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Mining and characterization of antimicrobials from plant growth-promoting rhizobacteria isolated from perennial ryegrass

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**Mining and characterization of
antimicrobials from plant growth-
promoting rhizobacteria isolated from
perennial ryegrass**

Zhibo Li

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university of
groningen

Mining and characterization of antimicrobials from plant growth- promoting rhizobacteria isolated from perennial ryegrass

PhD thesis

to obtain the degree of PhD at the
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1

Introduction

Perennial ryegrass

Perennial ryegrass (*Lolium perenne*) is an important forage grass growing in the temperate regions of the world [1, 2]. It is widely used as a pasture plant and for silage production because of the high digestibility, outstanding palatability, and rich nutritional value [1, 2]. However, the susceptