Olanrewaju et al., 2017). Biological N_2 fixation (BNF) is a process by which nitrogen-fixing bacteria convert nitrogen to ammonia utilizing a complicated enzyme system known as nitrogenase (Kim and Rees, 1994). Given the intricate nature of the interactions between soil, plant, and microorganisms, the use of BNF as an option to enhance efficiency and sustainability in the use of N in agriculture is critical. Phosphorus (P), the second most essential nutrient for plant development after nitrogen, is plentiful in soils in both organic and inorganic forms. The bulk of soil phosphorus is present in insoluble forms, whereas plants take it exclusively in two soluble forms, monobasic and dibasic ions. PGPR with phosphate solubilizing activity are rhizobacteria that solubilize phosphorus. They provide phosphate to plants in a more acceptable manner and are not harmful to the environment. They transform insoluble

organic and inorganic phosphate into a form that plants can use (Gupta et al., 2015). Siderophores are low molecular weight compounds that chelate the ferric ion (Fe^{3+}) with high specific activity and serve as carriers for the transfer of Fe (III) into a microbial cell under iron-limiting circumstances (Gupta and Gopal, 2008). Some PGPR generate siderophore compounds, which are chelating agents with a high affinity for ferric irons. This chemical chelates to the inaccessible form of iron, making it available to plant roots in the surrounding environment (Singh et al., 2020). Therefore, under iron restriction, siderophores operate as solubilizing agents for iron from minerals or organic molecules. In addition to iron, siderophores may form stable complexes with other environmentally hazardous metals such as Al, Cd, Cu, Ga, In, Pb, and Zn, as well as radionuclides such as U and Np 17, 18. Binding of the siderophore to a metal raises the concentration of soluble metal (Rajkumar et al., 2010). Plant growth and development are regulated by phytohormones, which are structurally unrelated tiny molecules found in nature and include auxin, abscisic acid, cytokinin, gibberellin, and ethylene (Maheshwari et al., 2015). With the synthesis of several phytohormones, PGPR can modify root architecture and stimulate plant growth (Kloepper et al., 2007). The amount of IAA generated by PGPR in the rhizosphere dictates how it affects the host plant, with high amounts causing developmental defects and driving the production of lateral and adventitious roots, and low levels favoring root elongation (Bal et al., 2013). Some literatures report that PGPR can promote plant growth by increasing the activity of the enzyme ACC deaminase, which stimulates plant growth by lowering plant ethylene levels. The enzyme converts ACC, the biosynthetic precursor of the hormone ethylene in plant tissues, to ammonia α -ketobutyrate, decreasing the degree of ethylene's inhibitory impact on root elongation and increasing plant development. Plants treated with ACC-deaminase producing PGPR,

can be significantly more resistant to the negative effects of produced ethylene which caused by stressful circumstances, promoting tolerance to a variety of biotic and abiotic stressors (Glick et al., 1998;Dimkpa et al., 2009;Zahir et al., 2009;Laslo et al., 2012).

The indirect effects of PGPR include production of antimicrobial compounds and stimulation of induced systemic resistance (ISR). Antimicrobial compounds produced by PGPR play an important role in the management of plant diseases. The process has been defined as the inhibition or suppression of pathogenic microorganisms via the production of low-molecular-weight compounds by other microorganisms (Kenawy et al., 2019). Plants possess latent defensive systems against diseases that can be systemically triggered when stressed or infected by pathogens. This is referred to as induced systemic resistance (ISR) (Tuzun, 1991;Baker et al., 1997). Disease incidence can be significantly decreased if systemic resistance is activated correctly prior to pathogen assault. Thus, ISR is a condition of increased defensive capability produced by a plant in response to certain biotic or chemical stimuli (Meena et al., 2020). ISR is activated by PGPR via the phytohormones jasmonic acid (JA) and ethylene (ET), resulting in a quicker and stronger immune response in the aftermath of pathogen assault (Constantin et al., 2019). It is thought that the microbe associated molecular patterns (MAMPs) of various beneficial bacteria are recognized by plant receptors, resulting in particular hormonal signals in the roots. MAMPs from beneficial microorganisms, such as flagellin, secondary metabolites, and lipopolysaccharides, activate MAMP-triggered immunity (MTI) and regulate hormonal signaling in plants (Jacobs et al., 2011;Hermosa et al., 2012;Zamioudis and Pieterse, 2012;Rashid and Chung, 2017).

Figure 1. The mechanisms of the plant growth promotion activity of PGPR for tomato plant. The black arrows indicate direct effects while the red arrows indicate indirect effects.

Beneficial *Bacillus* strains for plant growth

Many beneficial bacteria strains from different genera have been utilized as PGPR, including *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, and *Serratia* (Akinrinlola et al., 2018). Among them, beneficial bacteria from the genus of *Bacillus* are the most commercialized for application, since the heat and desiccation-

resistant spores produced by *Bacillus* strains can be formulated as a stable dry white powder with a long shelf life (Cho and Chung, 2020). For instance, *Bacillus velezensis* FZB42 is used commercially as biofertilizer and biocontrol agent in agriculture (Chowdhury et al., 2015a), and has been shown to have beneficial impacts on plant development and disease control in tomato, cucumber, cotton, tobacco, and lettuce (Grosch et al., 1999; Yao et al., 2006; Gül et al., 2008; Wang et al., 2009; Chowdhury et al., 2013; Chowdhury et al., 2015a; Chowdhury et al., 2015b). *Bacillus subtilis* NCD-2 can be used as biocontrol agent for cotton seedling damping-off and verticillium wilt caused by *Rhizoctonia solani* and *Verticillium dahliae*, respectively (Guo et al., 2014).

Antimicrobials to Fight Plant Pathogens

One of the most promising ways for more rational and safe crop-management strategies for fighting plant pathogens is the employment of beneficial microorganisms (Fravel, 2005). Antimicrobials, which are synthesized by biosynthetic gene clusters (BGCs) in those beneficial microorganisms genomes, have been the object of attention in past years as candidates for plant protection products (Montesinos, 2007), since they have function to kill and/or inhibit the growth of plant pathogens (Mullis et al., 2019). Many of the antimicrobials produced by beneficial microorganisms play an essential role in controlling plant pathogens and could be applied in agriculture (Olishevskaya et al., 2019).

Genome Mining for Secondary Metabolites of Bacteria

A new age in novel secondary metabolites discovery has begun with the advancements of contemporary DNA sequencing methods, which massively promote the development of genome sequencing in microorganisms (Vater et al., 2018). The key purpose of

genome mining for antagonistic compounds is to identify novel gene clusters of their biosynthesis (BGCs). BGCs encode biosynthetic pathways of naturally occurring natural products including Non-ribosomally synthesized peptides (NRPs), polyketides (PKs), hybrid polyketides non-ribosomally synthesized peptides (PKs-NRPs), ribosomally synthesized and post-translationally modified peptides (RiPPs) and terpene (Albarano et al., 2020). Genome mining is based on bioinformatics and computer technologies (Figure 2). A number of computing tools have been created to help scientists with this endeavor. These include antiSMASH 5.0 (Blin et al., 2019b), BAGEL 4 (van Heel et al., 2018), PRISM 4 (Skinnider et al., 2020), NP.searcher (Li et al., 2009), and NaPDos (Ziemert et al., 2012). Here we describe 2 bioinformatic tools.

antiSMASH 5.0 (<https://antismash.secondarymetabolites.org/#!/start>) employs pHMM's with a BGC-based profile detection that are specific to conserved core enzyme pathways for secondary metabolite production. Upon identifying the core enzymes, antiSMASH compares a set of manually curated BGC cluster criteria with co-located core genes (Blin et al., 2019a). The bioinformatic tool antiSMASH was additionally associated to the whole BLAST and Pfam genome analysis modules from the previously disclosed CLUSEAN framework, to allow more extensive manual genome analysis (Medema et al., 2011). The prediction of the products of the biosynthetic pathways was possible for NRPs, PKs, PKs-NRPs, RiPPs, terpene, etc (Blin et al., 2014).

BAGEL4 (<http://bagel4.molgenrug.nl/>) uses knowledge-based databases and motif databases to identify potential bacteriocin open reading frame (ORFs) in a DNA sequence (de Jong et al., 2006). BAGEL4 takes FASTA-format DNA nucleotide sequences as input.

These DNA sequences are simultaneously examined and compared using two distinct ways, one based on genes usually discovered in a bacteriocin gene cluster, and the other based on identifying the gene itself (van Heel et al., 2013). Currently, approximately 500 RiPPs (class 1), 230 unmodified (class 2) and 90 large (class 3) bacteriocins (> 10 kD) are in the database of BAGEL4 (van Heel et al., 2018).

Figure 2. The workflow of genome mining for biosynthetic gene clusters harbored by *Bacillus* sp.

Biosynthesis of NRPs and PKs by Megaenzymes

A large part of secondary metabolite manufacturing takes place via a host of metabolic intermediary biosynthesis pathways, including polyketide synthases (PKSs) and non-ribosomal peptide synthetases, two of the common and widely investigated megaenzyme families (Mullis et al., 2019). These two biosynthetic systems run independently in

assembly-line format to build polymeric chains and tailor their functions, which worked through units termed 'modules' (Weissman, 2015).

NRPSs synthesize peptides without RNA and ribosomal machinery messages (Walsh, 2016), which is just achieved by their own large multimodular enzymes (Strieker et al., 2010) (Figure 3). Different catalytic domains can also be classified according to their roles. They contain the adenylation (A) domains, thiolation/peptidyl carrier protein (T/PCP) domains, and condensation (C) domains. The A domain detects and activates specific amino acid or hydroxyl acid. The T/PCP domain which carries a phosphopantetheinyl on a conserved serine residue and provides substrates which activated by A domain to the respective active site. The C domain catalyzes the formation of amide bond between the thioester group and amino group to elongate peptide chain (Finking and Marahiel, 2004). The thioesterase (TE) domain is a key catalytic unit for product release which is situated in the end-module and either hydrolysis or macrocyclization catalyzes peptide release (Strieker et al., 2010). Besides, there are optional domains associated with NRPSs as well, such as the epimerization (E), reductase (R), cyclization (Cy), and oxidation (Ox) domains (Walsh et al., 2001). The E domain has been identified in NRPSs, where it catalyzes the conversion of an L-amino acid into a D-amino acid (Miller and Gulick, 2016). The R domain is involved in a peptide release alternate pathway, which catalyzes an NAD(P)H dependent two-electron reduction of the thioester to an aldehyde that can be further reduced to an alcohol (Tietze et al., 2020). The Cy domains in NRPSs catalyze the heterocyclization of cysteine and serine/threonine to thiazoline and oxazoline rings (Duerfahrt et al., 2004). The Ox domains oxidize peptide-bound intermediates to install important structural motifs, which usually convert previously generated thiazoline into

thioesterase.

Polyketides, encompassing a broad variety of bioactive chemicals characterized by alternating carbonyl and methylene groups, are synthesized by PKSs (Mullis et al., 2019) (Figure 4). PKSs biosynthesize polyketides (PKs) in a similar chain extension and modification way (Weissman, 2015). There are three types of naturally occurring PKSs modules, which are Type I, II, and III PKSs. Up to now, ketosynthase (KS), acyltransferase (AT), acyl carrier protein (ACP), and thioesterase (TE) are found in PKSs. Besides, auxiliary enzymes such as ketoreductase (KR), dehydratase (DH), and cyclase (CYC) are also found in most PKSs (Wong and Khosla, 2012). KS domain which catalyzes C-C bond formation (Broadhurst et al., 2003). The AT domain recruits a building block (usually methylmalonyl-CoA) and transfers its α -carboxyacetyl moiety through a thioester linkage to the 4'-phosphopantetheine (Ppant) arm of an ACP domain (Moretto et al., 2019). The ACP domain is responsible for tethering and shuttling the extender unit or the polyketide intermediate (Hwang et al., 2020). In PKSs, the TE domain hydrolyzes and /or cyclizes intermediates to produce the linear polyketide or macrocyclic polyketides (Hwang et al., 2020). Type I PKSs are made up of multiple modules, each of which is in charge of incorporating and modifying one acyl-CoA substrate in order to synthesis the polyketide product (Staunton and Weissman, 2001). They can be iterative, with a single set of biosynthetic enzymes catalyzing several rounds of an extension cycle, or modular, with many sets of enzymes catalyzing different cycles (Caffrey, 2012). Type II PKSs are multi-enzyme complexes made up of monofunctional proteins which are mostly found in bacteria and synthesize diverse aromatic polyketides (Hertweck et al., 2007). Type III PKSs only comprise homodimers (80-90 kDa) of KS domain-containing subunits with a single

KS domain that fulfills all of the activities of the important domains in type I/II PKSs (Shimizu et al., 2017).

Figure 4. The gene cluster for biosynthesis of difficidin. ACP, acyl carrier protein; KS, ketosynthase; DH, dehydratase domain; KR, ketoreductase; MT, methyltransferase; ER, enoyl reductase.

Biosynthesis of Bacteriocins by Ribosomes

Bacteriocins are a large family of ribosomally synthesized proteinaceous compounds produced by bacteria that have antimicrobial activity against other bacteria, sometimes closely related to the producer strain (Joerger, 2003). Bacteriocins are often divided into three classes: peptides that undergo extensive post-translational modifications (PTMs) (class I), unmodified peptides (class II) (Budisa, 2013) and large antimicrobial peptides (class III). Modified bacteriocins (class I) have molecular weights of < 5 kDa and all undergo PTMs, which are guaranteed by specialized enzymes expressed in the

bacteriocin gene cluster, these class I bacteriocins are belong to the RiPPs family (Zhao and Kuipers, 2016). Unmodified bacteriocins (class II) are small (<10 kDa) heat-stable, membrane-active peptides comprised only of unmodified amino acids; moreover, they are further classified into four subclasses, *Ila*, *Ilb*, *Ilc*, and *Ild* according to their detailed characteristics (Nazari and Smith, 2020). Large antimicrobial peptides (class III) include large peptides (>10 kDa). The BGCs of class III bacteriocins contain an immunity gene and a structure gene (Wang, 2010). Among the *Bacillus* strains, the RiPPs are abundantly produced bacteriocins. In this introduction, the biosynthesis of several kinds of RiPPs produced by *Bacillus* strains will be described.

Lanthipeptides are defined by the presence of lanthionine and/or methyllanthionine thioether cross-links, as well as the unusual amino acids, dehydroalanine and dehydrobutyrine (Goto et al., 2010), which can be subdivided into four classes by their modification enzymes. In class I lantipeptides dehydratase LanB and cyclase LanC are enzymes that catalyze the dehydration of serine and threonine residues, and the subsequent thioether linkage formation between dehydroalanine and dehydrobutyrine. In other classes lanthipeptides, bifunctional enzymes are responsible for both dehydration and cyclization processes (LanM for class II, RamC/LabKC for class III, and LanL for class IV) (Willey and van der Donk, 2007;Müller et al., 2010).

Head-to-tail cyclized peptides are cyclized between their N- and C-terminus and range in size between 30–70 amino acids (Arnison et al., 2013;Letzel et al., 2014). They are often highly hydrophobic and appear to exhibit antimicrobial action by targeting the cell membranes of other bacteria in order to generate holes and promote ion permeability,

resulting in cell death (Arnison et al., 2013). However, little is known yet about the circularization mechanism of Head-to-tail cyclized peptides compared to other peptides coming from ribosomal synthesis (van Heel et al., 2017).

Lasso peptides are characterized by their unique topology, which involves threading the C-terminal tail through an N-terminally to form macrolactam ring (Zhu et al., 2016). The lasso fold structure leads in exceptional resistance to proteolytic degradation and heat or chaotrope-induced denaturation. Two enzymes are required for lasso peptide biosynthesis: a lasso cyclase (C-protein) homologous to asparagine synthetase and a leader peptidase (B-protein) analogous to transglutaminase (Tietz et al., 2017).

Sactipeptides have a common feature that an intramolecular thioether bond are formed to crosslink the sulfur atom of a cysteine residue with the α -carbon of an acceptor amino acid, which is catalyzed by a highly specialized radical SAM enzyme (Flühe and Marahiel, 2013). Because of their unusual hairpin structures, and their potent, narrow-spectrum activity toward several human pathogenic bacteria, sactipeptides have become interesting for research (Hudson et al., 2019). Each sactipeptide biosynthetic gene cluster comprises at least one precursor peptide, one radical SAM enzyme per precursor peptide, one putative protease, and two proteins thought to be involved in sactipeptide export and immunity (Flühe and Marahiel, 2013).

Linearazole-containing peptides (LAPs) are defined by thiazol(in)e and (methyl)oxazol(in)e heterocycles formed by cyclization of Cys and Ser (Thr) side chains during the posttranslational modification of the precursor peptide (Arnison et al., 2013).

LAP biosynthetic gene cluster generally comprises genes for a YcaO-domain containing cyclodehydratase (gene D), YcaO-partner protein (gene C or gene F), dehydrogenase (gene B), which oxidizes azolines to azoles, and the efflux pump as well as the precursor peptide-coding gene (gene A) (Burkhart et al., 2017;Travin et al., 2019;Suzuki et al., 2021).

Secondary Metabolites of *Bacillus*

Strains from the *Bacillus* group are ubiquitous among Gram-positive bacteria and demonstrate unusual genetic adaptability, enabling it to colonize highly diverse habitats (Kaspar et al., 2019). The remarkable biosynthetic arsenal of this genus has been proven by analyses of many wild type strains. It has been estimated that 4-5% of their genome is dedicated entirely to antimicrobial compounds (AMCs) production (Stein, 2005). Their structures are usually cyclic and hydrophobic, with unusual moieties like D-amino acids (AA) or intramolecular thioether linkages (Caulier et al., 2019). Here, we will introduce several antimicrobial compounds produced by the genus of *Bacillus*, i.e., RiPPs, PKs, NRPs and hybrid PKs-NRPs.

Figure 5. The biosynthesis of subtilin by ribosome in *Bacillus* sp. The subtilin synthetase complex *SpaBTC* modifies, cleaves, and translocates the prepeptide *SpaS* across the cell membrane. Each functional complex of subtilin is transcribed together in an operon-like structure (*spaBTC*, *spaS*, *spaIFEG*, and *spaRK*).

RiPPs often have a narrow activity spectrum, making them potential clinical applications since they can avoid the off-target effects seen with broad spectrum antibiotics, which can disrupt the normal flora and open the door to undesired secondary infections by resistant organisms (Cotter et al., 2013). Subtilin, first unravelled by Gross & Kiltz, had been described as a pore-forming lantibiotic that causes dissipation of transmembrane proton motive force and release of cytoplasmic solutes (Gross and Kiltz, 1973; Parisot et al., 2008) (Figure 5). Subtilisin A is a head-to-tail peptide with 35 amino acid residues encoded by the *sbo-alb* operon of *Bacillus subtilis*. It has a circular peptide backbone that is held together by three unique thioether linkages formed by three cysteines and the α -

carbons of one threonine and two phenylalanines, respectively (Babasaki et al., 1985;Flühe et al., 2012). Paeninodin is a well characterized lasso peptide, which was purified from *Paenibacillus dendritiformis* C454. However, the roles of paeninodin and its phosphorylated version are yet unknown, and more research is needed to confirm their biological activities (Zhu et al., 2016;Hegemann et al., 2018). Plantazolicin, a polyheterocyclic, linear compound of the LAPs family with narrow-spectrum antibiotic activity produced by *Bacillus velezensis* FZB42 and *Bacillus pumilus*, is described as an antibiotic compound that inhibits Gram-positive organisms closely related to its producing organism, *Bacillus anthracis* (Scholz et al., 2011;Molohon et al., 2016).

PKs and hybrid PKs-NRPs form a varied structural group of secondary metabolites which show a broad range of biological activities including antibacterial, immunosuppressive, and antitumor. Difficidin is a highly unsaturated 22-membered macrocyclic polyene lactone phosphate ester that is encoded by a PKS BGC with 14 open reading frames ranging from *difA* to *difN* and *difY*, which was first identified from *Bacillus subtilis* ATCC 39320. Difficidin has a wide spectrum of anti-bacterial bioactivity (Aleti et al., 2015;Wu et al., 2015). Macrolactin, synthesized by the polyketide synthase (PKS), is a macrolide antibiotic with a 24-member lactone ring. It was first discovered in a marine *Bacillus* strain (Gustafson et al., 1989;Yuan et al., 2016). The gene cluster *mln*, which contains nine operons including *mlnA-I*, encodes cyclic macrolactins in *B. velezensis* FZB42. (Schneider et al., 2007). Bacillaene, a bacteriostatic antibiotic, is encoded by a hybrid PKs-NRPs BGC *bae* including five long open reading frames (ORFs) *baeJ*, *baeL*, *baeM*, *baeN* and *baeR*. Bacillaene was first discovered from *Bacillus subtilis* 3610 and 55422. It is notoriously unstable toward light, oxygen, and normal temperature (Patel et al.,

1995;Butcher et al., 2007;Wu et al., 2014;Li et al., 2021a).

NRPs, produced by *Bacillus* species, have particular antimicrobial activity against plant diseases, making them important in agriculture, biotechnology, and pharmaceuticals. Kurstakins, surfactins, iturins, and fengycins are the four major families of NRPs produced by *Bacillus* strains. Kurstakin, a partially cyclic heptalipopeptide first identified from *Bacillus thuringiensis* kurstaki HD-1, which was synthesized by *krs* BGC containing three genes *krsA*, *krsB*, and *krsC*. The purified kurstakin has biological activity against *Stachybotrys charatum* (Hathout et al., 2000;Béchet et al., 2012;Gélis-Jeanvoine et al., 2017). Surfactin was first discovered in 1968, as a secondary metabolite in *Bacillus subtilis* fermentation broth (Arima et al., 1968). Surfactin is a lipopeptide synthesized by the *srfA* BGC (*srfAA*, *srfAB*, *srfAC*, and *srfAD*), resulting a circular heptapeptide comprising glutamate acid (Glu), aspartate acid (Asp), valine (Val), and leucine, as well as a long chain fatty acid tail (Leu) (Nakano et al., 1991). Surfactin, a powerful lipopeptide biosurfactant, continues to pique the interest of applied microbiologists and biotechnologists all over the world, owing to its many bioactive characteristics, which include antifungal, antiviral, anticancer, insecticidal, and antimycoplasma activity (Sen, 2010). Iturin, a cyclic heptapeptide, is a strong antifungal lipopeptide generated mostly by *Bacillus* strains. Because of its low toxicity and absence of adverse effects on the host, iturin can be utilized as a possible biopesticide against plant pathogens as well as a treatment for human and animal mycoses (Dang et al., 2019). Iturin is synthesized by *itu* BGC consisting of four genes *ituD*, *ituA*, *ituB* and *ituC* (Tsuge et al., 2001). Genes *itu A-C* are responsible for incorporating different amino acid residues, and gene *ituD* encodes a malonyl-CoA transacylase (MCT-domain), which is related to the synthesis of fatty acids (Stein, 2005;Ongena and Jacques, 2008). Fengycin, also called plipastatin, is a lipodecapeptide

with an internal lactone ring in the peptidic moiety (Ongena and Jacques, 2008). The *fen* BGC is involved in the biosynthesis of fengycin including 5 genes, *fenA*, *fenB*, *fenC*, *fenD*, and *fenE* (Lu et al., 2016). Fengycin exhibits powerful antifungal activity against a wide range of phytopathogens such as *Rhizopus stolonifer*, *Gibberella zeae*, *Aspergillus niger*, *Mucor rouxii*, *Fusarium graminearum*, and *Sclerotinia sclerotiorum* (Liu et al., 2005; Ramarathnam et al., 2007; Ayed et al., 2014; Tang et al., 2014; Zhang and Sun, 2018).

Mode of action of Antimicrobial Peptides (AMPs)

Antimicrobial peptides (AMPs) have lately become more widely employed to combat the fast-rising incidence of bacterial resistance to conventional antibiotics. An extensive knowledge of AMPs' mode of action (MOA) is critical for developing more powerful and less toxic AMPs (Bechinger and Gorr, 2017; Seyfi et al., 2020). The net cationic charge and a high number of hydrophobic residues are two physical characteristics that many AMPs are sharing. The former promotes selectivity for negatively charged microbial cytoplasmic membranes, while the latter improves interactions with the fatty acyl chains of membrane phospholipids (Lohner, 2001; Yeaman and Yount, 2003; Nguyen et al., 2011; Schäfer and Wenzel, 2020).

Until now, most of AMPs exert their action by three ways, interaction with cell walls, cell membranes, and intracellular components (Li et al., 2021b). For the interaction with cell walls, the AMPs can target either peptidoglycan or lipid II on the cell wall resulting in inhibiting the biosynthesis of peptidoglycan and lipid II, which causes the lysis and death of cell due to the high cytoplasmic osmotic pressure and also allowing AMPs to anchor on the membrane components (Guilhelmelli et al., 2013). For example, vancomycin,

bacitracin and ramoplanin have been reported in view of their inhibition activity for the biosynthesis of peptidoglycan and lipid II (Fang et al., 2006; Allen, 2010; Malanovic and Lohner, 2016). For the interaction with cell membranes, the AMPs can target the lipopolysaccharide (LPS) from Gram-negative bacteria, resulting in penetration of the membrane barrier by neutralizing LPS, which causes the depolarization of the cell membrane potential and thus the death of bacteria (Sun and Shang, 2015). For instance, polymyxin and tyrocidine use this mechanism (Wenzel et al., 2018; Khondker et al., 2019). For the interaction with intracellular components, AMPs also can target some proteins, which leads to inhibition of the processes involving replication, transcription, and translation – essential for cell growth and multiplication (Corrêa et al., 2019). The antimicrobial peptide Tur1A inhibits the synthesis of bacterial proteins by attaching to the ribosome and preventing the transition from initiation to elongation (Mardirossian et al., 2018).

Scope of This Thesis

In this thesis, we isolated and characterized potential beneficial bacterial strains from healthy tomato plants. We investigated their antimicrobial biosynthetic potential and secondary metabolites production. Based on an interdisciplinary approach, combining *in vitro* and *in vivo* biological tests, including genome mining, high-performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and RNA-Seq technique the bioactive compounds involved in the interaction between beneficial bacteria and pathogens were identified and characterized. In **chapter 1** the research background on beneficial bacteria, plant-microbe interaction and biosynthesis of secondary metabolites are introduced. In **chapter 2**, we sequenced the genomes of

nine *Bacillus* strains and one *Paenibacillus* strain which were isolated from healthy tomato plants rhizosphere soil and their tissues. These strains showed outstanding biological activity against plant pathogens including bacterial, fungal, and oomycetal pathogens. In **chapter 3**, we characterized *Bacillus cabrialesii* BH5 for tomato grey mold biocontrol ability. Fengycin H (a new variant of fengycins) was isolated and identified to be responsible for cell wall integrity defects and finally the death of *Botrytis cinerea* hyphal cells. By transcription analysis, both strain BH5 and fengycin H can stimulate induced systemic resistance of tomato plants via the jasmonic acid signaling pathway and provide a strong biocontrol effect *in vivo*. In **chapter 4**, we isolated, identified and characterized medipeptins from *Pseudomonas* sp. EDOX. By LC-MS/MS analysis, medipeptins were identified as 22-amino-acid peptides. They showed biological activity against Gram-positive pathogens and Gram-negative pathogens. Furthermore, we investigated and characterized the mode of action of medipeptin A, which revealed that medipeptin A exerts its potent antimicrobial activity against Gram-positive bacteria via binding to both lipoteichoic acid (LTA) and lipid II as well as by forming pores in membranes. In **chapter 5**, we combined mass spectrometry, transcriptomic analysis, and *in vitro* bioactivity tests to study the bacteria-fungi interaction between *Priestia endophytica* FH5 and pathogen *Rhizoctonia solani*. We isolated and purified kurstakins produced by *P. endophytica* FH5, which showed biological activity against *R. solani* *in vitro*. Moreover, we showed that the expression of metabolic genes was altered during the interaction with *R. solani*. These studies highly improved our insights into the interaction between *P. endophytica* FH5 with the important fungal pathogen *R. solani* and the biocontrol potential for tomato damping-off.

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Chapter 2

Antimicrobial activity screening of rhizosphere soil bacteria from tomato and genome-based analysis of their antimicrobial biosynthetic potential

Lu Zhou¹, Chunxu Song^{1,2}, Zhibo Li¹ and Oscar P. Kuipers^{1*}

¹Department of Molecular Genetics, University of Groningen, Groningen, The Netherlands

²College of Resources and Environmental Sciences; National Academy of Agricultural Green Development; Key Laboratory of Plant-Soil Interactions, Ministry of Education, China Agricultural University, 100193 Beijing, China

** Corresponding author; o.p.kuipers@rug.nl*

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Abstract

Background: Tomato plant growth is frequently hampered by a high susceptibility to pests and diseases. Traditional chemical control causes a serious impact on both the environment and human health. Therefore, seeking environment-friendly and cost-effective green methods in agricultural production becomes crucial nowadays. Plant Growth Promoting Rhizobacteria (PGPR) can promote plant growth through biological activity. Their use is considered to be a promising sustainable approach for crop growth. Moreover, a vast number of biosynthetic gene clusters (BGCs) for secondary metabolite production are being revealed in PGPR, which helps to find potential anti-microbial activities for tomato disease control.

Results: We isolated 181 *Bacillus*-like strains from healthy tomato, rhizosphere soil, and tomato tissues. In vitro antagonistic assays revealed that 34 *Bacillus* strains have antimicrobial activity against *Erwinia carotovora*, *Pseudomonas syringae*; *Rhizoctonia solani*; *Botrytis cinerea*; *Verticillium dahliae* and *Phytophthora infestans*. The genomes of 10 *Bacillus* and *Paenibacillus* strains with good antagonistic activity were sequenced. Via genome mining approaches, we identified 120 BGCs encoding NRPs, PKs-NRPs, PKs, terpenes and bacteriocins, including known compounds such as fengycin, surfactin, bacillibactin, subtilin, etc. In addition, several novel BGCs were identified. We discovered that the NRPs and PKs-NRPs BGCs in *Bacillus* species are encoding highly conserved known compounds as well as various novel variants.

Conclusions: This study highlights the great number of varieties of BGCs in *Bacillus* strains. These findings pave the road for future usage of *Bacillus* strains as biocontrol agents for tomato disease control and are a resource arsenal for novel antimicrobial discovery.

Keywords: *Bacillus*; Plant Growth Promoting Rhizobacteria (PGPR); Genome mining;

Tomato; Rhizosphere; biosynthetic gene clusters; non-ribosomal synthesized peptides; polyketides; bacteriocins

Introduction

Tomato (*Solanum lycopersicum*) is the second most important vegetable crop worldwide after potato, based on the sizes of their growth areas (Olaniyi et al., 2010). However, tomato crops face serious threats of disease, partially due to the use of cultivars susceptible to diseases that are causing substantial production losses (Dias et al., 2017). The overuse of chemical pesticides has contaminated soils and has caused harmful effects on human beings (Asaka and Shoda, 1996b). Accordingly, putting biocontrol agents isolated from nature into the soil is environmentally friendly and useful for tomato crop disease control. One way to improve plant growth is by using plant growth-promoting rhizobacteria (PGPR), since PGPR have the ability to colonize the roots and express their plant growth promotion activities in the rhizosphere (Barea et al., 2005).

The rhizosphere, a narrow zone of soil that surrounds and is influenced by plant roots, gives home to an overwhelming variety of organisms, in particular microorganisms such as bacteria, fungi, oomycetes, archaea, protozoa and algae (Raaijmakers et al., 2009; Philippot et al., 2013). This complex microbial community has profound effects on plant growth since it facilitates nutrient absorption and provides health protection to plants (Lynch et al., 1990). Among all the microorganisms, PGPR has been largely described for their biocontrol capabilities. They can promote plant growth either indirectly by suppression of diseases with secreted antimicrobials or directly by the improvement of physiological metabolic processes such as N₂ fixation, phosphate

solubilization and IAA production (Whipps, 2001).

Among PGPR, the group of Gram-positive *Bacillus* strains has been studied less intensively, compared to widely used Gram-negative bacteria, like *Pseudomonas* strains (Silo-Suh et al., 1994). One of the most efficient Gram-positive bacteria that promote plant growth belongs to the genus *Bacillus*. *Bacillus subtilis* is used in agriculture to protect plants from several plant pathogens since it can either indirectly protect plants by inducing systemic resistance (ISR) against a broad range of pathogens or directly excrete antimicrobials (Ryu et al., 2004; Ongena et al., 2005; Choudhary and Johri, 2009; Wang et al., 2009). Besides, *Bacillus* species can produce hard, resistant endospores to allow them to resist adverse environmental conditions and permit easy formulation and storage of the commercial products (Perez-Garcia et al., 2011).

The *Bacillus* species offer a plethora of antagonistic compounds displaying a broad range of biological functions, which have good potential to be used as biocontrol agents for tomato disease control (Caulier et al., 2019). All the bioactive secondary metabolites are encoded by biosynthetic gene clusters (BGCs). Based on their products, BGCs are classified as ribosomally synthesized peptides (linear bacteriocin BGCs and ribosomally-produced posttranslationally modified peptides [RiPPs]), non-ribosomally synthesized peptides synthetases (NRPSs) BGCs and polyketide synthases (PKSs) BGCs (Crits-Christoph et al., 2018). Here, we set out to find novel BGCs in *Bacillus* strains which encode potentially active compounds to inhibit plant-pathogens. Based on genome mining, 10 selected (out of 181) promising *Bacillus* strains newly isolated from rhizosphere soils of healthy tomato plants and tissues were characterized with respect

to anti-pathogen activities. Subsequently, several novel BGCs were discovered, which have potential functions in tomato pathogens antagonism.

Methods

Sample Collection, Bacteria Isolation, and culture conditions

Healthy tomato plants (cultivar: Boludo) and their rhizosphere soil were carefully collected during spring (February 2017) from tomatoes grown in a garden in the village of Roden in the Netherlands and Almería in Spain, which were given to us by company Koppert and with their consent. The bacterial isolation was performed as described previously (Zhou et al., 2019). Briefly, 1 g rhizosphere soil was suspended in 9 ml of 10 mM sterilized MgSO_4 buffer. Then the suspension was diluted 10^3 - 10^6 times with 10 mM sterilized MgSO_4 buffer. After dilution, all the samples were heat-treated at 80 °C for 15 min. and subsequently spread on Lysogeny Broth (LB) agar plates. The plates were incubated at 28 °C for 24-48 h to obtain single colonies. For the isolation of endophytes, 1 g tomato leaves were surface-sterilized for 1 min. in 70% ethanol and 3 min. in 0.5% NaClO solution supplemented with one droplet Tween 80 per 100 ml solution and then rinsed 5 times with sterilized deionized water. After surface sterilization, the leaves were macerated in 9 ml of 10 mM sterilized MgSO_4 buffer with a sterilized mortar to obtain the suspension. The following steps were the same as isolation from rhizosphere soil. The surface-sterilization process was checked by spreading aliquots of the last rinsing deionized water on LB agar plates (if no organism growth was observed after 7 days, surface sterilization was considered to be successful). All the isolated strains were stored in 25% glycerol solution at -80 °C until further investigation.

Screening of Antimicrobial Activity

In vitro antagonistic activity assays were performed on dual culture plates as described before with slight modification (Chen et al., 2013). All the isolated strains were screened against different bacterial, fungal and oomycetal plant pathogens, such as *Erwinia carotovora*, *Pseudomonas syringae*; *Rhizoctonia solani*; *Botrytis cinerea*; *Verticillium dahliae* and *Phytophthora infestans*. All the isolated strains were tested with different pathogens in triplicates.

To test antibacterial activity, bacterial pathogens were mixed with pre-cooled LB agar media (around 55°C) at a final concentration of 1×10^6 cells/ml. Then the mixed media was poured into Petri dishes to obtain pathogen-fusion agar plates. 5 µl of 1×10^8 cells/ml overnight culture of each isolated strain was inoculated at the center of plates. All the plates were incubated at 28°C for 2 days before the clear halo surrounding the strain isolated was measured.

Antagonistic activity of isolated strains against *R. solani* was tested as follows. A 0.5-cm mycelium plug of 3-day-old *R. solani* was placed at the center of the 1/5th PDA plate, and 5 µl of 1×10^8 cells/ml overnight culture of each isolated strain was inoculated at a distance of 2 cm from the fungus. Plates were incubated at 28°C for 3 days and inhibition of fungal growth was recorded as the diameter of the inhibition zone (mm). As a control, LB media was used in place of the bacterial suspension.

Antagonistic activity determination of isolated strains against *B. cinerea*, *V. dahliae*, and *P. infestans* was performed similarly as the antibacterial activity assay. Spores of *B.*

cinerea and *V. dahliae* were collected respectively from 7-day-old and 20-day-old PDA plates with sterilized Mili-Q water by washing the mycelium. All the spores were counted using a Thoma chamber and were then mixed into 1/5th PDA media and adjusted to 1X10⁷ spores/ml. In addition, sporangia of *P. infestans* were harvested by washing the 30-day-old RSA plates with sterilized mili-Q water and then counted and mixed into 1/5th PDA media at the final concentration of 4000 sporangia/ml. Before mixing, the sporangia suspension was stimulated to release zoospores by chilling for 1-3 hours at 4 °C. Subsequently, the mixed 1/5th PDA media was poured into Petri dishes. 5 µl of 1X10⁸ cells /ml overnight culture of each isolated strain was inoculated at the center of plates. All the plates were incubated at 28°C. The clear halo surrounding each isolated strain was monitored and measured depending on the pathogens' growth rate.

Identification of bacterial strains

The isolated strains showing activity against pathogens were identified through partial 16S rRNA sequence homology analysis. The polymerase chain reaction (PCR) was carried out with bacterial-specific 16S rRNA primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') using genomic DNA as templates. The PCR (50 µl) contained following final concentrations: 10 µl of five-fold high fidelity Phusion buffer (Thermo Scientific), 0.4 mM of each of the four deoxynucleoside triphosphates (Thermo Scientific), 0.4 µM of each primer, 0.5 U of Phusion high fidelity DNA polymerase (Thermo Scientific), and ~50 ng of the isolated genomic DNA. The following thermal cycling scheme was used: initial denaturation at 98 °C for 3 min, 30 cycles of denaturation at 98 °C for 15 s, annealing at 56 °C for 15 s, extension at 72 °C for 50 s, followed by an additional extension step at 72 °C for 7 min. The PCR products were then purified with a

NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and sequenced at MacroGen Inc. The resulting 16S rRNA gene sequences were compared in a BLAST search to the NCBI database. Phylogenetic analysis was performed using the MEGA software package (Version X) (Kumar et al., 2018). The relationships between sequences were analyzed using the neighbor-joining method. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by analyzing 1,000 randomized data sets. The phylogenetic tree was visualized in iTOL version 4.4.2 (Letunic and Bork, 2019).

Genome Sequencing and Phylogenetic Analysis

Genomic DNA of isolates was extracted with a GenElute Bacterial Genomic DNA kit (Sigma) according to the manufacturer's protocol and sequenced at GATC Biotech (Germany) with an Illumina HiSeq sequencing system. The draft genomes were assembled and deposited in GeneBank. For the classification of species affiliations, dDDH and ANI values were calculated using the web tools TYGS (Meier-Kolthoff and Goker, 2019) and JSpeciesWS (Richter et al., 2016) respectively. Two genomes belonging to the same species would have a dDDH of at least 70%, which corresponds to at least 95% ANI (Orata et al., 2018). The whole genomes were then compared with other reference genomes using the GEGENEES tool (Agren et al., 2012) based on a fragmented nucleotides alignment with a setting of 200/100. A phylogenetic tree was generated with iTOL version 4.4.2 (Letunic and Bork, 2019) and Splitstree (Huson and Bryant, 2006).

Antimicrobial Compounds Mining among 10 Isolated Strains

For identification of biosynthesis gene clusters (BGCs) in isolated strains, each draft genome was assembled into a pseudomolecule using Medusa web server

(<http://combo.dbe.unifi.it/medusa>) (Bosi et al., 2015) based on multiple closely related strains as references. And then all the pseudomolecules were send to antiSMASH(Blin et al., 2019) and BAGEL4(van Heel et al., 2018) for BGCs mining.

Metabolite BGC Network Analysis among *Bacillus* strains

The complete genomes of 555 *Bacillus* strains from 60 species of *Bacillales* were downloaded from Genbank and analyzed by antiSMASH 5.0 (Blin et al., 2019). The similarity network between BGCs was calculated with BiG-SCAPE (<https://git.wageningenur.nl/medema-group/BiG-SCAPE>) (Yeong, 2016). A program that constructs sequence similarity networks of BGCs and groups them into Gene Cluster Families (GCFs) (Yeong, 2016). To visualize, the distance matrix between BGCs generated by BiG-SCAPE was exported and annotated in Cytoscape v3.7.0 (<http://www.cytoscape.org/>) (Saito et al., 2012). Default parameters were used for all software unless noted.

Results

Isolation of Bacteria and *In Vitro* Antagonistic Assay

A total of 181 *Bacillus*-like strains were isolated from healthy tomato rhizosphere soil and tomato plant tissue collected in either the Netherlands or Spain. Among them, 28 endophytic strains were isolated from healthy tomato plant tissues collected in Spain. 74 and 79 rhizosphere bacteria strains were isolated from tomato plants collected in Spain and the Netherlands, respectively. In order to identify potential PGPR strains, all the *Bacillus*-like strains were preliminarily screened by *in vitro* antagonistic activity against six major tomato plant pathogens, i.e. *Erwinia carotovora* (Alippi et al., 1997), *Pseudomonas*

syringae (Buell et al., 2003), *Rhizoctonia solani* (Asaka and Shoda, 1996a), *Botrytis cinerea* (El Oirdi et al., 2011), *Verticillium dahliae* (Bhat and Subbarao, 1999), and *Phytophthora infestans* (Legard et al., 1995). The results revealed that 34 *Bacillus*-like strains could inhibit different bacterial, fungal and oomycete plant pathogens growth on plates (Figure 1 and 2).

Figure 1. Neighbor-joining tree based on 16S rRNA genes of 34 isolated strains showing

antagonistic activity against tomato plant bacterial, fungal and oomycetal pathogens.

Red represents the inhibition halo size of bacterial pathogens and blue indicates the inhibition halo size of fungal and oomycetal pathogens. The red and blue scale bar represent the radius of inhibition halo observed (mm).

Figure 2. Pictures of antagonistic assay for each kind of pathogens in plates

We found that 34 *Bacillus*-like strains were distributed between three large major clusters of the neighbor-joining tree based on 16S rRNA genes. In the first cluster, strain EDO6 was clustered within the genus of *Paenibacillus*, which was related to type strain *Paenibacillus xylanexedens* B22a, with the percent identity value above 99.6% on 16S rRNA gene sequence. 10 isolated strains were grouped in the second cluster and were closely related to the type strains *Bacillus endophyticus* 2DT and MF126, *Bacillus firmus* IAM 12464, *Bacillus megaterium* NBRC 15308, or *Bacillus aryabhattai* B8W22. The third cluster consisted of 23 isolated strains. They were tightly related to reference strains *Bacillus cereus* ATCC 14579, *Bacillus velezensis* FZB42 or SQR9, *Bacillus subtilis* BSn5 and NCD-2.

Of all strains, seven strains (TH16, FH17, EH6, DH15, BH4, BH5 and BH6) showed inhibition on all pathogens. Among them, four strains (TH16, FH17, BH5 and BH6),

showing the biggest inhibition halo on all pathogens, were selected to sequence their genomes for further research. In addition, three strains (EH11, EDO6 and FH5), showing the highest inhibition activity (inhibition halo size >9.75mm against *P. infestans*), were also selected for genome sequencing. Besides, two strains (EH2 and EH5), showing the highest inhibition activity (inhibition halo size > 8.5 mm) against *B. cinerea*, were selected for genome sequencing as well. Strain DH12 was also selected for genome sequencing, because of the large inhibition halo size (>3.38 mm) measured on the plates against *Pseudomonas syringae*. In summary, a total of 10 strains (BH5, BH6, DH12, EH2, EH5, EH11, FH5, FH17, TH16 and EDO6) were genome sequenced for further research.

Genome Sequencing and Phylogenetic analysis

The genomes of 10 isolated strains were sequenced, assembled and annotated as described in a previous study (Zhou et al., 2019). Based on whole genome phylogenetic analyses, the 10 *Bacillus* strains were clustered into five clades as presented in Figure 3. All of them were tightly clustered together with reported PGPR strains from the *Bacillus* class, such as *B. subtilis* Bsn5, *B. velezensis* FZB42 and *P. polymyxa* E681. This suggests that they probably can promote plant growth as well, which needs to be further investigated. Moreover, to classify strains at the species level, Average Nucleotide Identity (ANI) and digital DNA-DNA Hybridization (dDDH) values were determined (Orata et al., 2018) (Additional file 1 Table S1). Strains DH12, EH2, EH5, and EH11 were exhibiting $\geq 98.21\%$ ANI and $\geq 86.70\%$ dDDH compared with reference genome *B. subtilis* Bsn5, therefore they were identified as *B. subtilis* species. Strains FH17 and TH16 were identified as *B. velezensis*, based on $\geq 98.14\%$ ANI and $\geq 85.30\%$ dDDH compared with reference genome of *B. velezensis* FZB42. Strains BH5 and BH6 were classified into *B.*

cabrialesii species because of exhibiting 96.60% ANI and 73.40% dDDH compared with the reference genome of *B. cabrialesii* TE3. Strain FH5 was identified as *B. endophyticus* based on 96.35% ANI and 73.40% dDDH compared with reference genome *B. endophyticus* KCTC 13922. Strain EDO6 could not be classified at the species level due to the low ANI and dDDH values (93.88% ANI and 57.60% dDDH), even compared with the closest species *P. xylanexedens* PAMC 22703, so we will name it *Paenibacillus* sp. EDO6.

Figure 3. Phylogenetic position of 10 isolated *Bacillus* and *Paenibacillus* strains with high significant antagonistic activity against tomato pathogens. A maximum likelihood (ML) tree was constructed based whole genome sequences analysis using Gegenee.

Biosynthesis Gene Cluster (BGC) Mining

A total of 120 BGCs were found, averaging 12 clusters per genome. All the BGCs were designated as those encoding NRPSs, PKSs, terpenes, hybrid NRPS/PKSs, bacteriocins, RiPPs and others (Table 1A and Additional file 1 Table S2). The BGCs encoding surfactin (Koumoutsis et al., 2004), fengycin (Koumoutsis et al., 2004), bacillibactin (Chen et al., 2009), subtilosin A (Kawulka et al., 2004), bacillaene (Moldenhauer et al., 2007), macrolactin (Schneider et al., 2007), difficidin (Chen et al., 2006), and subtilin (Klein et al., 1993) were discovered in the genomes. Besides, some BGCs encoding unknown compounds, were also identified (Table 1B). Most of the unknown BGCs (76.47%) are PKSs BGCs, which cannot be assigned to any known compounds. 73.07% bacteriocins BGCs encodes potential novel peptides. 27.78% and 27.27% of NRPSs and Hybrids BGCs are still unknown. These findings provide a great opportunity of new bioactive compounds discovery.

Table 1. Distribution of BGC totals in 10 isolated strains (A) and percentages of BGCs encoding unknown compounds identified in genome sequence (B).

A.							
Strains	Predicted	Hybrid					
	BGCs	NRPS	PKS	NRPS/PKS	Terpene	Bacteriocin	Other
<i>Bacillus cabrialesii</i> BH5	12	4	1	1	2	3	1
<i>Bacillus cabrialesii</i> BH6	12	4	1	1	2	3	1
<i>Bacillus subtilis</i> DH12	12	4	1	1	2	3	1
<i>Bacillus subtilis</i> EH2	10	3	1	1	2	2	1
<i>Bacillus subtilis</i> EH5	11	3	1	1	2	3	1
<i>Bacillus subtilis</i> EH11	12	4	1	1	2	3	1
<i>Bacillus endophyticus</i> FH5	10	2	1	1	2	3	1
<i>Bacillus velezensis</i> FH17	15	5	4	1	2	1	2
<i>Bacillus velezensis</i> TH16	12	4	4	1	1	1	1
<i>Paenibacillus</i> sp. EDO6	14	3	2	2	1	4	1
B.							
BGC Types	Total BGCs	% Unknown	Known compounds				
NRPSs	36	27.78	surfactin(8 BGCs), fengycin(8 BGCs), bacillibactin(10 BGCs)				
PKSs	17	76.47	macrolactin (2 BGCs), difficidin (2 BGCs)				
Hybrids	11	27.27	bacillaene (8 BGCs)				
Bacteriocin	26	73.07	subtilin (2 BGCs), subtilosin A (6 BGCs)				

Novel NRPs and PKs BGCs identified from the 10 strains

The majority of BGCs could be assigned to known compounds, whereas 5 clusters represented probably novel NRPs and PKs-NRPs hybrid BGCs for which no or low similarity BGCs could be identified in the MIBiG (Kautsar et al., 2020) database (Figure 4).

Figure 4. Novel Biosynthetic Gene Clusters (BGCs) identified from the isolated *Bacillus* and *Paenibacillus* strains. (a) an NRPs BGC discovered in *B. endophyticus* FH5. (b) a Type I PKs-NRPs hybrid BGC found in *B. endophyticus* FH5. (c. d) two trans-AT PKs-NRPs hybrid BGCs harbored by *Paenibacillus* sp. EDO6. (e) an NRPs BGC found

in both *B. velezensis* FH17 and TH16.

Two novel gene clusters were identified from *B. endophyticus* FH5. One NRPs (Figure 4a) BGC consists of three genes and has a total size of 25 kb. Three genes are encoding 24 domains, which includes 7 condensation (C) domains, 7 adenylation (A) domain, 7 thiolation (T) domain, 2 epimerization (E) domain and 1 thioesterase (TE) domain. All the domains are essential components in this BGC and catalyze primary formation of a lipopeptide product. This BGC is showing no similarity to any known BGCs reported. The other one (Figure 4b) is a Type I PKs-NRPs hybrid BGC with a size of approximately 30 kb. The PKs module consists of a ketosynthase (KS) domain, a acyltransferase (AT) domain, an acyl carrier protein (ACP) domain and a terminal reductase (TD) domain. It likely incorporates the polyketide moiety of malonyl-CoA, while the NRPs modules incorporate six amino acid residues. Based on antiSMASH analysis, only 28% genes show similarity to the known paenilamicin BGC. Paenilamicin (Garcia-Gonzalez et al., 2014), synthesized by pam BGC from *Paenibacillus larvae* DSM25430, has antibacterial and antifungal activity. The pam cluster consists of five NRPs genes, two Type I PKs genes, and two Type I PKs-NRPs hybrid genes, and has a size of ~60 kb. In contrast, the Type I PKs-NRPs hybrid BGC identified in *B. endophyticus* FH5 consists of only three NRPS genes and one Type I PKs gene. All of them differ from the pam cluster of *Paenibacillus larvae* DSM25430.

In the genome of *Paenibacillus* sp. EDO6, two novel trans-AT PKs-NRPs hybrid gene clusters (cluster 13 and cluster 12) were discovered, which have the sizes of almost 35 kb and 28 kb, respectively (Figures 4c and 4d). The order and domain of the genes of

both hybrid clusters differ from each other. Specifically, Cluster 13 has an additional dehydratase domain variant (Dht) playing an important role during polyketide biosynthesis through the dehydration of the nascent polyketide intermediate to provide olefins (Fiers et al., 2016), which cannot be found in cluster 12. In addition to the differences observed at the domain level of core biosynthetic genes, regulator and transporter genes are also different. Moreover, only 33% and 21% of the genes of cluster 13 and cluster 12 exhibit similarity to known pellasoren and xenocoumacin BGCs respectively. Pellasoren (Jahns et al., 2012) was isolated from *myxobacterium*, which has shown to possess potential anti-cancer activity. The known pellasoren BGC, is a Type I PKs-NRPs hybrid cluster identified from *Sorangium cellulosum* So ce38 and consists of six genes of Type I PKs and one single gene of NRPs as compared to the trans-AT PKs-NRPs hybrid gene (cluster 13) of *Paenibacillus* sp. EDO6, which in turn consists of four trans-AT PKs genes and one trans-AT PKs-NRPs hybrid gene. Xenocoumacin (Reimer et al., 2009) is the main anti-bacterial and anti-fungal compound produced by *Xenorhabdus nematophila*. The known xenocoumacin BGC, also being a Type I PKs-NRPs hybrid cluster, which was identified from *Xenorhabdus nematophila* ATCC 19061, consists of four genes of Type I PKs and two genes of NRPs, whereas cluster 12 from *Paenibacillus* sp. EDO6 consists of one single trans-AT domain gene, one gene of trans-AT PKs and one gene of trans-AT PKs-NRPs hybrid.

One novel NRPs BGC was discovered both in *B. velezensis* FH17 and TH16 (Figure 4e). This BGC contains seven genes with a size of approximately 33 kb. Whereas seven modules are only encoded by two core biosynthetic genes, seven amino acids are incorporated into the final product. This BGC shows no similarity to any known clusters.

Furthermore, a single heterocyclization (Cy) domain in the first module is found.

Novel Ribosomally synthesized and Post-translationally modified Peptides (RiPPs) identified in the 10 strains

A total of nine novel bacteriocin BGCs were identified from the 10 strains (Figure 5). All of them are belong to RiPPs (less than 10 kDa). These peptides are ribosomally synthesized, and undergo posttranslational modifications (PTMs), resulting in different structures and properties, mainly showing anti-bacterial activity against closely related producer strains (Zhao and Kuipers, 2016).

Figure 5. Novel bacteriocin Biosynthetic Gene Clusters (BGCs) identified from the isolated

***Bacillus* and *Paenibacillus* strains.** (a. b) two different class I lanthipeptide BGCs discovered in *B. subtilis* DH12 and *Paenibacillus* sp. EDO6 respectively. (c) class II lanthipeptide BGC found in both *B. cabrialesii* BH5 and BH6. (d. e) two different class II lanthipeptide BGCs discovered in *B. subtilis* EH5 and *B. endophyticus* FH5 respectively. (f. g) two different class III lanthipeptide BGCs found in *B. subtilis* EH2, *B. velezensis* TH16 respectively. (h) two different lasso peptide BGCs discovered in *Paenibacillus* sp. EDO6 and *B. endophyticus* FH5 respectively.

Two novel gene clusters were identified as class I lanthipeptide BGCs. One lanthipeptide BGC was identified from both *B. subtilis* DH12 and EH11 with a size of ~6 kb (Figure 5a). This BGC consists of four genes. The precursor peptide contains 59 amino acids, which shows no similarity to any known bacteriocins. Another one lanthipeptide BGC (Figure 5b) was identified from *Paenibacillus* sp. EDO6 with a size of ~9 kb. This BGC contains seven genes. The precursor peptide encoded by the core biosynthetic gene contains 59 amino acids, which also shows no similarity to any known bacteriocins.

Three novel BGCs were identified as class II lanthipeptide BGCs. All of them belong to two-component lanthipeptides consisting of two peptides. The individual peptides of two-component lanthipeptides only have little or no antimicrobial activity, but the two peptides act in synergy to exhibit significantly higher activity in equimolar concentrations (Xin et al., 2016). Both *B. cabrialesii* BH5 and BH6 harbor the same two-component lanthipeptide BGC (Figure 5c). It consists of six genes with a size of ~9 kb. This BGC has 70% of genes showing similarity to staphylococcin C55 α/β BGC (Navaratna et al., 1999). The presursors of two core biosynthetic genes (α and β) of this BGC identified contain 65

and 67 amino acids respectively. The C terminus (from C36 to K65) of the α precursor is belonging to the plantaricin C family of lantibiotics with a identity of 83.33% to the known peptide staphylococcin C55 α . Whereas the C terminus (from I38 to C67) of the β precursor shows 62.07% identity to lacticin 3147 A2 (Ryan et al., 1999). The second novel class II lanthipeptide BGC was discovered from *B. subtilis* EH5 (Figure 5d). This BGC has six genes with a length of ~ 9 kb. The presursors of two core peptide genes (α and β) contain 65 and 67 amino acids respectively. It is also showing 70% gene sequence similarity to staphylococcin C55 α/β BGC. The C terminus (from C36 to C64) of the α precursor has a similarity of 79.31% to the known peptide staphylococcin C55 α and the C terminus (from W38 to C63) of the β precursor is showing 72% identity to lacticin 3147 A2. The third BGC was identified from *B. endophyticus* FH5 (Figure 5e). It is comprised of nine genes with a size of ~ 10 kb. Its precursors of two peptides (α and β) contain 58 and 54 amino acids respectively. There is no similarity found to any known BGCs. The C terminal region (from A28 to C58) of the α precursor has a similarity of 53.33% to the known peptide plantaricin W α (Holo et al., 2001) and the C terminus (from A23 to D54) of the β precursor is showing 56.25% identity to haloduracin β (Lawton et al., 2007). Furthermore, the precursor β in this potential novel BGC found in *B. endophyticus* FH5 has four replicates, indicating potential high amount production of β peptide.

Two novel gene clusters were identified as class III lanthipeptide BGCs. This Class contains RiPPs that are modified by the multifunctional enzymes LanKC. LanKC firstly phosphorylates the Ser/Thr residues in the substrate peptide and then similarly catalyzes modification of the substrate to form the final product, as the class II lanthipeptide LanM enzyme (Zhang et al., 2012). The one identified from *B. subtilis* EH2

contains ten genes with a size of ~8 kb (Figure 5f). No similarity was found to any known BGCs. The full precursor contains 58 amino acids. The predicted cleavage site by antiSMASH is between T27 and G28. The C terminus (from G28 to N58) of the precursor has no identity to any known RiPPs. The other class III lanthipeptide BGC is harbored by *B. velezensis* TH16 (Figure 5g). This one contains five genes with a length of ~5 kb. The core biosynthetic gene encodes a 45-amino acid precursor peptide. 35% genes of this BGC show similarity to locillomycin (Luo et al., 2015), which is a cyclic lipopeptide (NRPs) discovered from *B. subtilis* 916. The predicted cleavage site is between V21 and D22 by antiSMASH and the C terminus (from D22 to C45) of the precursor has no identity to any known RiPPs.

Two novel lasso peptide BGCs were identified from the genomes of *Paenibacillus* sp. EDO6 and *B. endophyticus* FH5. The one from *Paenibacillus* sp. EDO6 contains eight genes with a size of ~8 kb (Figure 5h). It shows that gene sequences are 60% similar to that of the paeninodin BGC (Zhu et al., 2016). The precursor peptide contains 45 amino acids. The predicted cleavage site is between M22 and A23. The core peptide (from A23 to S45) shows 33.3% identity to the paeninodin (Zhu et al., 2016) from *P. dendritiformis* C454. Another novel lasso peptide BGC was mined from *B. endophyticus* FH5 (Figure 5i). This BGC comprised of six genes. It is showing 80% genes similarity to paeninodin. Its precursor peptide contains 45 amino acids. The cleavage site is between M20 and A21. The core peptide (from A21 to S45) has 76% identity to the paeninodin.

Large-scale genome-based analysis of the bioactive potential of *Bacillus*

Lipopeptides produced by the *Bacillus* genus are involved in the biocontrol mechanisms

of plant pathogens (Ongena and Jacques, 2008). To gain a general overview of BGCs distributed in the genomes of *Bacillus* genus, the diversity of BGCs in the genomes of *Bacillus* isolated was investigated. A total of 9459 BGCs were predicted and identified, which included NRPs (2377 BGCs), RiPPs (1564 BGCs), Type I PKs (517 BGCs), PKs-NRPs hybrids (309 BGCs), PKs (including Trans AT-PKs and Type III PKs) (1369 BGCs), Terpene (970 BGCs), Saccharide (62 BGCs) and Others (2291 BGCs).

Figure 6. The predicted Biosynthetic Gene Clusters (BGCs) Similarity network of 555 *Bacillus* strains genomes showing their diversity, distribution. The lines between the nodes represent genes shared between BGCs.

The similarity network of predicted BGCs revealed that a large number of BGCs are present in *Bacillus* strains, and are distributed throughout different kinds of secondary metabolites (Figure 6). Based on our investigation, some of the NRPs BGCs were conserved among the BGCs identified in the *Bacillus* species. 259 out of 2377 (10.85%) NRPs BGCs were encoding surfactin, 330 (13.88%) BGCs were encoding bacillibactin, 110 (4.63%) NRPs BGCs were encoding fengycin, 158 (6.65%) NRPs BGCs were encoding

petrobactin (Cendrowski et al., 2004). And 38 (1.60%) NRPs BGCs were encoding lichenysin (Veith et al., 2004). Thus, a total of ~38% of the NRPs BGCs are correlated to already reported compounds. Additionally, most of PKs-NRPs hybrid BGCs (67.64%) were encoding bacillaene. Unlike the well-described NRPs and PKs-NRPs hybrid BGCs, the PKs BGCs were mostly attributed to unknown products with the exception of macrolactin (Schneider et al., 2007) and difficidin (Chen et al., 2006). Notably, 1357 out of 1564 (87.76%) RiPPs BGCs were also unknown. Overall, the distribution of known and unknown BGCs vary dramatically across the different kinds of metabolites in *Bacillus* species, in which the NRPs BGCs are the most abundant ones, comprising 2377 BGCs. Many of them are conserved and already characterized, but still a large number of unknown NRPs BGCs are identified for further study.

Discussion

Bacillus strains attract more and more attention due to their ability to produce hard, resistant endospores and antibiotics which have the potential to be used as biocontrol agents. In this study, We found that 34 *Bacillus* strains (out of 181 *Bacillus*-like strains) have antagonistic activity against six major tomato plant pathogens (*E. carotovora*, *P. syringae*, *R. solani*, *B. cinerea*, *V. dahliae*, and *P. infestans*). These results suggest that these strains have potentials for tomato diseases control. 16S rRNA gene sequences comparison of 34 *Bacillus* strains highlighted the diversity of *Bacillus* strains exhibiting antagonistic activity against plant pathogens, suggesting that strains from rhizosphere soil encounter different plant pathogens, resulting in the acquisition of antagonistic activity during evolution. The 34 *Bacillus* strains were clustered into three large clades according to their 16S rRNA sequences. By comparing the inhibition activity on the

growth of each plant pathogen between these 34 strains, the most strongly inhibitory strains were closely related to *B. subtilis* and *B. velezensis*, suggesting that strains from *B. subtilis* and *B. velezensis* species possess strain-specific clusters of genes related to the biosynthesis of secondary metabolites, which play significant roles in pathogen suppression. In addition, among the 10 strains selected, two strains identified as *B. cabrialesii* BH5 and BH6 showed a growth reduction against all six major tomato plant pathogens (Figure 1). This finding is the first record of this species that could exhibit antagonistic activity against tomato pathogens, which serves as a basis to further identify and characterize the interaction mechanisms between species *B. cabrialesii* and tomato pathogens.

Based on the genome mining, a total of 14 novel BGCs were revealed from the genomes of 10 sequenced strains. Five novel clusters were identified as NRPs and PKs-NRPs hybrid BGCs. These categories of BGCs encode non-ribosomally synthesized peptides synthetases (NRPSs) and hybrid polyketides synthases and non-ribosomally synthesized peptides synthetases (PKS-NRPSs), which are modular multienzymes. NRPSs construct peptides from amino acids and PKS-NRPSs construct hybrid molecules from acyl-CoA moieties together with amino acids (Weissman, 2015). So far, some known bioactive compounds identified from *Bacillus* strains belong to these modular biosynthetic compounds, such as surfactin (Koumoutsis et al., 2004), iturin (Tsuge et al., 2001), bacillomycin (Koumoutsis et al., 2004), fengcin (Koumoutsis et al., 2004) and difficidin (Chen et al., 2006). All of them are antimicrobials which can be used for biocontrol in agriculture. As the economic loss caused by plant diseases is increasing in recent years, it is worthwhile to investigate the compounds produced by the five novel modular BGCs

identified. Due to the antagonistic assays *in vitro*, we speculate some of them have antibacterial and (or) antifungal activities. This needs to be characterized by experiments in the future. Especially, the novel NRPs BGC identified from *B. velezensis* FH17 and TH16 possess a single heterocyclization (Cy) domain in the first module, which could modify cystine (Cys) to form a thiazoline ring. This domain first catalyzes amide bond formation, and then the intramolecular cyclodehydration between the side chain of the first amino acid (Cys) and the backbone carbonyl carbon takes place to form a thiazoline ring (Bloudoff et al., 2017). This ring is important for the structure and function of this lipopeptide product. So far, many well-known drugs for anti-microbial and anti-cancer activity exhibit thiazoline rings (Kumawat, 2018), such as Sulfathiazole (anti-microbial drug), Ritonavir (anti-retroviral drug), Tiazofurin (anti-neoplastic drug) and Abafungin (anti-fungal drug) (Rouf and Tanyeli, 2015). These findings point to the potential anti-microbial activity of the compounds produced by this novel BGC in *B. velezensis* FH17 and TH16. Moreover, nine novel RiPPs BGCs were discovered. They are categorized into lanthipeptide I/II/III and lasso peptide BGCs. Lanthipeptides (also called lantibiotics for those with antibacterial activities) are ribosomally synthesized post-translationally modified peptides having thioether cross-linked amino acids, lanthionines, as a structural element (Ongey and Neubauer, 2016). They have potentials to be used as therapeutics. Subtilin, a lanthipeptide I, is one of the most studied bacteriocins from the *Bacillus* strains (Caulier et al., 2019). It is synthesized by *spa* BGC which is possessing strong antibiotic activities (Barbosa et al., 2015). Mersacidin, is produced by *mrs* BGC in *Bacillus* sp. HIL Y-85/54728 which is belonging to lanthipeptide II. It has activity against Gram-positive bacteria including *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecium* (Barbosa et al., 2015). However, to date, only Lanthipeptide I and II have been

reported from *Bacillus* strains. Here, we report two class III lanthipeptide BGCs from *Bacillus* strains (Figure 5f and 5g). Unlike other class III lanthipeptides, the one from *B. velezensis* TH16 only consists of one lanthionine/labionin moiety instead of two. Both *in vivo* and *in vitro* maturation of these peptides have value to be investigated in the future. With an increasing number of genomes, more and more lanthipeptide BGCs are certainly discovered, but only a few of them have been characterized by experimental research. Lasso peptides contain a macrocyclic linkage between an Asp or Glu side chain to the N-terminus of the core peptide. The C-terminal tail is threaded through the macrocycle, giving a lariat topology for which the lasso peptides are named (Tietz et al., 2017). Until now, some lasso peptides are reported with antimicrobial activity. i.e. lariatrin (Inokoshi et al., 2012), lassomycin (Gavrish et al., 2014) and microcin J25 (Wilson et al., 2003). However, we can not predict the compounds produced by these nine novel lanthipeptides and lasso peptides BGCs are antimicrobials or not, which also need to be investigated by further experiments. These 14 novel BGCs identified from *Bacillus* strains appear to have different NRPs modules, precursor peptides or extra genes that might lead to modification on the biosynthetic pathway, changing the compound's structure and their activity. Therefore, these novel BGCs are of great interest for antimicrobial activity and novel drug discovery. The expression of BGCs into biological active compounds, providing the real biocontrol effect of *Bacillus* sp., is still the main challenge. So far, it remains difficult to predict the exact compound products from genome sequence data only. Exploring the biosynthetic capacity of *Bacillus* sp. by genome mining in combination with advanced mass spectrometry techniques provide feasible solution for future compounds identification and characterization.

The result of large-scale genome mining of bioactive potential of *Bacillus* shows the distribution of known and unknown BGCs varying dramatically across the different kinds of metabolites in *Bacillus* species. NRPs and PKs-NRPs BGCs in *Bacillus* species are encoding highly conserved known compounds as well as various novel variants. These findings are consistent with Kirk J. Grubbs (Grubbs et al., 2017) reported. They found that majority of *Bacillus* natural products are comprised of a small set of highly conserved, well-distributed, known natural product compounds. The genus *Bacillus* is well known for the natural products with antibacterial and antifungal activities, which has a strong potential to be applied to agriculture for plant diseases control (Mohamad et al., 2018). Therefore, novel antimicrobials discovery is in need of identification and characterization of novel BGCs in *Bacillus* strains.

Conclusions

This work showed that 10 *Bacillus* and *Paenibacillus* strains, selected from 181 isolated *Bacillus*-like strains from the rhizosphere soil of healthy tomato plants and their tissues, have strong *in vitro* antagonistic activity against tomato bacterial, fungal and oomycetal pathogens. Based on genome mining, we identified a large number of BGCs from their genome sequences encoding known and unknown compounds, which form a great source for pharmaceutical compound discovery. Furthermore, a total of 14 novel BGCs were characterized in detail, including 2 NRPs, 3 PKs-NRPs hybrid and 9 RiPPs BGCs. In addition, from the large-scale bioinformatics analysis of the genomes from *Bacillus* genus, we found that NRPS and PKS-NRPS BGCs resources hidden in *Bacillus* species are frequently encoding highly conserved known compounds including surfactin, fengycin, bacillibactin, petrobactin, lichenysin and bacillaene.

Abbreviations

PGPR: plant growth promoting rhizobacteria; BGCs: biosynthetic gene clusters; ISR: inducing systemic resistance; NRPSs: non-ribosomally synthesized peptides synthetases; PKSs: polyketides synthases; NRPs: non-ribosomally synthesized peptides; PKs: polyketides; PKS-NRPSs: hybrid polyketides synthases and non-ribosomally synthesized peptides synthetases; RiPPs: ribosomally synthesized and post-translationally modified peptides; PTMs: posttranslational modifications; C: condensation; A: adenylation; T: thiolation; E: epimerization; TE: thioesterase; KS: ketosynthase; AT: acyltransferase; ACP: acyl carrier protein; TD: reductase; DHT: dehydratase domain variant; Cy: heterocyclization

Availability of data and materials

The whole genome data are available at DDBJ/EMBL/GenBank under the bioproject accession PRJNA503984 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA503984/>)

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Authors' contributions

LZ and OPK conceived the study and designed experiments. LZ and CS conducted the experiments. LZ performed the bioinformatic analysis and wrote the draft manuscript.

LZ, CS, ZL and OPK corrected the manuscript. All authors have read and approved the manuscript.

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Supplementary data

Table S1. Digital DNA-DNA Hybridization (dDDH) values (in upper triangle) and Average Nucleotide Identity (ANI) values (in lower triangle) amongst different strains

	<i>Bacillus cabrialesi</i> BH5	<i>Bacillus cabrialesi</i> BH6	<i>Bacillus subtilis</i> DH12	<i>Bacillus subtilis</i> EH2	<i>Bacillus subtilis</i> EH5	<i>Bacillus subtilis</i> EH11	<i>Bacillus subtilis</i> NCD-2	<i>Bacillus cabrialesi</i> TE3	<i>Bacillus subtilis</i> Bsn5	<i>Bacillus velezensis</i> FH17	<i>Bacillus velezensis</i> TH16	<i>Bacillus velezensis</i> DSM7	<i>Bacillus velezensis</i> FZB42	<i>Bacillus velezensis</i> SQR9	<i>Bacillus endophyticus</i> FH5	<i>Bacillus endophyticus</i> KCTC 13922	<i>Pseudobacillus</i> sp. EDO6	<i>Pseudobacillus polymyxa</i> EBB1	<i>Pseudobacillus polymyxa</i> SC2	<i>Pseudobacillus sydenhensis</i> PAMC 22703
<i>Bacillus cabrialesi</i> BH5		100.00	47.20	47.40	47.30	47.20	47.40	73.40	47.40	20.60	20.70	20.70	20.80	20.70	20.60	20.60	21.20	26.60	26.10	26.50
<i>Bacillus cabrialesi</i> BH6	100.00		47.10	47.40	47.30	47.10	47.40	73.40	47.40	20.60	20.70	20.70	20.80	20.70	20.50	20.60	21.10	26.50	26.10	26.50
<i>Bacillus subtilis</i> DH12	91.94	91.94		85.30	91.40	99.80	85.00	47.60	87.70	20.40	20.40	20.50	20.50	20.50	20.80	20.50	21.40	26.10	26.10	27.30
<i>Bacillus subtilis</i> EH2	92.08	92.09	98.17		85.80	85.30	84.70	47.80	86.90	20.40	20.40	20.50	20.60	20.50	21.90	20.80	21.40	25.90	26.00	27.30
<i>Bacillus subtilis</i> EH5	91.88	91.88	98.81	98.04		91.40	84.80	47.60	86.70	20.30	20.40	20.50	20.60	20.50	20.40	20.80	21.40	26.00	26.00	27.30
<i>Bacillus subtilis</i> EH11	91.93	91.93	99.96	98.03	98.86		84.90	47.60	87.70	20.40	20.40	20.50	20.60	20.50	20.80	20.50	21.50	26.30	26.30	27.50
<i>Bacillus subtilis</i> NCD-2	92.07	92.07	98.14	98.01	98.07	98.14		47.80	84.70	20.70	20.70	20.90	20.80	20.90	24.70	24.10	26.20	27.20	27.50	28.50
<i>Bacillus cabrialesi</i> TE3	96.60	96.60	92.20	92.12	92.22	92.20	92.17		47.80	20.40	20.60	27.40	28.70	28.10	20.90	21.40	21.20	26.70	26.10	26.60
<i>Bacillus subtilis</i> Bsn5	91.98	91.99	98.46	98.21	98.37	98.46	97.96	92.20		20.40	20.50	20.80	20.70	20.70	25.10	23.90	26.20	27.70	27.90	28.90
<i>Bacillus velezensis</i> FH17	76.42	76.42	76.12	76.06	76.08	76.11	76.17	76.22	76.06		84.70	55.50	85.30	84.20	21.60	21.60	26.00	28.40	28.70	32.00
<i>Bacillus velezensis</i> TH16	76.47	76.45	76.05	76.14	76.07	76.05	76.21	76.27	76.07	98.01		55.40	90.10	85.90	22.40	21.70	26.80	28.50	28.70	32.60
<i>Bacillus velezensis</i> DSM7	76.74	76.74	76.37	76.40	76.35	76.37	76.39	76.50	76.39	93.85	93.82		56.20	55.60	26.20	24.80	31.80	29.20	29.90	31.20
<i>Bacillus velezensis</i> FZB42	76.72	76.72	76.31	76.34	76.31	76.31	76.40	76.53	76.25	98.14	98.17	93.36		86.80	26.70	25.80	30.00	29.50	30.00	31.70
<i>Bacillus velezensis</i> SQR9	76.39	76.39	76.12	76.16	76.08	76.10	76.17	76.16	76.04	97.72	98.00	93.08	98.06		23.10	23.90	29.20	29.00	29.40	31.30

	<i>Bacillus cabrialesi</i> BH5	<i>Bacillus cabrialesi</i> BH6	<i>Bacillus subtilis</i> DH12	<i>Bacillus subtilis</i> EH2	<i>Bacillus subtilis</i> EH5	<i>Bacillus subtilis</i> EH11	<i>Bacillus subtilis</i> NCD-2	<i>Bacillus cabrialesi</i> TE3	<i>Bacillus subtilis</i> Ben5	<i>Bacillus velezensis</i> FH17	<i>Bacillus velezensis</i> TH16	<i>Bacillus velezensis</i> DSM7	<i>Bacillus velezensis</i> FZ942	<i>Bacillus velezensis</i> SQ9	<i>Bacillus endophyticus</i> FH5	<i>Bacillus endophyticus</i> KCTC 13922	<i>Paenibacillus</i> sp. EDO6	<i>Paenibacillus polymyxa</i> E681	<i>Paenibacillus polymyxa</i> SC2	<i>Paenibacillus xylanexedens</i> PAMC 22703
<i>Bacillus endophyticus</i> FH5	67.01	67.00	67.11	67.14	67.13	67.11	67.15	67.06	67.08	66.64	66.48	66.61	66.66	66.61		73.40	20.00	25.50	26.10	26.10
<i>Bacillus endophyticus</i> KCTC 13922	67.24	67.24	67.27	67.23	67.36	67.27	67.29	67.27	67.28	66.59	66.56	66.60	66.64	66.53	96.35		20.50	25.70	25.90	26.00
<i>Paenibacillus</i> sp. EDO6	64.51	64.51	64.34	64.35	64.34	64.33	64.38	64.51	64.31	64.32	64.37	64.39	64.36	64.44	64.74	64.68		25.20	25.30	57.60
<i>Paenibacillus polymyxa</i> E681	65.08	65.08	65.27	65.25	65.21	65.27	65.29	65.29	65.26	65.05	64.99	64.99	65.07	65.06	65.62	65.61	70.20		39.80	70.06
<i>Paenibacillus polymyxa</i> SC2	65.37	65.37	65.47	65.51	65.54	65.47	65.53	65.58	65.55	65.40	65.46	65.34	65.38	65.38	65.88	65.94	70.16	89.49		69.94
<i>Paenibacillus xylanexedens</i> PAMC 22703	65.16	65.16	65.20	65.19	65.17	65.20	65.13	65.18	65.14	65.21	65.19	65.31	65.24	65.27	65.50	65.57	93.88	70.06	69.94	

Table S2. All BGCs (including known and unknown) found in each genome of selected strains

Strains	cluster	Size(bp)	Type	Compounds
<i>B. cabrialesii</i> BH5	1	20264	Bacteriocin	Rhizoctin A (partial)
	2	63861	NRPS	Surfactin
	3	19891	Terpene	unknown
	4	66797	NRPS	unknown
	5	114089	Hybrid NRPS/PKS	Bacillaene
	6	111369	NRPS	Fengycin
	7	21590	Terpene	unknown
	8	27899	Bacterocin	Staphylococcin C55
	9	49743	NRPS	Bacillibactin
	10	21612	Bacteriocin	Subtilisin A
	11	41419	Other	bacilysin
	12	41098	Type III PKS	unknown
<i>B. cabrialesii</i> BH6	1	20264	Bacteriocin	Rhizoctin A (partial)
	2	63861	NRPS	Surfactin
	3	19891	Terpene	unknown
	4	66797	NRPS	unknown
	5	114089	Hybrid NRPS/PKS	Bacillaene
	6	111269	NRPS	Fengycin
	7	21590	Terpene	unknown
	8	27889	Bacterocin	Staphylococcin C55
	9	49743	NRPS	Bacillibactin
	10	21612	Bacteriocin	Subtilisin A
	11	41419	Other	bacilysin
	12	41098	Type III PKS	unknown
<i>B. subtilis</i> DH12	1	62295	NRPS	Surfactin
	2	20525	Terpene	unknown
	3	49742	NRPS	Bacillibactin
	4	114764	Hybrid NRPS/PKS	Bacillaene
	5	77493	NRPS	Fengycin
	6	41764	NRPS	Fengycin (partial)

Strains	cluster	Size(bp)	Type	Compounds
	7	21899	Terpene	unknown
	8	41098	Type III PKS	unknown
	9	26097	Bacteriocin	unknown
	10	26226	Bacteriocin	Subtilin
	11	21612	Bacteriocin	Subtilisin A
	12	41419	Other	Bacilysin
<i>B. subtilis</i> EH2	1	41419	Other	Bacilysin
	2	21612	Bacterocin	SubtilisinA
	3	49267	NRPS	Bacillibactin
	4	40889	Type III PKS	unknown
	5	20855	Terpene	unknown
	6	81195	NRPS	Fengycin
	7	114800	Hybrid NRPS/PKS	Bacillaene
	8	20807	Terpene	unknown
	9	26330	Bacteriocin	unkown
	10	65099	NRPS	Surfactin
<i>B. subtilis</i> EH5	1	26107	Bacteriocin	unknown
	2	41419	Other	Bacilysin
	3	21612	Bacteriocin	Subtilisin A
	4	49057	NRPS	Bacillibactin
	5	23117	Bacteriocin	unknown
	6	40666	Type III PKS	unknown
	7	21548	Terpene	unknown
	8	80494	NRPS	Fengycin
	9	114763	Hybrid NRPS/PKS	Bacillaene
	10	20804	Terpene	unknown
	11	64847	NRPS	Surfactin
<i>B. subtilis</i> EH11	1	62285	NRPS	Surfactin
	2	20525	Terpene	unknown
	3	26097	Bacteriocin	unknown
	4	114764	Hybrid NRPS/PKS	Bacillaene
	5	91208	NRPS	Fengycin
	6	42435	NRPS	Fengycin (partial)

Strains	cluster	Size(bp)	Type	Compounds
	7	21899	Terpene	unknown
	8	41098	Type III PKS	unknown
	9	49742	NRPS	Bacillibactin
	10	26226	Bacteriocin	Subtilin
	11	21612	Bacteriocin	Subtilisin A
	12	41419	Other	Bacilysin
<i>B. endophyticus</i> FH5	1	23854	Bacteriocin (Isso peptide)	unknown
	2	58658	NRPS	Bacillibactin
	3	65159	NRPS	unknown
	4	30327	Terpene	unknown
	5	82686	Hybrid NRPS/PKS	unknown
	6	18284	Terpene	unknown
	7	41089	Type III PKS	unknown
	8	20819	Terpene	unknown
	9	29091	Bacteriocin	unknown
	10	23546	Bacteriocin (LAP)	unknown
<i>B. velezensis</i> FH17	1	65263	NRPS	Surfactin
	2	40885	Other	unknown
	3	22183	Bacteriocin (LAP)	Plantazolicin
	4	41245	PKS-like	unknown
	5	17275	Terpene	unknown
	6	87810	trans AT-PKS	Macrolactin H
	7	64823	NRPS	unknown
	8	21884	Terpene	unknown
	9	136324	NRPS	Fengycin
	10	41101	Type III PKS	unknown
	11	106174	Hybrid NRPS/PKS	Difficidin
	12	50503	NRPS	Bacillibactin
	13	108797	Hybrid NRPS/PKS	Bacillaene
	14	41419	Other	Bacilysin
	15	2458	NRPS	unknown
<i>B. velezensis</i> TH16	1	106180	Hybrid NRPS/PKS	Difficidin

Strains	cluster	Size(bp)	Type	Compounds
	2	64842	NRPS	Surfactin
	3	17419	Terpene	unknown
	4	86391	trans AT-PKS	Macrolactin H
	5	109604	Hybrid NRPS/PKS	Bacillaene
	6	71730	NRPS	Fengycin
	7	41245	PKS-like	unknown
	8	41101	Type III PKS	unknown
	9	22616	Bacteriocin	unknown
	10	50949	NRPS	Bacillibactin
	11	41419	Other	Bacilysin
	12	68418	NRPS	unknown
<i>Paenibacillus</i> sp. EDO6	1	20873	Terpene	unknown
	2	34652	Type III PKS	unknown
	3	49099	NRPS	Bacillibactin
	4	22745	Bacteriocin	unknown
	5	17722	Other	unknown
	6	10243	Bacteriocin	unknown
	7	23968	Bacteriocin (lasso peptide)	unknown
	8	41210	NRPS-like	unknown
	9	26366	Bacteriocin	unknown
	10	41158	Type III PKS	unknown
	11	22961	Bacteriocin (LAP)	
	12	60514	trans-AT hybrid	PKs-NRPs unknown
	13	83731	trans-AT hybrid	PKs-NRPs unknown
	14	81118	NRPS	Polymyxin

Chapter 3

***Bacillus cabrialesii* BH5 protects tomato plants against *Botrytis cinerea* by production of specific antifungal compounds**

Lu Zhou¹, Chunxu Song^{1,2}, Claudia Y. Muñoz¹ and Oscar P. Kuipers^{1*}

¹Department of Molecular Genetics, University of Groningen, Groningen, The Netherlands

²College of Resources and Environmental Sciences; National Academy of Agricultural Green Development; Key Laboratory of Plant-Soil Interactions, Ministry of Education, China Agricultural University, 100193 Beijing, China

** Corresponding author; o.p.kuipers@rug.nl*

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Abstract

The grey mould caused by the phytopathogen *Botrytis cinerea* presents a threat to global food security. For the biological regulation of several plant diseases, *Bacillus* species have been extensively studied. In this work we explore the ability of a bacterial strain, *Bacillus cabrialesii* BH5, that was isolated from tomato rhizosphere soil, to control the fungal pathogen *Botrytis cinerea*. Strain *B. cabrialesii* BH5 showed a strong antifungal activity against *B. cinerea*. A compound was isolated and identified as a cyclic lipopeptide (CLP) of the fengycin family by high-performance liquid chromatography (HPLC) and tandem mass spectrometry (ESI-MS/MS), that we named fengycin H. The fengycin H-treated hyphae of *B. cinerea* displayed stronger red fluorescence than the control, which is clearly indicating that fengycin H triggered the hyphal cell membrane defects. Moreover, root inoculation of tomato seedlings with BH5 effectively promoted the growth of tomato plants. Transcription analysis revealed that both BH5 and fengycin H stimulate induced systemic resistance (ISR) of tomato plants via the jasmonic acid signaling pathway and provide a strong biocontrol effect *in vivo*. Therefore, the strain BH5 and fengycin H are very promising candidates for biological control of *B. cinerea* and the associated grey mould.

Keywords: *Bacillus*; tomato; phytopathogens; anti-fungal compounds; Induced Systemic Resistance (ISR)

Introduction

Tomato (*Solanum lycopersium*), ranking up to the second important vegetable, is a widely popular grown vegetable throughout the world (Yu et al., 2018). However, a number of diseases impact on the growth and yield of tomato. Grey mould, caused by airborne necrotrophic fungi *Botrytis cinerea*, is one of the most devastating diseases affecting tomato (Asselbergh et al., 2007; Toral et al., 2018). *B. cinerea* is able to attack more than 200 species causing lesions, and sporulating abundantly on aerial parts of plants (Boddy, 2016). Currently, repeated intensive fungicide sprays are necessary to control this pathogen especially in a mild and humid climate (Rupp et al., 2017), due to the lack of cultivars with resistance. Therefore, alternative approaches, less relying on chemicals and more environmentally friendly, are urgently needed for controlling grey mould (Soylu et al., 2010).

Biological control by beneficial microorganisms holds great promise for sustainable agriculture development (Myo et al., 2019). To date, more and more reports show that applying Plant Growth-Promoting Rhizobacteria (PGPR) into agriculture as biological control agents (BCAs) is a successful strategy for plant disease control (Ongena and Jacques, 2008). PGPR can influence plant growth by either facilitating resource acquisition or modulating plant hormone levels. Moreover, PGPR can decrease the various pathogenic effects, either by evoking induced systemic resistance (ISR) of plants or by producing antimicrobial compounds (Perez-Garcia et al., 2011; Berendsen et al., 2012; Olanrewaju et al., 2017). Various species from the genus *Bacillus* have been considered biologically safe, and their sporulation capacity makes it possible for them to live in diverse habitats and to be formulated as commercial products (Roh et al.,

2007;Ongena and Jacques, 2008;Arguelles-Arias et al., 2009;Perez-Garcia et al., 2011;Toral et al., 2018). *Bacillus* strains can secrete various types of antimicrobial compounds including non-ribosomally synthesized peptides (NRPs), polyketides (PKs), terpenes, and ribosomally synthesized and post-translationally modified peptides (RiPPs) which have shown distinct capacities to inhibit plant pathogens (Crits-Christoph et al., 2018). So far, the bioactive lipopeptides produced by *Bacillus subtilis* are mainly classified into three families: surfactin (Arima, 1968;Koumoutsis et al., 2004;Jiang et al., 2016), iturin (Besson et al., 1990;Tsuge et al., 2001), and fengycin (Vanittanakom et al., 1986;Wu et al., 2007;Zhang and Sun, 2018). All of them are cyclic lipopeptides (CLPs), which exhibit antimicrobial and antifungal activity against a broad range of phytopathogens. These CLPs, consisting of a fatty acid chain bound to a cyclic peptide ring, are produced by non-ribosomally synthesized peptide synthetases (NRPSs). Their broad antimicrobial and antifungal spectra offer great potential for plant disease control (Chowdhury et al., 2015). In addition, volatile organic compounds (VOCs) produced by *Bacillus* strains have also been proved to control plant pathogens, via inhibiting bacterial and fungal growth, promoting or inhibiting plant growth, triggering plant resistance and attracting other micro- and macro- organisms (Lim et al., 2017;Tahir et al., 2017a;Tahir et al., 2017b;Tyc et al., 2017;Avalos et al., 2018;Xie et al., 2018).

Plants also have their own mechanisms to deal with pathogens, and to coordinate the appropriate defense responses. A prominent defense mechanism is dependent on the jasmonic acid (JA) and ethylene (ET) signaling pathway or the salicylic acid (SA) signaling pathway in plants (Pieterse et al., 2009). Induced systemic resistance (ISR) is an important regulatory strategy of plants, which could activate appropriate cellular

defense responses before or upon pathogen attack (Akram et al., 2016). Different PGPRs have been shown to trigger ISR usually relying on the JA and ET signaling pathway (Pieterse et al., 2014). The JA and ET pathways associate with the responses against necrotrophic pathogens and chewing insects, while SA pathway associates with mainly biotrophic pathogens (Martinez-Hidalgo et al., 2015). In general, ISR could result in direct activation of the defense process including the increase of defense-related compound accumulation, and the priming of plant defensive capacity (van Hulten et al., 2006; Zamioudis and Pieterse, 2012).

Bacillus cabrialesii BH5, isolated from tomato rhizosphere soil, showed good antimicrobial activity against *Erwinia carotovora*, *Pseudomonas syringae*, *Rhizoctonia solani*, *Botrytis cinerea*, *Verticillium dahliae* and *Phytophthora infestans* among 181 other isolates in our previous study (Zhou et al., 2021). Strain BH5 showed antagonistic activity not only against tomato bacterial pathogens but also against tomato fungal pathogens. Therefore, we found it interesting to investigate the interaction mechanism between BH5 and pathogens of tomato plants. In this study, we demonstrate that *Bacillus cabrialesii* BH5 isolated from the rhizosphere soil of a healthy tomato plant, shows potent biocontrol activity against fungal pathogens via producing an antifungal compound, that is a variant of fengycin that we named fengycin H, with a different lipid moiety attached. We also found that *B. cabrialesii* BH5 is able to promote tomato plant resistance to *B. cinerea* by activating the jasmonic acid signaling pathway. These findings demonstrate the feasibility of *B. cabrialesii* BH5 to be applied in practice for tomato disease control.

Materials and Methods

Strains and culture conditions

Strain *Bacillus cabrialesii* BH5 was isolated from the rhizosphere soil of the healthy tomato cultivar Boludo during the Spring (February 2017), grown at Almería in Spain (Zhou et al., 2019; Zhou et al., 2021). BH5 was grown on Lysogeny Broth (LB) agar plates or LB liquid media at 28 °C, 220 rpm. All phytopathogens (*Botrytis cinerea* B05.10, *Fusarium culmorum* PV, *Pythium ultimum* P17, *Rhizoctonia solani* AG2-2 IIIB) were cultured on Potato Dextrose Agar (PDA) plates or Potato Dextrose Broth (PDB) media at room temperature. Plant pathogens *B. cinerea*, *F. culmorum*, *P. ultimum*, and *R. solani* used in our study, were kindly provided by Prof. Jos Raaijmakers of Netherlands Institute of Ecology (NIOO-KNAW) and Asst. Prof. Jan van Kan of Wageningen University & Research (WUR) as gifts.

Antifungal activity test

The plant pathogens (*B. cinerea*, *F. culmorum*, *P. ultimum*, and *R. solani*) were grown on PDA plates for 3 days at room temperature. Then a mycelial plug of 5 mm diameter of each pathogens was placed in the center of a new PDA plate. 5 µl of BH5 culture (OD₆₀₀ = 1.0) was spotted 2 cm away from the plug. LB media only was inoculated as control. The plates were sealed with parafilm and incubated at room temperature for 5 days. Hyphal growth was monitored depending on the pathogens' growth rate. The percentage inhibition of pathogens growth values were determined according to the equation as below:

$$\text{inhibition rate (\%)} = \frac{\text{Diameter of control} - \text{Diameter of test}}{\text{Diameter of control}} \times 100$$

Effect of VOCs from BH5 on plant Pathogens

The effect of BH5 VOCs on the growth of fungal and oomycetal plant pathogens, such as *B. cinerea*, *F. culmorum*, *P. ultimum*, and *R. solani* was investigated using bottoms of two 90-mm-diameter Petri dishes as described before (Cordovez et al., 2015). These two bottoms were sealed facing each other by parafilm and incubated at room temperature. One bottom was used to spread and grow BH5 with LB agar media, another bottom was used to grow fungal or oomycetal pathogen with PDA media. In order to prevent colonies of BH5 falling down on the plates with fungus, the plates of pathogens were turned to the upside once the plug of pathogens stuck on the PDA plates after 2 days. The LB medium plate without BH5 was considered as negative control. Fungal and oomycetal growth inhibition was monitored and measured as the diameter growth of fungal and oomycetal hyphae. Percentage inhibition of pathogens growth values were determined according to the equation as described before.

Identification of antifungal compounds

A single colony of BH5 was inoculated in 5 ml LB broth and incubated at 28 °C, 220 rpm for overnight. The overnight culture was diluted at 1:100 in 200 ml LB broth and incubated at 28 °C, 220 rpm for 24 hours. The supernatant was then collected by centrifugation (10,000 x g, 10 min) and precipitated with ammonium sulfate to 40% saturation. The precipitates were dissolved in Milli-Q water and desalted by 10 g C18 silica gel (Sigma-Aldrich). The crude extracts were filter sterilized with 0.45 µm Durapore™ membrane and then subjected to a reverse high-performance liquid chromatography (HPLC) equipped with Aeris widepore 3.6µ XB-C18 250 x 4.6mm column for purification. The mobile phases were HPLC-grade water supplemented with 0.1%

trifluoroacetic acid (TFA) (solvent A) and acetonitrile supplemented with 0.1% TFA (solvent B). A two-step gradient of solvent B from 5 to 40% in 10 min and from 40 to 95% in 30 min at a flow rate of 1 ml/min was applied for compound purification. A UV-detector set at a wavelength of 214 nm was used to monitor the compounds elution. All the peaks were collected for MALDI-TOF analysis and tandem MS (MS/MS).

Viability and integrity changes of *Botrytis cinerea* hyphal cells caused by the fengycin H compound produced by BH5

Cell viability and cellular integrity were measured by propidium iodide (PI) staining, as described previously with slight modification (OuYang et al., 2018; Zhang and Sun, 2018). To produce *B. cinerea* mycelium cells, the mycelial plug of 5 mm diameter of *B. cinerea* from PDA plates was inoculated in 50 ml PDB media. After incubation at 28 °C for 3 days with shaking at 100 rpm. the purified fengycin H were dissolved in methanol, and were injected into 50 ml PDB culture at a final concentration of 20 µM. The resulting cultures were incubated for 48 hours. The same amount of methanol was used as the control. The hyphal cells were collected and resuspended with 10 mM phosphate-buffered saline (PBS), and then stained with PI at 28 °C for 20 min in the dark. The cell viability and integrity changes were observed and recorded by a fluorescence microscope with a filter of 535 nm/615 nm.

Characterization of PGPR related traits of strain BH5

Siderophore production was investigated using the overlay chromeazuroil S assay (O-CAS assay) reported before with slight modification (Perez-Miranda et al., 2007). Briefly, iron deprived LB media was made by adding 5 g chelex 100 resin into 100 ml LB and stirring

for 1.5 hours. The overlay of CAS was applied over iron deprived LB agar plates containing cultivated strain BH5. The ability of siderophore production was evaluated by the size of the orange halo surrounding the colony of isolates after one hour.

Protease activity detection was achieved on 3% (W/V) skimmed milk agar plates (Dinesh et al., 2015). 5 μ l 1×10^8 cells/ml overnight culture of BH5 was spot-inoculated at the center of skimmed milk agar plates. After 3 days incubation at 28 °C, the radius of the halo zone around the strain was considered as an activity of the protease produced.

Phosphate solubilization ability assay was performed on Pikovskaya's media (PVK media) (Ali et al., 2020). The PVK media plates were spot-inoculated at the center of 5 μ l 1×10^8 cells/ml overnight culture of BH5 and incubated at 28 °C for 7-10 days. The zone of Phosphate solubilization surrounding the colonies was measured.

The Indole-3-acetic acid (IAA) production assay was carried out with the protocol reported previously (Bric et al., 1991; Goswami et al., 2013; Goswami et al., 2014). The capacity of IAA biosynthesis of isolates was estimated by the Salkowski colorimetric technique with slight modifications. BH5 was grown in LB media supplemented with 5 mM tryptophan for 3 days. Subsequently, supernatants were collected by centrifugation at 10,000 x g for 30 min. 2 ml supernatant was mixed with 10 μ l of orthophosphoric acid and 4 ml Salkowski reagent. After 25 min incubation at room temperature, the intensity of pink color was detected at 530 nm spectrophotometrically and the amount of IAA was calculated based on the standard curve.

Swarming motility was investigated on LB plates supplemented with 0.7% agar (Gao et al., 2016). 5 μ l 1×10^8 cells/ml overnight culture of BH5 was spot-inoculated at the center of 0.7% agar swarming plates and incubated at 28 °C overnight and the zone of colonization on 0.7% agar swarming plates was measured.

The biofilm formation ability was tested according to a previous protocol (Oliveira et al., 2015;Zhao et al., 2015). The growth of biofilm was quantified by the crystal violet staining method in 96-well polystyrene plates. Briefly, an overnight culture (1:100) of BH5 was inoculated in 100 µl LB media and incubated at 30 °C for 24 hours. Then the culture was stained with 40 µl 0.25% crystal violet for 15 min. After a three times wash with PBS buffer, to each well was added 200 ul 33% acetic acid to dissolve the biofilm for 15 min. The optical density was measured at OD₅₉₅ and the value was used as a proxy for biofilm formation.

***In vivo* plant growth promotion**

Tomato seeds (Moneymaker) were surface-disinfected in 70% ethanol for 1 min and 2% NaClO solution for 10 min. Subsequently, tomato seeds were rinsed 5 times with sterilized deionized water. Seeds were pre-germinated horizontally on 15 cm Petri dishes with Murashige and Skoog (MS) agar in a growth chamber (16 hours day time at 24+/- 2 °C, 8 hours dark time at 21+/-2 °C) for 2 days. Pre-germinated seeds were transferred to new MS agar plates and incubated vertically in a growth chamber for 2 days. BH5 were grown in LB media at 28°C and 220 rpm for overnight. Cells were harvested and then washed with Phosphate buffer solution (PBS) and diluted to a final concentration of 1×10^7 cells/ml with appropriate PBS. 5-day-old root tips of seedlings were inoculated with 5 µl of cell suspension. The effects of bacteria on the tomato plants were evaluated 3 weeks post-inoculation.

Assessment of BH5 protective effect on tomato plants against *B. cinerea*

One-month-old tomato (Moneymaker) plants were used for antifungal bioassays. These plants were grown in plastic pots of 1 L capacity, filled with sterilized commercial substrate and placed in a growth chamber at 24/21 °C with a 16/8 hours photoperiod and 60% humidity. The inoculation was performed 3 days before pathogen challenge. 5 ml of BH5 suspension (10^9 cells/ml) in PBS buffer or 5 ml purified pure fengycin H (20 µM) in PBS buffer was carefully brought in the soil near the roots of the tomato plant using a micropipette, while the same amount of PBS buffer was used as control. All the treatments were divided into two groups. One group of treatments was sampled at 0, 24, 48, 72, 96 and 120 h for the measurements of the relative expression of JA signaling pathway-related genes. Another group was subjected to *B. cinerea* challenge. For antifungal assay *in vivo*, after 3 days inoculation of BH5 or the fengycin H, tomato leaves were collected and washed with sterilized MilliQ water, then subjected to antifungal bioassay. Three leaves of each plant were placed in plastic trays on wet paper and challenged with *B. cinerea* by applying 5 mm diameter plugs of one-week-old PDA culture plates. The leaves were then placed in a growth chamber. The disease symptoms were scored on each leaf after 2 days of infection and thus recorded every 2 days over a 15-day period. The diameter of the necrotic lesions formed by the fungal hyphae in the leaflets was measured. The biocontrol efficacy was calculated by the formula reported before (Soylu et al., 2010). Biocontrol Efficacy = $[(Lc-Lt)/Lc] \times 100$, where Lc indicating lesion diameter in control, Lt indicating lesion diameter in treatment.

Quantitative Real-Time PCR (qRT-PCR) analysis

A 100 mg sample of frozen leaves in powder form was used to extract the total RNA with RNeasy Plant Mini Kit (QIAGEN, Germany). The total RNA was dissolved in 30 µl of RNAase-free water, quantified by microspectrophotometry, and stored at -80 °C. In brief, 1 µg total RNA was used for cDNA synthesis by using SuperScript™ III Reverse transcriptase (Invitrogen) following the instruction manual. After the cDNA was diluted 10 times, 2µl diluted cDNA was used for quantitative real-time PCR with the following program: 95 °C 3 min, followed by 40 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. The relative gene expression for each sample was normalized and calibrated to the housekeeping gene *SIEF* (encoding for the tomato elongation factor-1 α) Ct value, and counted using the formula $2^{-\Delta\Delta Ct}$ (Cheng et al., 2017). All experiments were run in triplicate with different cDNAs synthesized from three biological replicates. Three (*SLLoxD*, *SPI II*, and *SIJAZ1*) genes related to JA signaling pathway were selected for qRT-PCR analysis. Primers for qRT-PCR are listed in Table 1. The *SLLoxD* gene (encoding for a JA-inducible lipoyxygenase) is related to the biosynthesis of JAs, involving in the generation of endogenous jasmonic acid (JA), and regulating the expression of plant defense genes and resistance to high temperature and pathogen attack (Hu et al., 2015; Beris et al., 2018). The *SPI II* gene (encoding for proteinase inhibitor II), mainly regulated by JA and MeJA, is a marker of JA/ET-dependent pathways (Xin et al., 2014). The role of *SPI II* gene is making a contribution in plant defense against herbivores and pathogens (Rehman et al., 2018). *SIJAZ1*, which is a negative gene in the JA signaling pathway (Thines et al., 2007; Chico et al., 2008), encoding for key repressors in the JA signaling pathway, and acting as a primary role of tomato in cell death associated to pathogen infections (Ishiga et al., 2013; Chini et al., 2017).

Table 1. Sequences of specific primers used for qPCR analysis

Gene name	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
<i>SIEF</i> (housekeeping)	X53043	GGTGCATCAAATTCTGCCTGT	GGCGACTTGTTGTCACTCT
<i>SILoxD</i>	NC_015440.3	GGCACGGTGTCAGTGTATT	CATAAGGCTGTGTTGCGTCC
<i>SIP1 II</i>	NM_0012477698	TTCGGGATATGCCCACGTTC	GGTGCAAGCATTGTCCTTT
<i>SIIAZ1</i>	NM_001247954	AGCCAACAAACAGAACCCCA	AATTCCGTCTCGCGATTGGT

Results

Antifungal activity of *B. cabrialesii* BH5

Antifungal activity was detected by the dual culture method on PDA plates. *B. cabrialesii* BH5 showed a strong ability to inhibit the growth of four test pathogens (Figure 1A). The mycelial inhibition rates were 61%, 66%, 50%, and 63% towards *Botrytis cinerea*, *Fusarium culmorum*, *Pythium ultimum*, and *Rhizoctonia solani*, respectively.

Figure 1. Antifungal activity of *Bacillus cabrialesii* BH5 towards four plant fungal pathogens (A) and the antifungal activity of volatile compounds (VOCs) produced by BH5 (B)

Antifungal activity of VOCs Produced by *B. cabrialesii* BH5 on Agar Media

To investigate the effect of VOCs produced by BH5 on plant pathogens, we performed the so-called sandwich assay (Cordovez et al., 2015). Once the hyphae reached the edge of the plates in control plates, the hyphal growth of *B. cinerea*, *F. culmorum*, *P. ultimum*, and *R. solani* was measured and recorded. BH5 showed the inhibition on the hyphal growth of pathogens (Figure 1B). The inhibition rates were 87%, 32%, 14%, and 32% towards *B. cinerea*, *F. culmorum*, *P. ultimum*, and *R. solani*, respectively.

Figure 2. Two families of lipopeptides produced by *Bacillus cabrialesii* BH5. The ensembles found in the mass ranges m/z 1008-1072 (A) and m/z 1435-1477 (B) were

attributed to the well-known surfactins and fengycins. Effect of surfactins and fengycins produced by BH5 on four different plant pathogens (C.)

Antifungal activity of the lipopeptides produced by *B. cabrialesii* BH5

Two product families were detected in the extracts (Figure 2). They were found in the mass ranges m/z 1008-1072 (Figure 2A) and m/z 1435-1477 (Figure 2B) and were attributed to the well-known surfactins and fengycins. These two families of lipopeptides were then subjected to investigate the activity with phytopathogens *in vitro*. Only the fengycins showed bioactivity against the hyphal growth of *B. cinerea*, *F. culmorum*, and *R. solani* (Figure 2C). To further characterize the antifungal compounds, all the peaks were applied to LC-MS/MS analysis. A fengycin-like compound (m/z 1433.8) was found in the extract (Figure 3A and 3B). This compound showed identical amino acid composition as fengycin A but had a different fatty acid chain. It is different from fengycins discovered until now, because the lipid portion of fengycins consists of a β -hydroxy fatty acid (β -OH FA), with its-COOH group amide bonded to α -NH₂ of Glu1 (Figure 3C). However, there is no β -hydroxyl at the fatty acid chain in the fengycin-like compound produced by BH5 as shown in Figure 3D, which makes the chain more hydrophobic. We denoted this variant as fengycin H.

A.

B.

C.

D.

Figure 3. Characterization of fengycin H produced by *Bacillus cabrialesii* BH5. (A) Tandem MS analysis of fengycin H. (B) Tandem MS analysis of hydrolyzed fengycin H (fengycin H was hydrolyzed with 2M NaOH). (C) The structure of C15 fengycin (D) The structure of fengycin H produced by BH5

Effect of fengycin H on *B. cinerea* hyphal cell viability and cellular integrity

Hyphal cell viability and cellular integrity were investigated by the fluorescent dye propidium iodide (PI) which is excluded from viable cells. Cells with a compromised membrane integrity take up the probe and appear as red-labeled upon binding of the dye to DNA as shown in Figure 4. The fengycin H-treated hyphae of *B. cinerea* displayed

stronger red fluorescence than the control, which is clearly indicating that fengycin H triggered the hyphal cell membrane defects and cell death.

Figure 4. Effect of fengycin H from *Bacillus cabrialesii* BH5 on viability and membrane integrity of *B. cinerea* hyphal cells, observed by light microscopy (A and C) and fluorescence microscopy (B and D). Live fungal hyphal cells with intact membranes show no fluorescence under fluorescence microscopy (B); the damaged cellular membranes of fungal hyphae showed red fluorescence (D). Methanol served as the control treatment.

BH5 was screened for diverse PGPR traits such as siderophore production, protease activity, phosphate solubilization, IAA production, swarming motility and biofilm formation ability. As shown in Supplementary Table S1 and Supplementary Figure S1, BH5 was positive for siderophore production, protease production, phosphate solubilization, swarming motility, and biofilm formation. In addition, the IAA production by BH5 was measured by Salkowski assay, and IAA was produced at $5.8 \pm 0.2 \mu\text{g/ml}$.

Tomato plant growth promotion by *B. cabrialesii* BH5

In Figure 5A, the effect of BH5 on the growth of tomato seedlings was monitored by measuring the shoot/root length, shoot/root fresh weight and dry weight. *B. cabrialesii* BH5 showed a significant increase of fresh and dry weight both of the shoot and root. The fresh weight of shoot and root significantly increased by about 97 and 30 mg respectively as compared to the control. The dry weight of shoot and root significantly increased by about 7.1, 1.3 mg respectively as compared to the control. The length of the root significantly increased by about 5.18 cm as compared to the control, while the length of the shoot showed no significant increase as compared to the control.

Transcription analysis of genes involved in the JA signaling pathway in tomato leaves

To investigate induced systemic resistance (ISR) stimulated by BH5 or fengycin H in our study (Figure 5B, 5C and 5D), the expressions of three genes related to the JA signaling pathway of tomato at 0, 24, 48, 72, 96 and 120 h after treatment were measured. For the JA signaling pathway related gene *SLLoxD*, the results (Figure 5B) showed that the expression of *SLLoxD* gene in fengycin H or BH5 treatment was always significantly higher than that in control from 48 to 96 h, and the expression of the *SLLoxD* gene in fengycin

H treatment peaked at 24 h and then decreased. Meanwhile, the expression level of *S/LoxD* gene under BH5 treatment started to increase at 48 h, whereas the expression of *S/LoxD* in fengycin H treatment showed no significant difference compared with the control at 120 h. For the JA signaling pathway related gene *S/PI II* (Figure 5C), we found that the transcript levels of *S/PI II* gene were significantly higher in plants inoculated with fengycin H or BH5 from 48 to 120 h, and both of them peaked at 48 h. Remarkably, the relative expression of *S/PI II* gene of tomato plants inoculated with fengycin H were significantly higher than that inoculated with BH5 at 24h and significantly lower than that inoculated with BH5 from 48 to 120h. Additionally, for the JA signaling pathway related gene *S/JAZ1* (Figure 5D), the variable patterns of *S/JAZ1* expressions in fengycin H and BH5 treatments tomato plants significantly decreased from 24h.

Figure 5. Plant growth promotion activity of *Bacillus cabrialesii* BH5 on tomato seedlings (A) and effects of fengycin H and BH5 treatment on JA signaling-related gene expressions: (B) *SiLoxD*, (C) *SIPI II*, and (D) *SIJAZ1*. Significant differences between 20 μM fengycin H or strain BH5 treatment group and control group were compared by Duncan's multiple range test. Significant differences at $P < 0.01$ and $P < 0.05$ were marked as () and single (*), respectively. The data (mean $\pm$ SD) were calculated using five replicate assays. ns means no significant difference was detected. Test of *B. cinerea* pathogenicity on detached leaves over 5 days (E)**

Bioassay against *B. cinerea* on detached tomato leaves

The efficiency of BH5 and fengycin H for controlling *B. cinerea* in tomato plants were assessed on detached leaves. An obvious difference in virulence between the control

and treatments was observed (Figure 5E). Results from the bioassay showed that BH5 and fengycin H prevented disease symptoms caused by *B. cinerea* on tomato leaves. After two days infection, the leaves turned yellow and exhibited necrotic spots in control. In contrast, leaves treated with BH5 or fengycin H only started to appear small necrotic spots after 6 days infection. The biocontrol efficacies were 71.4 % for BH5 treatment, and 57.1 % for fengycin H treatment. The average lesion diameter was 7.0 ± 0.3 mm in the control, while 2.0 ± 0.3 and 3.0 ± 0.2 mm was measured both on BH5 and fengycin H treatments.

Discussion

The definition of PGPR is well established and it is therefore worth considering the relationship between PGPRs and biocontrol. By suppressing well-known diseases caused by major pathogens or reducing the deleterious effects of pathogens, PGPR indirectly increases plant growth (Whipps, 2001; Aktan and Soyulu, 2020). Moreover, PGPR also increases plant development directly, such as by associative N_2 fixation (Khan, 2005), solubilizing nutrients (phosphate solubilization) (Jeon et al., 2003), producing siderophore (Bhattacharyya and Jha, 2012), releasing phytohormones (IAA production) (Marques et al., 2010), and decreasing the toxicity of heavy metals (Bhattacharyya and Jha, 2012). For plant disease control, earlier research has repeatedly documented that natural antagonistic microorganisms can regulate many plant diseases through the production of antipathogenic compounds, competition for space and other necessities or stimulating the host defense mechanism (Cook and Baker, 1983; Cook et al., 2002).

In this work, we report the biocontrol potential of *B. cabrialesii* BH5 for grey mould in tomato as well as its plant growth promoting ability. BH5 shows positive traits of PGPR (Table S1.) and can promote tomato seedlings growth in the climate chamber (Figure 5A). These findings are the first records of *B. cabrialesii* that shows both *in vivo* and *in vitro* antagonistic activity against *B. cinerea*. Besides, we also demonstrate both BH5 and its antifungal compound (fengycin H) can stimulate induced systemic resistance of tomato plants (Figure 5B,5C and 5D).

The synthesis of non-ribosomally synthesized peptides (NRPs) such as iturin (Besson et al., 1990), fengycin (Wu et al., 2007), and surfactin (Arima, 1968) is one of the major factors linked to the antifungal activity of members of the genus *Bacillus* (Soylu et al., 2005;Romero et al., 2007;Ongena and Jacques, 2008;Pramudito et al., 2018). Q-TOF MS analysis was performed to detect the lipopeptides released by BH5, showing that strain BH5 produces all lipopeptides except iturin. Some of these macromolecules have been shown to be formed by *B. velezensis* strains (Rabbee et al., 2019). In our research, only fengycins showed good antifungal activity against phytopathogens, including *B. cinerea*, *F. culmorum* and *R. solani* (Figure 2C). The PI staining assays further indicate that fengycin H discovered from BH5 was responsible for cell wall integrity defects and finally the death of these hyphal cells (Figure 4). Overall, our findings indicate that fengycin H causes significant plasma membrane damage in the fungal pathogen *B. cinerea*, which results in the death of cells. Furthermore, our study of the BH5 and its fengycin H treatments of tomato plants before *B. cinerea* challenge showed that both BH5 and fengycin H could stimulate induced systemic resistance of tomato plants by JA signaling pathway (Figure 5B, 5C and 5D). JAs are one of the most essential plant pathogen

defense hormones, and previous studies have shown that plants can activate the pathway of JA signaling in resistance to necrotrophic pathogens (Trusov et al., 2009; Nie et al., 2017). Mutant plants with JA signaling defects were more susceptible to *B. cinerea* (Rossi et al., 2011). The *SLOxD* gene (encoding for a JA-inducible lipoxygenase) (Beris et al., 2018) is related to the biosynthesis of JAs. The *SIP1 II* gene (encoding for proteinase inhibitor II) (Xin et al., 2014), mainly regulated by JA and MeJA, is a JA-dependent gene. In our research, up-regulated expression of *SLOxD* and *SIP1 II* genes in tomato plants treated with fengycin H and BH5 showed that both fengycin H and BH5 may boost gene expression associated with biosynthesis of JAs to promote disease resistance in tomato plants. Furthermore, decreased transcript levels of *SIJAZ1*, which is a negative gene in the JA signaling pathway (Thines et al., 2007; Chico et al., 2008), in fengycin H and BH5 treated tomato plants compared with the control further confirmed that both fengycin H and BH5 could activate JA signaling pathway to enhance disease resistance to *B. cinerea* in tomato plants. Interestingly, *SLOxD* and *SIP1 II* genes of tomato plants under BH5 treatment always exhibit 24 hours later an up-regulated or down-regulated expression as compared to fengycin H treatment, which is likely to suggest that BH5 will take two days after inoculation to successfully colonize the tomato root. This is relatively quicker than other PGPR, with this vigorous capacity for occupation that could effectively allow BH5 colonize the root area against plant exudates and other rhizobacteria. When *B. cinerea* was applied to detached leaves after 3 days inoculation of fengycin H or BH5, disease severity is significantly reduced. This result demonstrates that the durable systemic resistance induced by BH5 or fengycin H could continuously provide a disease control ability for tomato. Altogether, the *in vivo* and *in vitro* investigation of biocontrol ability of fengycin H and strain BH5 suggest an important role of BH5 for use as

biocontrol agent against *B. cinerea* as well as a biofertilizer for promoting tomato growth. Various *Bacillus* spp. strains stimulating plant defense reactions have been reported. The existence of molecular determinants secreted by these Gram-positive bacteria responsible for the elicitation of the phenomenon of induced systemic resistance (ISR) is, however, still little understood. This study shows that the fengycin H lipopeptide may be involved in this process of elicitation. We demonstrated that the ISR-mediated protective effect on tomato plants can be achieved by pure fengycin H in tomato plants via the JA signaling pathway. This finding is consistent with a previous study which reported that pure fengycins and surfactins can provide a significant ISR-mediated protective effect on bean plants (Ongena et al., 2007). By transcription analysis, we provide the evidence that fengycin H from BH5 stimulates the ISR in tomato plants via the JA signaling pathway.

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Supplementary data

Table S1. Summary of PGPR traits of strain *B. cabrialesii* BH5

Strain	PGPR traits					
	Siderophore production	Protease production	Phosphate solubilization	IAA production	Swarming	Biofilm formation
<i>B. cabrialesii</i>	+	+	+	5.75 ±	+	+
BH5				0.19		
				µg/mL

Figure S1. Pictures of *B. cabrialesii* BH5 with clear halo on SMA, PVK or CAS agar plates.

Chapter 4

Identification, isolation and characterization of medipeptins, antimicrobial peptides from *Pseudomonas mediterranea* EDOX

Lu Zhou, Anne de Jong, Yunhai Yi, Oscar P. Kuipers*

*Department of Molecular Genetics, University of Groningen, Groningen, The
Netherlands*

** Corresponding author; o.p.kuipers@rug.nl*

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Abstract

The plant microbiome is a vastly underutilized resource for identifying new genes and bioactive compounds. Here, we used *Pseudomonas* sp. EDOX, isolated from the leaf endosphere of a tomato plant grown on a small farm in the Netherlands. To get more insight into its biosynthetic potential, the genome of *Pseudomonas* sp. EDOX was sequenced and subjected to bioinformatic analyses. The genome sequencing analysis identified strain EDOX as a member of the *Pseudomonas mediterranea*. *In silico* analysis for secondary metabolites identified a total of five non-ribosomally synthesized peptides synthetase (NRPS) gene clusters, related to the biosynthesis of syringomycin, syringopeptin, anikasin, crochelin A and fragin. Subsequently, we purified and characterized several cyclic lipopeptides (CLPs) produced by NRPS, including some of the already known ones, which have biological activity against several plant and human pathogens. Most notably, mass spectrometric analysis led to the discovery of two yet unknown CLPs, designated medipeptins, consisting of a 22-amino-acid peptide moiety with varying degrees of activity against Gram-positive and Gram-negative pathogens. Furthermore, we investigated the mode of action of medipeptin A. The results show that medipeptin A acts as a bactericidal antibiotic against Gram-positive pathogens, but as a bacteriostatic antibiotic against Gram-negative pathogens. Medipeptin A exerts its potent antimicrobial activity against Gram-positive bacteria via binding to both lipoteichoic acid (LTA) and lipid II as well as by forming pores in membranes. Collectively, our study provides important insights into the biosynthesis and mode of action of these novel medipeptins from *P. mediterranea* EDOX.

Keywords: *Pseudomonas*, genome mining, biosynthesis, cyclic lipopeptides (CLPs), mode

of action

Introduction

Pseudomonas bacteria are found in abundance in nature and have a fascinating metabolic diversity, which is linked to their ability to colonize a diverse range of ecological niches (Gotze and Stallforth, 2020). *Pseudomonas* species have a wide metabolic range and can produce diverse metabolites, including cyclic lipopeptides (CLPs), 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), pyoluteorin (PLT), phenazines (PHZs), 2,5-dialkylresorcinol, gluconic acid, quinolones, rhamnolipids, siderophores, and hydrogen cyanide. It is obvious that the bacteria from the genus *Pseudomonas* represent a rich source of natural products (Gross and Loper, 2009; D'ae et al., 2010; Masschelein et al., 2017; Zhao et al., 2019).

With the progress made by up-to-date nucleotide-sequencing techniques, a new age of natural products discovery has begun, greatly accelerated by the pace of genome sequencing of microorganisms. This advancement has paved the way for the effective prediction of gene clusters encoding new bioactive compounds, as well as the understanding of the underlying biosynthetic processes (Vater et al., 2018). Genome mining studies of *Pseudomonas* species have shown a wide variation of biosynthetic gene clusters (BGCs) between genera, species, and even strains within the same species (Loper et al., 2012; Van Der Voort et al., 2015). *Pseudomonas* species produce non-ribosomally synthesized peptides (NRPs), which are oligopeptides (8-25 amino acids) that are N-terminally acylated with a fatty acid. This kind of bacterial natural products are formed by non-ribosomally synthesized peptide synthetases (NRPSs), which may insert non-

canonical amino acids into extending peptides (Schwarzer et al., 2003; Weissman, 2015; Gotze and Stallforth, 2020). The vast majority of *pseudomonal* lipopeptides are not linear, but rather have a macrolactone ring shaped between the peptide's C-terminus and the hydroxyl group of a serine or threonine. The macrocycles vary in size from four to ten amino acids (Gotze and Stallforth, 2020).

All known CLPs produced by *Pseudomonas* species can be classified into 14 major groups, according to the length and structure of the oligopeptide. The majority of them have antifungal and antibacterial properties, while others have antioomycete, antiviral, antiprotozoan, and antitumor properties (Geudens and Martins, 2018). CLPs inhibit bacterial growth by affecting either cell wall biosynthesis or cell membrane integrity. In brief, CLPs can insert into plasma membranes and alter their microdomain membrane fluidity. This can result in a rearrangement of plasma membrane architecture, which can cause depolarization and ion leakage or influx, both of which can cause cell death or compromised proliferation (Muller et al., 2016). In general, most CLPs from *Pseudomonas* species tested exhibit activity against Gram-positive bacteria in the low micromolar range, but not against Gram-negative bacteria (Raaijmakers et al., 2006; Reybroeck et al., 2014).

Materials and Methods

Isolation of Bacteria

The isolation of bacteria from leaves was conducted according to a previous protocol (Zhou et al., 2021). In brief, 1 g tomato leaves were surface-sterilized for 1 min. in 70% ethanol and 3 min. in 0.5% NACLO solution combined with one droplet Tween 80 per 100

ml solution. After that, leaves were rinsed 5 times in sterilized deionized water. Subsequently, the leaves were macerated in 9 ml of 10 mM MgSO₄ buffer with a mortar to get the tissue suspension. After dilution 10³-10⁶ times with 10 mM MgSO₄ buffer, the suspension was spread on Lysogeny Broth (LB) agar plates. To obtain single colonies, the plates were incubated at 28 °C for 24–48 hours. Surface sterilization was validated by spreading aliquots of the final rinsing by deionized water on LB agar plates (if no organism growth was detected after 7 days, surface sterilization was deemed successful).

Whole Genome Sequencing and Assembly

Genomic DNA was extracted from a culture grown from a single colony using a GenElute bacterial genomic DNA kit (Sigma) according to the manufacturer's instructions. The genomes were sequenced at GATC Biotech (Germany) using an Illumina HiSeq sequencing system. Raw reads were validated by FastQC version 0.11.5 (Andrews, 2010). Trimmomatic version 0.38 was used to delete low-quality reads (Bolger et al., 2014). Subsequently, the reads were assembled de novo using SPAdes version 3.11.1 (Bankevich et al., 2012). Default parameters were used for all software unless specified. The draft genomes were then annotated by the Rapid Annotations using Subsystems Technology (RAST) server. The dDDH and ANI values were determined using the online applications TYGS (Meier-Kolthoff and Goker, 2019) and JSpeciesWS (Richter et al., 2016), respectively, for the categorization of species affiliations.

Genome Mining of Biosynthetic Gene Clusters

In order to identify biosynthesis gene clusters (BGCs) in strain EDOX, the draft genome sequence was constructed into a pseudomolecule using the Medusa web server

(<http://combo.db.e.unifi.it/medusa>) (Bosi et al., 2015) with multiple closely related strains as references. The pseudomolecules were then sent to antiSMASH 5.0 (Blin et al., 2019) and BAGEL 4 (van Heel et al., 2018) for BGC mining.

Purification of Antimicrobial Compounds from the EDOX Strain

For antimicrobial compounds purification, seed culture of strain EDOX was cultivated in 4 ml King's B (KB) media contained in glass tube and shaken on a rotary shaker for 24 h at 28°C, 210 rpm. Subsequently, the seed culture was inoculated in 2 L flasks containing 400 ml KB media at 210 rpm for 48 hours. The supernatant was collected by centrifugation (10,000 x g, 30 min), and acidified to pH 2 with 6 N hydrochloric acid (HCl) and kept overnight at 4°C for precipitation of CLPs. After that, the precipitates were collected by centrifugation (10,000 x g, 30 min) and extracted using methanol. To get crude CLPs extracts, methanol was evaporated at room temperature. The crude extracts were dissolved in Milli-Q water and filtered with 0.45 µm Durapore™ membrane and then subjected to a reverse high-performance liquid chromatography (HPLC) equipped with Aeris widepore 3.6 µm XB-C18 250 x 4.6mm column for purification. The mobile phases were HPLC-grade water supplemented with 0.1% trifluoroacetic acid (TFA) (solvent A) and acetonitrile supplemented with 0.1% TFA (solvent B). An elution gradient of solvent A/solvent B (75:25 to 0:100) was applied at a flow rate of 1 ml/min, for compound purification. A UV-detector set at a wavelength of 214 nm was used to monitor the compounds elution.

Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) Analysis

A Q-Exactive Orbitrap™-based mass spectrometer equipped with an Ultimate 3000 UPLC,

an ACQUITY BEH C18 column (2.1 50 mm, 1.7 μ m particle scale, 200; Waters), a HESI-II ion source, and an Orbitrap detector was used to perform LC-MS/MS to obtain insight into the molecular configurations of the peptides. In each run, a 30-minute gradient of 5-90 % MeCN/Milli-Q water (v/v) with 0.1 % formic acid (v/v) was used with a flow rate of 0.35 mL/min. The doubly and triply charged ions of the compound of interest were selected by MS/MS in a separate run in PRM mode.

Determination of the Minimum Inhibitory Concentration (MIC)

MIC tests were conducted in triplicate using liquid growth inhibition microdilution assays in sterile polypropylene microtiter plates according to the Clinical and Laboratory Standards Institute (CLSI) guideline (Wiegand et al., 2008; Hindler and Richter, 2016). The pathogenic indicator strains were streaked on LB agar plates first, and then single colonies were selected and incubated in LB broth overnight. The final bacteria inoculum was prepared in CAMHB (cationic adjusted Mueller-Hinton broth, Sigma-Aldrich) at 5×10^5 CFU/mL. The antimicrobials were serially diluted two times, and 50 μ L of diluted bacterial suspension was applied to each well to make the final volume 100 μ L. Without stirring, the microtiter plates were incubated for 48 hours at 30 °C. The OD₆₀₀ was measured using a microplate reader (Tecan Infinity F200) to determine growth inhibition. The MIC value is described as the lowest concentration of antimicrobials that inhibits a pathogen's visible growth.

Time-Kill Assay

This assay was performed according to a previously described protocol with small modification (Ling et al., 2015). An overnight culture of either *Staphylococcus aureus*

533R4 or *X. translucens* pv. graminis LMG587 was diluted 50-fold in MHB and incubated at 30 °C with the shaker at 220 rpm. Bacteria were grown to an OD₆₀₀ of 0.5, and then the concentration of cells was adjusted to $\approx 5 \times 10^5$ cells per ml for *X. translucens* and $\approx 2 \times 10^8$ for *S. aureus*. Bacterial cultures were then exposed to 10X MIC antimicrobials at 30 °C and 220 rpm. Bacteria not treated with medipeptin A were used as a negative control. Bacteria treated with vancomycin or colistin were used as a positive control. At desired time points (0, 1, 2, 3, 4, 5, and 6 hour), 10 µl culture was taken and tenfold serially diluted. Subsequently, each sample was plated on MHA plates. After incubation at 30 °C for 48 hours, colonies were counted and the number of CFU per ml was calculated. Each experiment was performed in triplicate.

Membrane Permeabilization Assays

In order to investigate whether medipeptin A could permeabilize the cell membranes, a commercial LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) was used. Cells of *S. aureus* were grown in LB overnight and diluted to an OD₆₀₀ of 0.2. Compound medipeptin A was added at a concentration of 40 µg/ml (5X MIC) in 1 ml culture, while nisin was added at a concentration of 2.5 µg/ml. The same amount of DMSO was added to the control. Cells were treated at room temperature for 1 hour before harvest. The harvested cells were washed and resuspended in 200 µl 0.85% saline (NaCl) solution. Two different dyes (3.34 mM SYTO®9 and 20 mM propidium iodide) were added at a ratio of 1:1 (v/v). Cells were stained in the dark for 30 min and a 2 µl sample was mounted on a 1% agarose pad before being imaged using a DeltaVision Elite microscope.

Lipoteichoic Acid (LTA) Binding Assay

Cultures of *S. aureus* grown overnight were diluted to an OD₆₀₀ of 0.05 in a 96-well plate and incubated in a microplate spectrophotometer (1,000rpm at 30 °C). When the OD₆₀₀ reached 0.1, medipeptin A (dissolved in DMSO) was added to each well at a final concentration of 24 µg/ml (3X MIC), and the same volume of DMSO was used as a negative control. Nisin (at a final concentration of 2 µg/ml) was used as a positive control for *S. aureus*. Lipoteichoic acid (LTA) (at a final concentration of 100 µg/ml) was added to look into the binding activity of medipeptin A. The growth curve was recorded using the microplate spectrophotometer under the same circumstances for the next 16 hours, with four replicates for each treatment. LTA is negatively charged and is thought to influence Gram-positive bacteria's sensitivity to cationic antimicrobial peptides like defensin and nisin via electrostatic contact (Yang et al., 2017). The LTA added in each sample could compete with LTA on cell envelopes for binding with cationic antimicrobial peptides, which could cause the reduction of antimicrobial activity of peptides. Comparing with only peptides treatment, medipeptin A binding to LTA is indicated by the restoration of cell growth under treatment of LTA and peptides together.

Lipid II Binding Assay

An overnight culture of *S. aureus* was mixed with pre-cooled LB agar (around 55 °C) at a final concentration of 0.1% (v/v), and then the mixed media was poured onto Petri dishes to obtain pathogen-fusion agar plates. Binding of medipeptin A and lipid II was further evaluated by spotting of purified lipid II (300 µM, 4 µl) to the edge of the halo caused by each compound. Briefly, medipeptin A or vancomycin were loaded to the agar plate, and after the solution drops had dried, purified lipid II was spotted to the edge (identified by

a pre-experiment) of the inhibition halo formed by antibiotics. After the lipid II solution drops had dried, the plates were transferred to a 30 °C incubator for overnight incubation. The decrease in halo size indicates the binding of lipid II and medipeptin A.

Results

Isolation and Identification of Strain EDOX

The strain *Pseudomonas* sp. EDOX, isolated from the healthy tomato leave endosphere, showed antagonistic activity against both bacterial and fungal pathogens in our preliminary research. The genomic DNA was isolated and sequenced. The draft genome consists of a 6,142,102 bp. A total of 5,335 CDSs and 66 RNA genes including rRNA, tRNA and ncRNA genes were identified. The general features of the genome of strain EDOX are summarized in Table 1. To classify at the species level, ANI and dDDH values were determined. Strain EDOX exhibited $\geq 99.27\%$ ANI and $\geq 96.80\%$ dDDH compared with the closest related species, i.e. *Pseudomonas mediterranea* CFBP 5447, which is above the gold standard threshold value of 95% ANI and 70% dDDH for the delineation of species. Therefore, strain EDOX was identified as *Pseudomonas mediterranea* EDOX. This whole genome analysis result has been deposited at DDBJ/ENA/GenBank under the accession number [GCA_016735085.1](#).

Table 1. Draft genome features of *P. mediterranea* EDOX

Characteristics	Genome
Size (bp)	6,142,102
G+C content (%)	61.4
N50 (bp)	422,748
Genes number	5,401
Contigs	83
CDS number	5,335
rRNA	4
tRNA	58
ncRNA	4

Automated Searching for Secondary Metabolite Clusters Using the antiSMASH Pipeline

By bioinformatic evaluation of the genome sequence of EDOX using antiSMASH 6.0, 12 biosynthetic gene clusters were identified (Table2). Five of these are NRPs BGCs predicted to related to the biosynthesis of syringomycin (Scholz-Schroeder et al., 2003), syringopeptin (Scholz-Schroeder et al., 2001), anikasin (Götze et al., 2017), crochelin A (Baars et al., 2018) and fragin (Jenul et al., 2018). Two were identified as type I PKs-NRPs hybrid BGCs and related to the biosynthesis of lankacidin C (Mochizuki et al., 2003) and entolysin (Vallet-Gely et al., 2010). One siderophore BGC and One Ripp-like BGC were also found in the genome. In addition, four other BGCs were discovered. Type 'other BGC' is a cluster containing a secondary metabolite-related protein that does not fit into any other category.

Table 2. Secondary metabolite and antibiotic gene clusters identified from *P. mediterranea* EDOX using antiSMASH 6.0 and BAGEL4

BGCs cluster	Type	Most similar known BGCs	MIBiG BGC ID	Mass spectrometry Identification
1	Type I PKs-NRPs hybrid	Lankacidin C (13% of genes show similarity)	BGC00011 00	not detected
2	NRPs	Syringomycin (100% of genes show similarity)	BGC00004 37	Cormycins (Strano et al., 2015)
3	NRPs	Syringopeptin (100% of genes show similarity)	BGC00004 38	edox-1, edox- 2*
4	Siderophore	-	-	not detected
5	NRPs	Anikasin (22% of genes show similarity)	BGC00015 09	not detected
6	Type I PKs-NRPs hybrid	Entolysin (17% of genes show similarity)	BGC00003 44	not detected
7	NRPs	Crochelin A (15% of genes show similarity)	BGC00020 01	not detected
8	Other	Fengycin (13% of genes show similarity)	BGC00010 95	not detected
9	Other	-	-	not detected
10	RiPP	-	-	not detected
11	Other	APE Vf (45% of genes show similarity)	BGC00008 37	not detected
12	NRPs	Fragin (37% of genes show similarity)	BGC00015 99	not detected

Hyphens (-) were indicating that no similarity was found with known biosynthetic gene clusters (BGCs) in MIBiG repository. *edox-1 and edox-2 were later renamed as medipeptin A and medipeptin B.

Purification and Identification of Lipopeptides

Two compounds initially named edox-1 and edox-2, were isolated and purified from strain EDOX (Table 2). The compounds were subjected to LC-MS and LC-MS/MS, which showed m/z values of 1041.60 $[M+2H]^{2+}$ (Figure 1B) and 1026.09 $[M+2H]^{2+}$ (Figure 1C) respectively. As shown in Figure 1, these two lipopeptides were identified as 22-amino acid cyclic lipopeptides, i.e., Dhb-Pro-Ala-Ala-Ala-Val-Val-Dhb-Thr-Val-Ile-Dha-Gly-Ala-Ala-Val-Dhb-Thr-Ala-Dab-Ser-Ile and Dhb-Pro-Ala-Ala-Ala-Val-Val-Dhb-Gly-Val-Ile-Dha-Ala-Ala-Ala-Val-Dhb-Thr-Ala-Dab-Ser-Ile, respectively. Analyses using high-resolution LC-MS/MS, the primary ions at m/z 2082.20 $[M+H]^+$ and 2051.18 $[M+H]^+$ showed a distinct sequence tag for 16 amino acid residues with the sequences Pro-Ala-Ala-Ala-Val-Val-Dhb-Thr-Val-Ile-Dha-Gly-Ala-Ala-Val-Dhb and Dhb-Pro-Ala-Ala-Ala-Val-Val-Dhb-Gly-Val-Ile-Dha-Ala-Ala-Ala-Val-Dhb, respectively. Furthermore, the mass spectrum showed two larger fragments of m/z 254.167 and m/z 473.264. The m/z 254.167 fragment's molecular formula corresponded to a Dhb residue linked to 3-OH decanoic acid also known as monomer C10:0-OH(3), which is similar to the structure of corpeptin (Emanuele et al., 1998). The m/z 473.264 fragment's molecular formula has a Thr-Ala-Dab-Ser-Ile cyclic tail, which was consistent with nunapeptin (Michelsen et al., 2015; Hennessy et al., 2017) and corpeptin. Lipopeptides edox-1 and edox-2 showed

A.

B.

Figure 1. Characterization of Purified CLPs produced by strain EDOX. (A) HPLC chromatographic profile of crude extract from supernatant of strain EDOX. (B) *De novo* sequencing of medipeptin A (m/z 2082.20) predicted from gene cluster 3 obtained by genome mining of *P. mediterranea* EDOX by using LC-MS/MS. (C) *De novo* sequencing of the medipeptin B (m/z 2051.18) encoded by gene cluster 3 by using LC-MS/MS. (D) The proposed chemical structure of medipeptin A.

some structural resemblance to nunapeptin from *P. fluorescens* In5 (Michelsen et al., 2015), to braspeptin from *Pseudomonas* sp. 11K1 (Zhao et al., 2019) and to corpeptin A from *P. corrugate* CFBP 5454 (Emanuele et al., 1998;Strano et al., 2015) as well as to thanapeptin from *Pseudomonas* sp. SH-C52 (Van Der Voort et al., 2015) (Figure 2). Therefore, in agreement with the naming of corpeptin produced by *P. corrugate*, we designated the edox-1 and edox-2 as medipeptin A and medipeptin B respectively. Based on the genome mining data, these two lipopeptides purified from the bacteria culture are obviously in accordance with cluster 3 identified by antiSMASH 6.0 (Figure 3).

medipeptin A	2082.22 [M+H ⁺]	C ₁₀ H ₁₉ O ₂	Dhb	Pro	Ala	Ala	Ala	Val	Val	Dhb	Thr	Val	Ile	Dha	Gly	Ala	Ala	Val	Dhb	Thr	Ala	Dab	Ser	Ile
medipeptin B	2051.21 [M+H ⁺]	C ₁₀ H ₁₉ O ₂	Dhb	Pro	Ala	Ala	Ala	Val	Val	Dhb	Gly	Val	Ile	Dha	Ala	Ala	Ala	Val	Dhb	Thr	Ala	Dab	Ser	Ile
corpeptin	2095.21 [M+H ⁺]	C ₁₀ H ₁₉ O ₂	Dhb	Pro	Ala	Ala	Ala	Val	Val	Dhb	Hse	Val	Ile	Dha	Ala	Ala	Ala	Val	Dhb	Thr	Ala	Dab	Ser	Ile
nunapeptin	2109.22 [M+H ⁺]	C ₁₂ H ₂₃ O ₂	Dhb	Pro	Ala	Ala	Ala	Val	Ala	Dhb	Ser	Val	Ile	Dha	Ala	Val	Ala	Val	Dhb	Thr	Ala	Dab	Ser	Ile
thanapeptin	2120.22 [M+H ⁺]	C ₁₀ H ₁₉ O ₂	Dhb	Pro	Ala	Ala	Pro	Val	Val	Dhb	Thr	Val	Ile	Dha	Ala	Ala	Ala	Val	Dhb	Thr	Ala	Dab	Ser	Ile
syringopeptin	2144.20 [M+H ⁺]	C ₁₀ H ₁₉ O ₂	Dhb	Pro	Val	Val	Ala	Ala	Val	Val	Dhb	Ala	Val	Ala	Ala	Dhb	Thr	Ser	Ala	Dhb	Ala	Dab	Dab	Tyr

Figure 2. Comparison of medipeptins with similar CLPs. The macrolactone ring shaped between the C-terminus of each peptide and a hydroxyl group of a threonine is remarked by square area. The different parts in medipeptins, compared with other CLPs, was marked in red. non-canonical amino acids are abbreviated as follows: Dhb dehydrobutyrine; Hse homoserine; Dha dehydroalanine; Dab 2,4-diaminobutyric acid.

Biosynthesis of Medipeptins by *Pseudomonas* sp. EDOX

Based on the genome mining, a total of 12 BGCs were discovered in the genome of strain EDOX. We identified and characterized the gene cluster for medipeptin biosynthesis

(Figure 3). This BGC contains 11 genes, including three large biosynthetic genes (*mepA-C*), six genes associated with export of peptides (*mepD*, *pseA-C*, *pseE* and *pseF*), one gene related to biosynthesis regulation of medipeptins (*mepP*) and one gene encoding a thioesterase (TE) domain. Based on the structure of medipeptins, we determined that medipeptin biosynthesis is catalyzed by genes *mepA-C* in a colinear manner, which is identical to the biosynthesis of corpeptin (Strano et al., 2015). Moreover, medipeptin A and B show two amino acid residues difference to corpeptin and nunapeptin, one amino acid residue difference to thanapeptin and 16 amino acids residues different to syringopeptin (Figure 2). All these differences may lead to distinctive antimicrobial activity of each lipopeptide.

Figure 3. Characterization of biosynthetic gene cluster (BGC) of medipeptins. Transporter genes are indicated in blue; Core biosynthetic genes are shown in red; Regulator genes is labeled in pink. Three core biosynthetic genes (*mepA-C*) contain 22 modules including adenylation (A), condensation (C), Thiolation (T) and Thioesterase (TE) domains. The predicted amino acids sequences are compared with identified medipeptins below. Abbreviation: FA, fatty acid; Nrp, uncertain amino acid.

Determination of MIC

The antimicrobial activity of medipeptin A against pathogenic bacteria was determined by MIC assays. In our study, purified medipeptin A showed potent antimicrobial activity against several tested Gram-negative pathogens, including *X. translucens*, *P. syringae*, and *X. campestris*, with a MIC value of 4 to 16 µg/mL (Table 3). In addition, purified medipeptin A also showed potent antimicrobial activity against Gram-positive bacteria, including *M. flavus*, *S. aureus* and *B. cereus*, with a MIC value of 2 to 8 µg/mL (Table 3).

Table 3. MIC value of medipeptin A against pathogenic bacteria

Type of pathogens	Pathogens	MIC (µg/mL)		
		medipeptin A	Colistin	Vancomycin
Gram-negative	<i>Xanthomonas campestris</i> pv. <i>campestris</i> NCCB92058	16	1	nd
	<i>Xanthomonas translucens</i> pv. <i>graminis</i> LMG587	4	1	nd
	<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	8	1	nd
	<i>Ralstonia syzygii</i> subsp. <i>Syzygii</i> LMG6969	>32	1	nd
	<i>Escherichia coli</i> ET8	>32	2	nd
	<i>Klebsiella pneumoniae</i> LMG20218	>32	2	nd
	<i>Acinetobacter baumannii</i> LMG01041	>32	2	nd
Gram-positive	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> 5334R4	8	nd	0.5
	<i>Bacillus cereus</i> ATCC14579	8	nd	0.5
	<i>Micrococcus flavus</i> (not pathogenic)	2	nd	0.25

nd was indicating that MIC values was not detected.

Medipeptin A Acts as a Bactericidal Antibiotic against Gram-positive Pathogens but as a Bacteriostatic Antibiotic against Gram-negative Pathogens

The time dependency of antibiotic action is a sound method for determining whether a compound is bacteriostatic or bactericidal. In this study, we monitored the killing kinetics of medipeptin A against *X. translucens* and *S. aureus*. Lipopeptide medipeptin A showed bactericidal capacity against *S. aureus*, and it killed all the *S. aureus* in 4 h at a desirable concentration (10X MIC) (Figure 4A). However, the growth of *X. translucens* cells treated with medipeptin A just stopped or showed limited growth when compared to the untreated cells (Figure 4A). These results demonstrate that medipeptin A may act as a bactericidal antibiotic against *S. aureus* and acts as a bacteriostatic antibiotic against *X. translucens*.

Medipeptin A Permeabilizes the Cell Membrane of Gram-positive Bacteria

We analysed whether medipeptin A can permeabilize the cell membrane of *S. aureus* using a commercial LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen). Cells with an intact membrane will stain green, whereas cells with a compromised membrane will stain red. The results show that medipeptin A disrupts the membrane of *S. aureus* cells when compared to the control treated with DMSO, which showed no influence on the membrane permeability of *S. aureus* cells (Figure 4B). These results demonstrate that medipeptin A acts as a potent antibiotic against Gram-positive bacteria by disrupting the cytoplasmic membrane.

Figure 4. Investigation of the mode of action of medipeptin A. (A) Time killing curve of medipeptin A (10X MIC) to *S. aureus* and *X. translucens* pv. *graminis*. (B) Membrane permeability of medipeptin A (5X MIC) to *S. aureus*. Nisin was added at a concentration of 2.5 $\mu\text{g/ml}$, the same amount of DMSO was treated as control. Cells with a compromised membrane integrity were stained in red, while cells with intact membrane were stained in green. (C) Medipeptin A binds to LTA. Medipeptin A (24 $\mu\text{g/ml}$ 3X MIC) was added to each well, and the same volume of DMSO was used as a negative control. Nisin (2 $\mu\text{g/ml}$) was used as a positive control. (D) Medipeptin A binds to lipid II (Gram-positive lipid II, lipid II-Lys). Medipeptin A was added at a concentration of 40 $\mu\text{g/ml}$; Vancomycin was added at a concentration of 200 $\mu\text{g/ml}$; *, the position of lipid II-Lys

added (300 μ M, 4 μ l)

Medipeptin A Binds to Lipoteichoic Acid (LTA) and Lipid II

To assess if medipeptin A has LTA binding action, bacterial growth assays were performed in 96-well plate with or without LTA. The results showed that cells treated with medipeptin A at a concentration of 3X MIC value grew as well as untreated cells when both of them were exposed to LTA (Figure 4C), which demonstrates that medipeptin A has an LTA binding capacity. Furthermore, the addition of lipid II originating from Gram-positive bacteria (lipid II-Lys) decreased the inhibition zone of medipeptin A toward *S. aureus* (Figure 4D), suggesting that medipeptin A binds to Gram-positive-type lipid II as well.

Discussion

In this study, we isolated and characterized *Pseudomonas mediterranea* EDOX from tomato leaf endosphere. This strain can produce medipeptins to exert strong inhibitory activity against plant and human pathogens. In addition, we sequenced the genome of strain EDOX and conducted in silico analysis of BGCs. Furthermore, two CLPs, designated as medipeptins, were purified and characterized and their encoding gene cluster was identified. Their predicted sequence is shown in Figure 3. Subsequently, we investigated the mechanisms of medipeptins against Gram-positive and Gram-negative pathogens. Medipeptin A, binding to both LTA and lipid II as well as forming pores in membranes, is bacteriocidal against Gram-positive bacteria (*Staphylococcus aureus* subsp. *aureus* 5334R4). In contrast, medipeptin A acts as a bacteriostatic antibiotic against Gram-negative pathogens (*Xanthomonas translucens* pv. *graminis* LMG587). These findings are

suggesting that not only *P. mediterranea* EDOX have potential to be used as biocontrol agent for controlling plant pathogens but also medipeptins contribute to develop new antibiotics and provide alternatives treatments for pathogens. Interestingly, *P. mediterranea* is a pathogenic species for plants such as *P. mediterranea* CFBP 10906 (Alippi and López, 2010). While our strain *P. mediterranea* EDOX is isolated from leave endosphere of a healthy tomato plant which indicates that *P. mediterranea* EDOX may be a true endophyte and play a beneficial role in tomato plants. This phenomenon is reasonable since the different behavior of same species can be explained by rather subtle differences (e.g., the expression of genes involved in plant defense reactions) (Sheibani-Tezerji et al., 2015) and the influences from the host and/or the environmental conditions (Brader et al., 2017). For instance, *P. syringae* is a well-known pathogen worldwide, while *P. syringae* 260-02 was shown to promote plant-growth and exert biocontrol of *P. syringae* pv. tomato DC3000, *Botrytis cinerea* and *Cymbidium Ringspot* (Passera et al., 2019). The biocontrol ability and its mechanisms of *P. mediterranea* EDOX need to be further research.

Genome mining has become a powerful tool for the discovery of microbial natural products and understanding the activity profiles of bacteria. One of the advantages of this approach is that it unveils the full biosynthetic capacity of a microorganism (Jahanshah et al., 2019). The ever-increasing availability of genome-sequencing data and the bioinformatic annotation of biosynthetic gene clusters (BGCs) facilitates the discovery of novel natural products. The inspection of a new *Pseudomonas* genome allowed us to predict the peptide sequence of multiple new variants and to identify potential producers of CLPs.

The BGC for biosynthesis of medipeptins in *P. mediterranea* EDOX is quite similar to that of some known CLPs BGCs in *Pseudomonas* strains, including corpeptin BGC and nunapeptin BGC (Michelsen et al., 2015; Strano et al., 2015), but the structure and function of the potential CLPs produced by strain EDOX have not yet been investigated. The biosynthesis of medipeptin A and B are catalyzed by three NRPS genes (*mepA*, *mepB*, and *mepC*) in a collinear manner (Figure 3). By combining genome mining and LC-MS/MS analysis, we have determined the molecular structure of medipeptin A and medipeptin B. Medipeptins were produced as a family of cyclic lipopeptides of 22 amino acid residues with molecular masses 2082.20 [M+H]⁺ (medipeptin A) and 2051.18 [M+H]⁺ (medipeptin B) (Figure 1).

Even though there are some reports showing the antimicrobial and/or antifungal activity of similar CLPs (corpeptin, nunapeptin, etc.), the antimicrobial mode of action of them is still not clear. To this end, we investigated the antimicrobial mechanisms of medipeptin A. In this study, we show that the newly discovered medipeptin A exerts its potent antimicrobial activity against Gram-positive pathogens via binding to both LTA and lipid II and probably by subsequently forming pores in membranes. LTA is a crucial constituent of Gram-positive cell envelopes, consisting of a poly (GroP) backbone linked to a glycolipid membrane anchor (Edgar et al., 2019). LTA, which is anchored to the cell envelope, could control autolysin activity. The interaction of CLPs with LTAs can result in loss of regulation of autolysins, which then causes autolysis by hydrolysing peptidoglycan chains and peptide bridges of murein (Bierbaum and Sahl, 1985). In addition, the interaction between LTA and CLPs is believed to facilitate penetration of CLPs through the thick peptidoglycan layer, allowing the CLPs to reach and act on the cytoplasmic

membrane (Yasir et al., 2019). In our study (Figure 4C), the added LTAs affect the activity of medipeptin A against *S. aureus*, which suggests that medipeptin A binds to LTA. Since lipid II is the bactoprenol-bound precursor of the bacterial cell wall, binding to lipid II could block the incorporation of lipid II into peptidoglycan, resulting in slow cell lysis and/or enhances the stability of pore formation, which increases the activity of the peptide (Bauer and Dicks, 2005). Based on the lipid II binding assay, and the fact that the antimicrobial activity of medipeptin A decreased after treatment of the cells with lipid II (Figure 4D), we suggest that medipeptin A can also bind to lipid II to exert its activity against *S. aureus*. In addition, we observed that cells of *S. aureus* could be stained red after treated with medipeptin A, indicating that medipeptin A shows pore-forming activity in biological membranes of *S. aureus* (Figure 4B).

To conclude, we isolated *P. mediterranea* EDOX from tomato endosphere. One BGC encoding the novel medipeptin biosynthesis cluster was identified from the genome sequence of EDOX. The purified medipeptin A has bactericidal activity against Gram-positive pathogens, but a bacteriostatic activity towards Gram-negative pathogens. We also show that medipeptin A combats Gram-positive pathogens through binding both to LTA and lipid II, as well as by forming pores in membranes. This study provides novel insights into the antimicrobial mode of action of medipeptin A, which broadens the array of candidates for drug development and biocontrol agents.

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Author's contributions

LZ and OPK conceived the study and designed experiments. ADJ and YY assembled the draft genome. LZ performed the experiments and wrote the draft manuscript. LZ and OPK corrected the manuscript. All authors have read and approved the manuscript.

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Chapter 5

Characterization of the interaction between *Priestia endophytica* FH5 and *Rhizoctonia solani*: biocontrol potential against tomato damping-off

Lu Zhou, Anne de Jong, Oscar P. Kuipers*

*Department of Molecular Genetics, University of Groningen, Groningen, The
Netherlands*

** Corresponding author; o.p.kuipers@rug.nl*

Abstract

Rhizoctonia solani is a soil-borne necrotrophic fungus that causes damping-off in tomato and poses a severe threat to its production. Typically, these infections are managed by chemical pesticides, which pollute the environment and may lead to medication resistance if used for an extended period of time. In addition, pesticides are not permitted to be used in organic agriculture either. Currently, biological control for the prevention of phytopathogen infection has steadily gained attention. To control damping-off of tomato in an alternative way, we conducted this study. *Priestia endophytica* FH5, that we isolated from healthy tomato rhizosphere soil, is a strain from the *Bacillaceae* and has biological activity against several plant pathogens, especially *R. solani* as we show. In order to understand the interaction between strain FH5 and *R. solani*, we purified the compounds produced by strain FH5 and identified them as kurstakins. By *in vitro* assays on plates and microscopic glass slides, purified kurstakins showed strong biological activity against *R. solani*. Furthermore, we conducted a transcriptomic analysis of strain FH5 with or without exposure to *R. solani*. We provide evidence that strain FH5 altered the expression of genes associated with metabolism, in response to the fungal pathogen *R. solani*, including genes related to amino acid transport, carbohydrate transport, energy production and conversion, and inorganic ion transport and metabolism. Besides, we found that genes encoding three novel ribosomally synthesized and post-translationally modified peptides (RiPPs) showed an upregulated expression level, which indicates the production of novel compounds with possible antagonistic activity towards *R. solani*. We also show that *Priestia endophytica* FH5 can significantly decrease the disease incidence of *R. solani* ($P < 0.05$) when compared with the control *in vivo*. Altogether, our study generates more insights into the

interaction between *Priestia endophytica* FH5 with the fungal pathogen *R. solani* and highlights its good biocontrol potential to fight tomato damping-off.

Keywords: *Priestia endophytica*, *Rhizoctonia solani*, kurstakin, antifungal compounds, transcriptomics, tomato, damping-off

Introduction

Tomato plants (*Solanum lycopersicum*) are members of the *Solanaceae* family (Dorais et al., 2008), and they belong to the most important horticultural crops in the world (Vos et al., 2014). Up to now, tomato production has been hampered by the adoption of cultivars that are sensitive to diseases and pests, resulting in significant output losses (Singh et al., 2017). Tomato plants are frequently attacked by fungi from the genus *Rhizoctonia* (Ghosh et al., 2021). *Rhizoctonia solani* is an anamorphic soil-borne pathogen, which causes tomato damping-off that significantly reduces yield. In crop growing areas, the pathogen lives as mycelia or resistant structures known as sclerotia in plant detritus and on weeds (Carling et al., 2002;Tsrer, 2010). Chemical substances (fungicides) have mostly been used to manage plant diseases. Many efficient chemical compounds have been employed against a variety of plant diseases, but owing to health and environmental risks, residue persistence, and chemical resistance in pathogens and pests, this has not been deemed a long-term solution (Zaidi et al., 2009;Damalas and Eleftherohorinos, 2011). Plant growth-promoting rhizobacteria (PGPR) have the potential to replace chemical pesticides as biocontrol agents, lowering the amount of unwanted chemical residues in agriculture. Plant growth promotion, plant disease suppression, and rhizosphere competence (colonization and survival on plant roots) are all key requirements for commercial product development (Perez-Garcia et al., 2011;Qiao et al., 2017).

Microorganisms are capable of producing a wide spectrum of antibacterial and (or) antifungal compounds. These compounds are mainly derived from secondary metabolism (Bulgarelli et al., 2013). *Bacillus* strains have been known for decades to produce a wide variety of secondary metabolites that mediate antibiosis (Oliševska et al., 2019). It is presently predicted that at least 4–5 percent of the genome of every *Bacillus* strain is dedicated to the synthesis of antimicrobial chemicals (AMCs) (Stein, 2005). Antimicrobial peptides make up the majority of these compounds (AMPs). Their structures are often cyclic, hydrophobic, and contain unusual residues and moieties like D-amino acids (AA) and intramolecular thioether linkages and can also be lipidated. Aside from AMPs, volatile metabolites also form a broad class of antimicrobials that play a variety of metabolic and functional roles (Caulier et al., 2019).

The use of RNA-Seq to analyze the entire transcriptome has provided detailed insight into gene regulation and how it changes as a result of interactions between beneficial bacteria and pathogens, which can lead to the identification of antimicrobial drugs and help to elucidate biocontrol mechanisms (Ribeiro da Cunha et al., 2019). Moreover, genome-based expression profiling provides a valuable tool for antimicrobial discovery, enabling further functionality studies of unknown secondary metabolites genes, mechanism-of-action (MOA) studies, and development of novel types of antimicrobial screening (Freiberg et al., 2004). Today's genome mining efforts often depend on the postulation that the products of BGCs can remain obscure because of insufficient levels of gene expression (Chiang et al., 2011; Rutledge and Challis, 2015; Okada and Seyedsayamdost, 2017). Comparative transcriptomics provides a new level of understanding to genome mining efforts by revealing which BGCs are expressed and

which are silent (Amos et al., 2017). Therefore, transcriptome profiles can be used to effectively prioritize strains for biosynthetic research.

Priestia endophytica FH5 (formerly named *Bacillus endophyticus* FH5), displays potent bioactivity against various phytopathogens, and was isolated in a previous study (Zhou et al., 2021). Some known and unknown biosynthetic gene clusters (BGCs) were identified from its genomic data. In order to thoroughly investigate the effects of the interaction between strain FH5 and *R. solani*, and to identify biocontrol compounds, we performed this study.

Materials and Methods

Strains and Culture Condition

The microorganism *Priestia endophytica* FH5 was grown on Lysogeny Broth (LB) agar or LB broth at 28 °C. The fungal pathogen *Rhizoctonia solani* was cultured on Potato Dextrose Agar (PDA) at room temperature (Zhou et al., 2019).

Extraction and Purification of Secondary Metabolites from Strain FH5

For purification of secondary metabolites, the previous protocol was used with modification (Yu et al., 2019). Strain FH5 was cultivated in 400 ml LB media at 28 °C with shaking at 210 rpm for 48 hours from a seed culture. The supernatant was then collected by centrifugation (10,000 x g, 30 min), and filtered through a 0.45 µm cellulose acetate membrane. The filtrate was precipitated with ammonium sulfate at 40% (w/v) saturation and the pellets were harvested by centrifugation (10,000 x g, 30 min). The pellets were dissolved in Milli-Q H₂O and filtered through a 0.45 µm cellulose acetate membrane to

obtain crude extracts. The crude extracts were then subjected to a reverse high-performance liquid chromatography (HPLC) equipped with an Aeris widepore 3.6 μ XB-C18 250 x 4.6mm column for purification. HPLC-grade water supplemented with 0.1 percent trifluoroacetic acid (TFA) (solvent A) and acetonitrile supplemented with 0.1 percent TFA (solvent B) were used as mobile phases (solvent B). An elution two-step gradient of solvent A/solvent B (from 70:30 to 50:50 in 40 min and from 50: 50 to 5:95 in 2min) was applied at a flow rate of 0.5 ml/min, for compound purification. A UV-detector, set at a wavelength of 214 nm, was used to monitor the elution of the compounds.

Identification of Lipopeptides Produced by Strain FH5

To obtain insight into the molecular configurations of the peptides, LC-MS/MS was performed using a Q-Exactive OrbitrapTM-based mass spectrometer equipped with an Ultimate 3000 UPLC, an ACQUITY BEH C18 column (2.1 50 mm, 1.7 μ m particle scale, 200; Waters), a HESI-II ion source, and an Orbitrap detector. A 30-minute gradient of 5-90 % MeCN/Milli-Q water (v/v) with 0.1 % formic acid (v/v) was used in each run.

Microscopic Inhibition of *R. solani* Using Purified Compounds *in vitro*

The biological activity of *P. endophytica* FH5 CLPs against *R. solani* was tested under *in vitro* microscopic conditions (Oni et al., 2019). In brief, sterilized microscopic glass slides were coated with a thin, flat layer of water agar and put in a plastic Petri dish with moist sterilized filter paper. Each glass slide was infected with an agar plug (diameter = 5 mm) obtained from an actively growing Potato Dextrose Agar (PDA) plate of *R. solani*. Stock solutions (100 mM) of pure compound solutions were prepared in DMSO, and dilutions

were prepared in sterile MilliQ water. 20 µl each of compounds solution (10 mM) were dropped at two sides of the glass slide (about 2 cm from the mycelium plug). All plates were incubated at 28°C for 4 days. Microscopic slides were examined for hyphal leaking and branching under an Olympus MVX10 microscope.

Interaction Assay between Strain FH5 and *R. solani*

P. endophytica FH5 was grown overnight in LB broth (28 °C). Cells were collected and resuspended in phosphate-buffered saline (PBS buffer). The OD₆₀₀ of cells was adjusted to 1.0. A 0.5-cm mycelium plug of 3-day-old *R. solani* was placed at the center of a PDA plate, and 5 µl of cell suspension was inoculated at a distance of 2 cm from the fungal plug. Plates were incubated at 28 °C for 3 days. As a control, *P. endophytica* FH5 was grown on PDA plates without *R. solani* in the same condition. Scraped colonies of FH5 were suspended in 400 µl DEPC-treated water before being frozen in liquid nitrogen for RNA isolation.

RNA Extraction

Total RNA was extracted with the Roche RNA isolation kit with some modifications. *P. endophytica* FH5 cells previously suspended in 400 µl DEPC-treated water were used for RNA isolation. The 500 µl chloroform/isoamyl alcohol (24:1, v/v), 50 µl of 10% SDS, and 0.5 g glass beads (0.5 µm) were added to cells suspension. The samples were homogenized using a Mini-BeadBeater (BioSpec, United States) for three 50 s pulse and were chilled on ice between pulses. Samples were centrifuged at 10,000 rpm (4 °C) and the supernatant was mixed with 500 µl chloroform/isoamyl alcohol (24:1, v/v). After centrifugation at 4°C for 8 min, the upper phase was transferred to a fresh tube and two

volumes of lysis/binding buffer from the Roche RNA isolation kit were added. The mixture was transferred to the columns from the kit and centrifuged for 30 s at 8,500 rpm. 10 µl DNaseI and 5 µl Ribolock were added to each column and incubated at room temperature for 30 min. After two washing steps with the wash buffer (Roche RNA isolation kit), the RNA was eluted with 50 µl elution buffer (Roche RNA isolation kit) and stored at -80°C before sequencing. The Bioanalyzer (Agilent Technologies, Wilmington, DE, United States) was used to assess the RNA quality.

RNA Sequencing and Data Analyses

RNA sequencing was conducted at the University Medical Center Groningen (UMCG). FastQC (Andrews, 2010) was used to check the raw RNA-Seq reads, and Trimmomatic (Bolger et al., 2014) was used to trim them. STAR (Dobin et al., 2013) matched the sequences to the *P. endophytica* FH5 reference genome sequence. The *P. endophytica* FH5 core genome's RPKM (Reads Per Kilobase Million) table was created. Differential gene expression analyses were conducted with the web server pipeline T-Rex2 (de Jong et al., 2015), in which the RPKM values of the *P. endophytica* FH5 grown in the presence of *R. solani* was compared with that grown in the absence of *R. solani*. The thresholds for significance were p-value < 0.05 and fold-change > 2 ("TopHits" in T-Rex). The Genome2D web server (Baerends et al., 2004) was used to do functional classification, which included GO (Ashburner et al., 2000), InterPro (Hunter et al., 2009), KEGG (Kanehisa and Goto, 2000), and PFAM domains (Bateman et al., 2004). This study's RNA-seq data have been uploaded to the NCBI Gene Expression Omnibus (GEO3) under the accession number GSE179605.

In vivo* Evaluation of Biocontrol Activity of FH5 on *R. solani

The plant assay was conducted in a climate chamber as protocols reported before with modification (Manganiello et al., 2018;Zhu et al., 2020). To examine the effect of the biocontrol ability of *P. endophytica* FH5 on disease incidence of tomato seedlings infected with *R. solani*, we initiated this investigation. The treatments were as follows: tomato plant treated with *R. solani* (P+R); tomato plants treated with *R. solani* + *B. velezensis* FZB42 (P+R+B); tomato plants treated with *R. solani* + *P. endophytica* FH5 (P+R+P). Tomato seeds (cultivar: Moneymaker) were surface disinfected and pre-germinated horizontally on 15 cm Petri dishes with Murashige & Skoog (MS) agar in a growth chamber (16 hours daytime at 24+/- 2 °C, 8 hours darktime at 21+/-2 °C) for 2 days. Pre-germinated seeds were transferred to plastic pots filled with sterilized soil and incubated for 3 weeks before being inoculated with bacterial strains. Strains FZB42 and FH5 were grown in LB media at 28°C with 220 rpm for overnight. Cells were then harvested and washed with Phosphate buffer solution (PBS) and diluted to a final concentration of 1×10^7 cells/ml with PBS. 30 ml of each bacterial suspension (10^7 cells/ml) in PBS buffer was carefully brought into the soil near the roots of the tomato plant using a micropipette, while the same amount of PBS buffer was used as control. After 4 days of inoculation with FH5 and FZB42, each seedling was inoculated in the soil with *R. solani* (30 ml of *R. solani* biomass homogenized in PBS at 2% w/v). All plants were placed in a growth chamber under controlled conditions, temperature (25°C), RH 70% and photoperiod (16 h of light/8 dark). Thirty tomato seeds were employed for each treatment and sown in pots containing sterile soil, with three independent biological replicates used for each treatment. The disease development was evaluated every 24 h for a 5-day period after pathogen inoculation, and disease incidence was recorded and calculated according to

the following formula:

Disease Incidence (%) = (the number of damping-off diseased seedlings in each treatment/total number of seedlings in each treatment) × 100.

Results

Purification and Identification of Kurstakins

P. endophytica FH5 showed antibacterial and antifungal activity in our previous study (Zhou et al., 2021). To identify and characterize the bioactive compounds, the supernatant of *P. endophytica* FH5 was precipitated with ammonium sulfate and substances further purified by HPLC. Two major peaks that eluted from HPLC were collected for LC-MS/MS to figure out their structure. The two peaks had m/z value of 892.44 and 906.46, corresponding to a singly protonated molecule $[M + H^+]$ respectively (Figure 1A.). The mass difference between these two molecules amounts to 14.02 Da, which is compatible with a CH_2 at the fatty acid chain. As shown in Figure 1B, 1C, and 1D., the two molecules are cyclic lipopeptides, consistent with the presence of seven identical amino acid residues. Thr-Gly-Ala-Ser-His-Gln-Gln attached to a C12:0/iC12:0 ($C_{12}H_{22}O$) or iC13:0 ($C_{13}H_{24}O$) fatty acid tail, respectively. The lactone ring of two molecules were formed between the C-terminal Gln and the Ser residue. By comparison with known compounds, these two molecules were designated as kurstakins.

A.

B.

C.

D.

Figure 1. Characterization of kurstakins purified from supernatant of *Priestia endophytica*

FH5. (A) HPLC chromatogram of compounds kurstakins extracted from FH5 and molecular structure of C12 kurstakin. (B) LC-MS/MS analysis of C12 kurstakin (m/z 892.43) and the proposed C13 kurstakin. (C) LC-MS/MS analysis of C13 kurstakin (m/z 906.45) and the proposed C13 kurstakin. (D) LC-MS/MS analysis of linear C13 kurstakin and amino acids residue composition.

Antifungal Activity of Kurstakins Against *R. solani*

The two compounds purified from the supernatant of *P. endophytica* FH5 were tested for biological activity against bacterial and fungal pathogens. The antagonistic activity was only observed on *R. solani* (Figure 2A.). Subsequent microscopic analysis showed that purified kurstakins caused either extensive branching or lysis of the mycelia of *R. solani* (Figure 2B.) which inhibits the growth of *R. solani*.

Figure 2. Effect of purified kurstakins on *R. solani*. (A) Inhibitory interaction between purified kurstakins and *R. solani* on PDA agar plates for 2 or 4 days at 28 °C. * indicates the point of position where kurstakins or DMSO added. (B) Effect of kurstakins on *R. solani* mycelium. Hyphal distortion and branching of *R. solani* caused by C12 kurstakin and C13 kurstakin under microscopy observation.

Global Transcriptional Profiles of *P. endophytica* FH5 in Response to *R. solani*

The filtered reads from the transcriptome analysis of FH5 with or without the presence of *R. solani* were mapped on the genome of strain *P. endophytica* FH5 to deduce the RPKM value of each gene. The RPKM values of the core genes (5,546 genes) were used to compare the transcriptomes of the strains under study using the T-REx pipeline.

Significant differentially expressed genes (DEGs) were shown in the TopHits genes close to cutoff values (p-value 0.05 and fold-change of 2) (Figures 3A). During the interaction with *R. solani*, 641 genes were significantly changed. Of those, 376 genes were upregulated, and 307 genes were downregulated (Figure 3B). 145 out of 641 altered genes were represented in the COG database and hence used to perform ortholog analyses. As shown in Table 1, the expression of genes associated with metabolism, including genes related to amino acid transport, carbohydrate transport, energy production and conversion, and inorganic ion transport and metabolism was found in significant change.

Figure 3. Analysis of differentially expressed genes (DEGs) of *Priestia endophytica* FH5 in response to tomato pathogen *R. solani*. (A) Volcano diagrams of DEGs of *Priestia endophytica* FH5 with or without exposure to *R. solani*. Spots in the right and left quadrant indicate genes that are significantly up or down regulated, respectively. Spots in the gray area indicate neither over- nor under-expressed genes. (B) The number of up and downregulated DEGs of *Priestia endophytica* FH5 with or without exposure to *R. solani*.

Table 1. Functional categories of genes regulated of FH5 exposed to *R. solani* according to COGs (Clusters of Orthologous Groups).

Class	Description	No. of genes
ID		(up/down regulated)
M	CELLULAR PROCESSES AND SIGNALING; Cell wall/membrane/envelope biogenesis	4/1
T	CELLULAR PROCESSES AND SIGNALING; Signal transduction mechanisms	2/1
L	INFORMATION STORAGE AND PROCESSING; Replication, recombination and repair	4/0
C	METABOLISM; Energy production and conversion	8/10
S	POORLY CHARACTERIZED; Function unknown	20/3
I	METABOLISM; Lipid transport and metabolism	6/2
E	METABOLISM; Amino acid transport and metabolism	16/10
V	CELLULAR PROCESSES AND SIGNALING; Defense mechanisms	2/3
Q	METABOLISM; Secondary metabolites biosynthesis, transport, and catabolism	4/2
K	INFORMATION STORAGE AND PROCESSING; Transcription	8/2
P	METABOLISM; Inorganic ion transport and metabolism	11/5
G	METABOLISM; Carbohydrate transport and metabolism	19/2

Transcriptional Analysis of Biosynthetic Gene Clusters (BGCs) Harbored by *P. endophytica* FH5 in Response to *R. solani*

As shown in Table 2, five gene clusters relevant to secondary metabolites were

downregulated from 1.2 to 3.3 folds in response to *R. solani*. These gene clusters were non-ribosomal peptide synthetases (cluster 1), a lasso peptide BGC (cluster 2), terpene BGC (cluster 4), non-ribosomal peptide synthetase (cluster 6), and type I PKS-NRPs hybrid BGC (cluster 7). In addition, another five gene clusters associated with the biosynthesis of class II lanthipeptides (cluster 3), Type III PKS (cluster 5), LAP (cluster 8), terpene (cluster 9), and class III lanthipeptide (cluster 10) were upregulated from 1.5 to 3.0-fold in response to *R. solani*. The kurstakin BGC was downregulated around 1.5-fold, ($P > 0.05$) in response to *R. solani* by analysis of RNASeq data, which suggests that the biosynthesis of kurstakin from FH5 is not significantly affected by the interaction with *R. solani*. However, three novel bacteriocins BGCs were significantly upregulated under *R. solani* treatment, including Cluster 3 (class II lanthipeptide BGC), Cluster 8 (LAP BGC), and Cluster 10 (class III lanthipeptide BGC), which indicates that these three BGCs could produce potential novel compounds with biological activity towards *R. solani*. Furthermore, the precursor of upregulated unknown LAP BGC was initially predicted by antiSMASH (Blin et al., 2019) into the gene FH5_02068 (Zhou et al., 2021). However, by combining the RNAseq analysis and manual search, the precursor ORF was found upstream of the three modification genes (Figure S1). The precursor of the LAP encoded by this unannotated gene contains 92 amino acid residues with numerous Cys and Ser residues, which is a distinguishing feature of LAPs (Arnison et al., 2013; Banala et al., 2013; Travin et al., 2019). The core peptide is anticipated to be the final 53 residues.

Table 2. Gene expression changes of secondary metabolites of biosynthetic genes of FH5 to *R. solani*

Locus	Gene Cluster		Fold Change
	Gene	Product	(Treatment/CK)
Cluster 1 (NRPs BGC)			
FH5_00584	non-ribosomal peptide synthetase	bacillibactin	-3.3
Cluster 2 (lasso peptide BGC)			
FH5_00650	ABC transporter	paeninodin-like	-1.2
FH5_00651	ATP-dependent cysteine protease		1.1
FH5_00652	ATP-dependent cysteine protease		-1.5
FH5_00653	putative kinases		-1.4
FH5_00654	precursor peptide		-1.6
FH5_00655	asparagine synthase		-1.3
Cluster 3 (class II lanthipeptide BGC)			
FH5_02831	precursor	unknown	2.6
FH5_02832	lantibiotic biosynthesis protein LanM		2.4
FH5_02833	ABC transporter		2.3
FH5_02834	precursor		1.5
FH5_02837	precursor		2.5
FH5_02838	lantibiotic biosynthesis protein LanM		2.0
Cluster 4 (terpene BGC)			
FH5_02884	Squalene/phytoene synthase	unknown	-2.2
FH5_02885	Sugar (and other) transporter		1.3

Locus	Gene Cluster		Fold Change (Treatment/CK)
	Gene	Product	
Cluster 5 (Type III PKS BGC)			
FH5_03164	Chalcone and stilbene synthases	unknown	1.5
FH5_03165	methyltransferase		2.4
Cluster 6 (NRPs BGC)			
FH5_03968	major facilitator transporter	kurstakin	-1.2
FH5_03969	non-ribosomal peptide synthetase		-1.4
FH5_03970	non-ribosomal peptide synthetase		-1.6
FH5_03971	non-ribosomal peptide synthetase		-1.5
Cluster 7 (type I PKS-NRPs hybrid BGC)			
FH5_04973	non-ribosomal peptide synthetase	unknown	-2.4
FH5_04974	cyclic peptide export ABC transporter		-2.3
FH5_04975	non-ribosomal peptide synthetase		-2.0
FH5_04976	type I polyketide synthase		-2.6
FH5_04977	non-ribosomal peptide synthetase		-2.3
Cluster 8 (LAP BGC)			
FH5_02065	SagB-type dehydrogenase domain	unknown	1.7
FH5_02066	SagD-thiazole/oxazole-forming maturase	peptide	2.1
FH5_02067	thiazole-containing protein maturation		2.1
unannotated	Precursor		1.8
Cluster 9 (terpene)			

Locus	Gene Cluster		Fold Change (Treatment/CK)
	Gene	Product	
FH5_02281	squalene-hopene cyclase	unknown	3.0
Cluster 10 (class III lanthipeptide BGC)			
FH5_03700	Precursor	unknown	2.7
FH5_03701	lantibiotic biosynthesis protein LanKC		2.4
FH5_03702	ABC transporter		2.2

Biocontrol Effect of *P. endophytica* FH5 on Damping-off Caused by *R. solani*

As shown in Table 3, all of the tested treatments significantly decreased the disease incidence of *R. solani* ($P < 0.05$) when compared with the control, but the disease incidence between the treatment with the well-known model strain FZB42 and strain FH5 did not show significant differences with each other at each time point. In the treatment with strain FH5, means of disease incidence reduction were 23.7% after 120 hours while the treatment with FZB42, means of disease incidence reduction were 30.0% after 120 hours. This result showed that *P. endophytica* FH5 has good potential of biocontrol activity for damping-off caused by *R. solani*.

Table 3. Effect of *P. endophytica* FH5 on damping-off caused by *R. solani* on two different tomato cultivars.

Cultivar	Treatments	Disease Incidence (DI, %)				
		24 h	48 h	72 h	96 h	120 h
Moneymaker	P+R	0	49.0±1.7a	95.3±4.0a	100±0.0a	100±0.0a
	P+R+B	0	31.0±1.7b	53.3±3.5b	67.7±5.0b	70.0±3.0b
	P+R+P	0	32.0±5.6b	50.0±3.0b	74.0±1.7b	76.3±3.5b

Values (mean ± standard error) in the same column followed by different letters indicate significant differences (p < 0.05). There are 30 seedlings per treatment and three independent biological replicates used for each treatment.

P+R: tomato plant treated with *R. solani*; P+R+B: tomato plants treated with *R. solani* + *B. velezensis* FZB42; P+R+P: tomato plants treated with *R. solani* + *P. endophytica* FH5.

Discussion

Priestia is a genus of Gram-positive mostly rod-shaped bacteria in the *Bacillaceae* family of the *Bacillales* order (*Priestia flexa* stains Gram-variable and *Priestia koreensis* stains Gram-negative) (Gupta et al., 2020). *Priestia endophytica* FH5 was isolated in our previous study and showed significant antifungal activity *in vitro* (Zhou et al., 2021). Secondary metabolites biosynthetic gene clusters (BGCs) harbored by *Priestia endophytica* FH5 were also identified. Notably, most of the BGCs remained unknown. To identify compounds produced by strain FH5, we purified the lipopeptides from the supernatant of cell culture. Only one family of compounds was discovered, and they were identified as kurstakins by LC-MS/MS (Figure 1). Kurstakins were initially identified in

Bacillus thuringiensis kurstaki HD-1 and were made up of partly cyclic heptalipopeptides with just different fatty acid chains (Hathout et al., 2000). The biosynthetic gene cluster for kurstakins lipopeptides encoded 3 kurstakin synthetases (*KrsA*, *KrsB*, and *KrsC*) (Béchet et al., 2012;Gélis-Jeanvoine et al., 2017).

Kurstakins have previously been discovered in bacterial cells, notably on spores, but not in the culture supernatant of generating strains (Hathout et al., 2000;Price et al., 2007;Abderrahmani et al., 2011). Kurstakins are secreted molecules with strong affinity for membranes. The presence of the basic amino acid histidine give the lipopeptide a small cationic charge and therefore is increasing its electrostatic interaction with cell membrane phospholipids, which may account for the high affinity (Béchet et al., 2012;Dubois et al., 2012). Purified kurstakins displayed a good antifungal activity against *Stachybotrys charatum* (Hathout et al., 2000) and might be pore-forming molecules with a narrow spectrum of activity. However, there is no link between antifungal activities and the presence of kurstakins, as certain strains that generate kurstakins do not show antifungal activity, while others that do not produce kurstakins do show antifungal activities (Abderrahmani et al., 2011). In addition to their antifungal activities, kurstakins can be used as biosurfactant to increase biodegradation of diesel oil (Diallo et al., 2019). In our study, we found that purified kurstakins have antifungal activity against *R. solani* based on a microscopic assay (Figure 2), suggesting that kurstakins have the potential to be used as biocontrol agents to prevent damping-off in tomato plants.

Global transcriptional analysis by RNA-Seq of FH5 in response to *R. solani* reveals clear differences in gene expression changes between with and without *R. solani* treatment (Figure 3 and Table 1). The genes represented by COG classes with known function were

mostly categorized in amino acid transport and metabolism, carbohydrate transport and metabolism, energy production and conversion, and inorganic ion transport and metabolism. This finding shows that the presence of pathogenic fungus *R. solani* might generate a stressful environment for FH5 growth and development. In addition, via investigation of secondary metabolites BGCs (Table 2), one novel class II lanthipeptide BGC (cluster 3), one novel linear azol(in)e-containing peptide (LAP) BGC (cluster 8), and one novel class III lanthipeptide BGC (cluster 10) were significantly upregulated. Among them, the novel class II lanthipeptide BGC (cluster 3), encoding a two-component lanthipeptide, was found with no similarity to any known BGCs. The α precursor shows a similarity of 53.33% to plantaricin W α , and the β precursor shows a similarity of 56.26% to haloduracin β (Zhou et al., 2021). Plantaricin W (Plw), a two-component bacteriocin, from *Lactobacillus plantarum*, inhibits a large number of Gram-positive bacteria. Plantaricin W α shows low antimicrobial activity individually but acted synergistically with plantaricin W β (Holo et al., 2001). The antimicrobial activity of haloduracin from *Bacillus halodurans* C-125 also depends on the presence of both modified α and β peptides (Lawton et al., 2007). This finding suggests that the two-component lanthipeptide in FH5 may synergistically exert antifungal activity towards *R. solani*. The novel linear azol(in)e-containing peptide (LAP) BGC (cluster 8) found in FH5 (Figure S1), encoding a precursor peptide with 92 amino acid residues, has no similarity to any known BGCs. The last 53 residues were predicted to be the core peptide. Linear azol(in)e-containing peptide (LAP) is distinguished by virtue of containing a variety of heterocyclic rings of thiazole and (methyl)oxazole which are derived from cysteine, serine, and threonine residues via ATP-dependent cyclodehydration and subsequent flavin mononucleotide-dependent dehydrogenation (Melby et al., 2011). A well-known peptide belonging to the LAP family,

discovered from plant growth promotion bacteria, is plantazolicin (Scholz et al., 2011). It is derived from *Bacillus velezensis* FZB42 and *Bacillus pumilus* (Molohon et al., 2016). The core peptide consists of 14 amino acid residues (RCTCTTISSSTF), with 10 of them (Cys, Ser/Thr) potentially serving as heterocycle formation sites. Whereas the precise biological role of PZN has not to be determined, it is only known to be a narrow-spectrum antibacterial molecule (Scholz et al., 2011). The class III lanthipeptide BGC (cluster 10), encoding a precursor peptide (FH5_03700) with 38 amino acid residues, shows no similarity to any known BGCs. The last 19 residues were predicted to be the core peptide. LanKC, a bi-functional modifying enzyme responsible for both dehydration and cyclization, modifies the precursor peptides of class III lantipeptides, which has three domains: an N-terminal phosphothreonine lyase, a central protein kinase, and a C-terminal putative cyclase domain (Arnison et al., 2013). Until now, class III lanthipeptides have been found to exhibit minimal or weak antibacterial, antiallodynic/antinociceptive, and antiviral activity (Takano et al., 2017). Taken together, although the compounds biosynthesized by these novel BGCs were not identified from the culture of FH5, the upregulation of these BGCs suggests that these compounds may contribute to the antifungal activity against *R. solani*, which needs to be further investigated by a mutagenesis and/or overproduction study.

In addition, the results of *in vivo* plant assay reveal that strain FH5 has good biocontrol ability for damping-off caused by *R. solani* (Table 3). Under *R. solani* infection, tomato seedlings treated with strain *P. endophytica* FH5 and *B. velezensis* FZB42 can reduce the diseases incidence 23.7% and 30.0%, respectively. Since *B. velezensis* FZB42 is utilized as a biofertilizer and a biocontrol agent in agriculture, it is being especially efficient against

fungal and bacterial pathogens (Fan et al., 2018). Our strain *P. endophytica* FH5 can achieve a similar biocontrol efficacy on *R. solani* compared with the model strain FZB42, which suggests that *P. endophytica* FH5 is a promising biocontrol strain with good application possibilities.

Taken together, we have investigated the response of *P. endophytica* FH5 to *R. solani* and the biocontrol activity for damping-off of tomato seedlings. According to our findings, *P. endophytica* FH5 changed the expression of genes associated to secondary metabolites. We isolated and characterized the FH5-produced chemicals (kurstakins) that can inhibit or disrupt *R. solani* proliferation. Furthermore, *P. endophytica* FH5 can significantly decrease the disease incidence of *R. solani* ($P < 0.05$) *in vivo*. Our study shows the potential biocontrol ability of *P. endophytica* FH5 for *R. solani*, helps us to better understand the interaction between FH5 and *R. solani*, as well as provides the possibility for identification of potential new secondary metabolites which are expected to have a good ability as anti-*R. solani* compounds.

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Supplementary data

Figure S1. LAP gene cluster of FH5 upregulated in response to *R. solani*. Genes labeled in green is precursor peptide. Genes labeled in red is modification and maturation enzymes. The cleavage point between leader and core peptide is indicated by a red arrow.

Chapter 6

General Discussion

Lu Zhou

*Department of Molecular Genetics, University of Groningen, Groningen, The
Netherlands*

Food Security and Sustainable Agriculture

678 million people were undernourished in 2018, and the situation is getting worse (Organization, 2020). Based on scientific projections, the global number of undernourished people in 2030 would exceed 840 million (Organization, 2020). By 2050, the world's population will have surpassed 9 billion people, resulting in an increased demand for accessible water, food, and arable land. Nutritional deficiencies, food safety issues, customer attitudes, post-harvest losses, climate change and inconsistencies in legislation are all huge challenges that must be addressed in order to ensure food security (Khan et al., 2021). Plant diseases caused by various phytopathogens can reduce crop yields and have a serious impact on food security. The traditional way for disease control by chemicals not only causes environmental problems but also harms the health of humankind and animals which stimulated researchers and growers/breeders to explore new and eco-friendly modes of disease control. The use of beneficial bacteria in agricultural production is an environmentally-friendly strategy for sustainable agriculture (Kannoja et al., 2019).

Beneficial Bacteria for Plant Growth

Use of beneficial bacteria with plant growth promoting activity has been investigated as a sustainable alternative to the use of chemical fertilizers and pesticides to improve agricultural yields since the 1980s, and effective plant growth promoting bacteria have been isolated from different parts of plants (e.g. the endosphere, phyllosphere, and rhizosphere) (Rilling et al., 2019).

Root-associated microbial communities provide their hosts with particular benefits, by

modulating growth, health, and productivity (Korenblum et al., 2020). For instance, plant growth promoting rhizobacteria (PGPR) exhibit multiple plant growth promoting activities including disease suppression with antimicrobial compounds, stimulation of induced systemic resistance (ISR), and physiological metabolic process improvement (Zhou et al., 2021). An understanding of the basic mechanisms of how plants and PGPR interact and benefit each other can provide new strategies to improve plant productivity and thereby help to maintain food security.

Natural Products Discovery

Microorganisms form a promising reservoir of a huge range of natural products that have contributed significantly to human, plant and veterinary life, since some microorganisms devote up to 15% of their genomic content to the production of specialized microbial metabolites (Davies, 2013).

The most well-known examples of natural products are antibiotics (Demain, 1999). Although antibiotics no longer refer only to natural products, in fact the majority of recent commercially available antibiotics are still based on natural chemotypes (Newman et al., 2003). The development of penicillin, which is produced by *Penicillium rubens*, and was discovered in 1928, as well as streptomycin, chloramphenicol, and tetracycline, which are produced by soil bacteria, heralded in the antibiotic era between 1945 and 1955 (Clardy et al., 2009). After this golden age, the efficiency of antibiotic research declines, and rediscovery is more frequent. Only a few novel antibiotics are identified, and the vast majority of new compounds are variants of known compounds (Bérdy, 2012). However, a new era in novel antibiotics discovery has begun with the

development of up-to-date DNA sequencing techniques, which vastly accelerates the research of biosynthetic potential in microorganisms (Vater et al., 2018) (Figure 1). The work presented in this thesis thus aimed to isolate beneficial bacteria from tomato rhizosphere soil, mine genome sequences for novel biosynthetic gene clusters (BGCs), identify and characterize novel antimicrobial compounds produced as well as investigate the biocontrol mechanisms between tomato plant and beneficial bacteria (Figure 2).

Figure 1. Timeline of natural products discovery.

Biosynthetic Potential of Antimicrobial Compounds produced by *Bacillus*

When it comes to crop protection, growth stimulation, and soil health improvement, PGPR will play an important role (Fernando et al., 2005). The main biocontrol mechanism for PGPR is based on the synthesis of antimicrobial compounds. In chapter 2, we describe the sequencing of the genomes of 10 isolated strains with antagonistic activities from tomato rhizosphere soil and their plant tissues (Zhou et al., 2019). In

chapter 3, we explored the biosynthetic potentials of these strains using genome mining tools, including antiSMASH 5.0 (Blin et al., 2019) and BAGEL 4 (van Heel et al., 2018). Briefly, we isolated 351 bacterial strains from rhizosphere soil of healthy tomato plants and their tissues, 181 of which are *Bacillus* sp. Among them, 34 *Bacillus* species showed antimicrobial efficacy against *Erwinia carotovora*, *Pseudomonas syringae*, *Rhizoctonia solani*, *Botrytis cinerea*, *Verticillium dahliae*, and *Phytophthora infestans*, according to *in vitro* antagonistic tests. *Bacillus* and *Paenibacillus* strains with good antagonistic activity have their genomes sequenced. We discovered 120 BGCs encoding NRPs, PKs-NRPs, PKs, terpenes, and bacteriocins using genome mining techniques, including well-known chemicals like fengycin (Vanittanakom et al., 1986), surfactin (Nakano et al., 1991), bacillibactin (Zhou et al., 2018), and subtilin (Parisot et al., 2008). Apart from discovery of some known BGCs, numerous novel BGCs were discovered. Furthermore, we also found that the NRPs and PKs-NRPs BGCs of *Bacillus* species encode highly conserved recognized chemicals as well as new variations by using previous reported genome sequences of *Bacillus* for genome mining. Our study highlights the large number of BGCs in *Bacillus* strains. These findings direct the way for the future use of *Bacillus* strains as biocontrol agents for the control of tomato diseases and as resources for the development of new antimicrobials.

Figure 2. A summary of each works conducted to study the natural products produced by beneficial bacteria as well as the plant-microbe interactions. In chapter 2, we isolated beneficial strains from rhizosphere soil and plant tissues of tomato plants and further we investigated the antimicrobial biosynthetic potential. In chapter 3, we characterized the biocontrol ability of *B. cabrelesii* BH5 for grey mould of tomato plants which can be caused by *B. cinerea*. In chapter 4, we identified and characterized novel antimicrobial lipopeptides, medipeptins which are produced by *Pseudomonas* sp. EDOX. In chapter 5, we studied the interaction between *P. endophytica* FH5 and *R. solani* and further investigated the biocontrol potential for damping-off in tomato plants.

***B. cabrelesii* BH5, a Biocontrol Agent to Fight Tomato Grey Mould**

Plants have their own pathogen management mechanisms. A prominent defensive mechanism relies on jasmonic acid (JA) and ethylene (ET) or salicylic acid (SA) signaling pathway for plants. Induced systemic resistance (ISR) is an essential plant regulation

strategy that can trigger cell defense responses before or after a disease attack (Pieterse et al., 2009). The ISR generally depending on JA and ET signals has been shown within several PGPRs (Akram et al., 2016). **In chapter 4**, we investigated the biocontrol mechanism of *Bacillus cabrialesii* BH5 for *Botrytis cinerea*. *B. cabrialesii* BH5 was shown to have potent antifungal properties against *B. cinerea*. Using high-performance liquid chromatography (HPLC) and tandem mass spectrometry (ESI-MS/MS), we extracted and identified fengycin H, a cyclic lipopeptide (CLP) from the fengycin family. Fengycin H was shown to be responsible for cell wall integrity deficiencies and, ultimately, the death of *B. cinerea* hyphal cells, according to analyses employing propidium iodide (PI) staining. Furthermore, root-inoculated tomato seedlings with BH5 successfully enhanced tomato plant growth. Both BH5 and fengycin H promote induced systemic resistance (ISR) in tomato plants via the jasmonic acid signaling pathway and have a significant biocontrol impact *in vivo*. As a result, strain BH5 and fengycin H are both excellent options for biological control of *B. cinerea* and its associated grey mold.

Antimicrobial Peptides from *Pseudomonas* sp. EDOX

Strains from the genus of *Pseudomonas* are a rich natural products resource. Most cyclic peptides from *Pseudomonas* species tested exhibit activity against Gram-positive bacteria in the low micromolar range (Raaijmakers et al., 2006; Reybroeck et al., 2014).

In chapter 5, we utilized *Pseudomonas* sp. EDOX, isolated from the leaf endosphere of a tomato plant. A number of cyclic lipopeptides (CLPs) generated by NRPS were isolated and characterized. In particular, the mass spectrometric analysis led to the identification of medipeptin, a 22-amino-acid peptide moiety with variable degrees of action against Gram-positive and Gram-negative pathogens. Moreover, we observed that medipeptin

A can be used as a bactericidal antibiotic for Gram-positive pathogens, but as a bacteriostatic antibiotic for Gram-negative pathogens. The powerful antibacterial action of Medipeptin A is carried out with Gram-positive bacteria via a binding process to lipoteichoic acid (LTA) and lipid II and the formation of holes in membranes. Our study gives new insight into medipeptin A's antimicrobial mode of action, which extends the range of possibilities for the creation of drugs and biocontrol agents.

Interaction between *Priestia endophytica* FH5 and *Rhizoctonia solani*

Bacteria and fungi often co-occur and interact, whereas relatively little is known about the mechanisms about bacterial–fungal interaction, especially the interaction between beneficial bacterial and fungal pathogens (Boer et al., 2005; Zhao et al., 2021). In **chapter 6**, We looked at the interaction between *P. endophytica* FH5 and *R. solani*. The beneficial strain *P. endophytica* FH5 showed biological activity against *R. solani* in our previous study. We have purified and identified kurstakins generated by strain FH5. In an *in vitro* test on plates and small glass slides, kurstakins exhibited good biological activity against *R. solani*. In addition, we did a transcriptomic analysis of FH5 with or without *R. solani* exposure, which showed that strain FH5 altered many genes associated with metabolism in response to fungal pathogen *R. solani*, including genes related to amino acid transport, carbohydrate transport, energy production and conversion, and inorganic ion transport and metabolism. Notably, *P. endophytica* FH5 can significantly decrease the disease incidence of *R. solani* ($P < 0.05$) when compared with the tomato without *P. endophytica* FH5 treatment *in vivo*. Altogether, our study has significantly improved insights into the interaction between *P. endophytica* FH5 with the fungal pathogen *R. solani* and demonstrated its biocontrol potential for tomato damping-off.

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Summary

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Acknowledgements

Summary

Environmentally friendly biotechnological ways for plant disease and pest control, such as the application of microbial biocontrol agents, provide alternatives to traditional chemicals. The usage of bioformulations, which are made from plant growth promoting rhizobacteria (PGPR) with biocontrol activity, is promising for sustainable agriculture. Beneficial bacteria from the genus of *Bacillus* are now the most commonly utilized microorganisms on the biopesticide market due to the lengthy shelf life of their endospores. In addition, the antimicrobial compounds produced by *Bacillus* strains are large structural diversity and with broad biological activities, such as antimicrobial, antiviral, immunosuppressive, and antitumor activities, that enable the natural products discovery for pharmaceutical development. In this thesis, we aimed to find potential biocontrol strains for tomato diseases control as well as novel antimicrobial compounds produced by themselves.

Bacillus strains are well-known as manufactories of a wide range of antimicrobial compounds with different structures. To look for the *Bacillus* with antimicrobial biosynthetic potential, we isolated strains from tomato rhizosphere soil and tissues (**chapter 2 and 3**). A total of 351 bacterial strains (181 of which are *Bacillus* sp.) were isolated from rhizosphere soil, and tissues of healthy tomato plants. 34 *Bacillus* strains were shown to exhibit biological activity against phytopathogens. Through genome mining, 120 BGCs that encode NRPs, PKs-NRPs, PKs, terpenes, and bacteriocins, including well-known chemicals like fengycin, surfactin, bacillibactin, subtilin, and others were identified. In addition, numerous novel BGCs were also discovered. Furthermore, by large-scale bioinformatics analysis, the NRPs and PKs-NRPs BGCs in

Bacillus species were revealed encoding highly conserved recognized chemicals as well as some new variations. In **chapter4**, fengycin H was isolated and identified from *Bacillus cabrelesii* BH5 by high-performance liquid chromatography (HPLC) and tandem mass spectrometry (ESI-MS/MS). Analyses using propidium iodide (PI) staining revealed that fengycin H was responsible for cell wall integrity defects and ultimately the death of *Botrytis cinerea* hyphal cells. Transcription analysis revealed that both BH5 and fengycin H stimulate induced systemic resistance (ISR) of tomato plants via the jasmonic acid signaling pathway and provide a strong biocontrol effect *in vivo*. In **chapter 6**, we investigated the interaction between *Priestia endophytica* FH5 and *Rhizoctonia solani*. To this end, a lipopeptide kurstakin with biological activity against *R. solani* was purified and identified from *P. endophytica* FH5. When *P. endophytica* FH5 interacts with *R. solani*, FH5 altered the expression level of genes associated with metabolism in response to fungal pathogen, including genes related to amino acid transport, carbohydrate transport, energy production and conversion, and inorganic ion transport and metabolism. Apart from these, *P. endophytica* FH5 can significantly decreased the disease incidence of *R. solani in vivo*. In **chapter 5**, a 22-amino-acid peptide moiety with varying degrees of activity against Gram-positive pathogens and Gram-negative pathogens, designated as medipeptin, was purified and identified from *Pseudomonas* sp. EDOX. Medipeptin A acts as a bactericidal antibiotic against Gram-positive pathogens, but as a bacteriostatic antibiotic against Gram-negative pathogens, which have ability of binding to both lipoteichoic acid (LTA) and lipid II as well as forming pores in membranes in our investigation.

In summary, this thesis provides important insights into the antimicrobial biosynthesis

potential of *Bacillus* as well as the potential for application as biocontrol agents. Some of the approaches such as genome mining, mass spectrometry and RNA-Seq, were combined to discover novel antimicrobial compounds produced by bacteria. Our study serves as a foundation for further investigation of the beneficial bacteria-pathogens interaction of *Bacillus* strains. However, many questions remain unanswered, and more research is required to completely clarify the mechanisms of biocontrol capability of *Bacillus*.

Samenvatting

Milieuvriendelijke biotechnologische manieren voor de bestrijding van plantenziekten en plagen, zoals de toepassing van microbiële biologische bestrijdingsmiddelen, bieden alternatieven voor traditionele chemicaliën. Het gebruik van bioformuleringen, die zijn gemaakt van plantengroeibevorderende rhizobacteriën (PGPR) met biocontrole-activiteit, is veelbelovend voor duurzame landbouw. Bevorderende bacteriën van het geslacht *Bacillus* zijn nu de meest gebruikte micro-organismen op de markt voor biopesticiden vanwege de lange houdbaarheid van hun endosporen. Bovendien hebben de antimicrobiële verbindingen geproduceerd door *Bacillus*-stammen een grote structurele diversiteit en diverse biologische activiteiten, zoals antimicrobiële, antivirale, immunosuppressieve en antitumoractiviteiten, die de ontdekking van natuurlijke producten voor farmaceutische ontwikkeling mogelijk maken. In dit proefschrift doelen we op het vinden van potentiële biologische bestrijdingsstammen voor de bestrijding van tomatenziekten, evenals de ontdekking van nieuwe antimicrobiële stoffen geproduceerd door deze stammen.

Bacillus-stammen staan bekend als fabrikanten van een breed scala aan antimicrobiële verbindingen met verschillende structuren. Voor de zoektocht naar een *Bacillus*-stam met antimicrobieel biosynthetisch potentieel, hebben we stammen geïsoleerd uit de bodem en weefsels van de tomatenrhizosfeer (**hoofdstuk 2 en 3**). In totaal werden 351 bacteriestammen (waarvan 181 *Bacillus* sp.) geïsoleerd uit de bodem rhizosfeer en weefsels van gezonde tomatenplanten. 34 *Bacillus*-stammen bleken biologische activiteit te vertonen tegen fytopathogenen. Door middel van genoom-mining werden 120 BGC's geïdentificeerd die coderen voor NRP's, PK's-NRP's, PK's, terpenen en

bacteriocines, waaronder bekende chemicaliën zoals fengycine, surfactine, bacillibactine, subtiline en andere. Daarnaast werden ook tal van nieuwe BGC's ontdekt. Bovendien werden door grootschalige bio-informatica-analyse de NRP's en PK's-NRP's BGC's in *Bacillus*-soorten onthuld die coderen voor zowel zeer geconserveerde chemicaliën, als voor enkele nieuwe variaties op deze moleculen. In **hoofdstuk 4** werd fengycine H geïsoleerd en geïdentificeerd uit *Bacillus cabrelesii* BH5 door middel van high-performance vloeistofchromatografie (HPLC) en tandem massaspectrometrie (ESI-MS/MS). Analyses met behulp van propidiumjodide (PI) kleuring onthulden dat fengycine H verantwoordelijk was voor defecten in de celwandintegriteit en uiteindelijk de dood van *Botrytis cinerea* hyfencellen. Transcriptieanalyse onthulde dat zowel BH5 als fengycine H de geïnduceerde systemische resistentie (ISR) van tomatenplanten stimuleert via de jasmonzuur signaalpathway en een sterk biocontrole-effect *in vivo* biedt. In **hoofdstuk 6** onderzochten we de interactie tussen *Priestia endophytica* FH5 en *Rhizoctonia solani*. Hiertoe werd het lipopeptide kurstakin met biologische activiteit tegen *R. solani* gezuiverd en geïdentificeerd uit *P. endophytica* FH5. Wanneer *P. endophytica* FH5 een interactie aangaat met *R. solani*, veranderde FH5 het expressieniveau van genen geassocieerd met metabolisme als reactie op schimmelpathogenen, waaronder genen gerelateerd aan aminozuurtransport, koolhydraattransport, energieproductie en -omzetting, en anorganisch ionentransport en metabolisme. Daarnaast kan *P. endophytica* FH5 de ziekte-incidentie van *R. solani* *in vivo* aanzienlijk verminderen. In **hoofdstuk 5** werd een 22-aminozuurtellend peptide met verschillende mate van activiteit tegen Gram-positieve pathogenen en Gram-negatieve pathogenen, aangeduid als medipeptine, gezuiverd en geïdentificeerd uit *Pseudomonas* sp. EDOX. Medipeptine A werkt als een bacteriedodend antibioticum

tegen Gram-positieve pathogenen, maar als een bacteriostatisch antibioticum tegen Gram-negatieve pathogenen. Daarbij kan medipeptine A zowel aan lipoteichoïnezuur (LTA) als lipide II binden en poriën in het membranen vormen.

Samenvattend biedt dit proefschrift belangrijke inzichten in het antimicrobiële biosynthesepotentieel van *Bacillus*, evenals het potentieel voor de toepassing van *Bacillus* als biologische bestrijdingsmiddel. Verschillende benaderingen, zoals genoom-mining, massaspectrometrie en RNA-Seq, werden gecombineerd om nieuwe antimicrobiële verbindingen te ontdekken die door bacteriën worden geproduceerd. Onze studie dient als basis voor verder onderzoek naar gunstige bacterie-pathogenen interactie van *Bacillus*-stammen. Er blijven echter veel vragen onbeantwoord en er is meer onderzoek nodig om de mogelijkheden voor toepassing van *Bacillus* voor biocontrole en de bijbehorende onderliggende mechanismen volledig te verduidelijken.

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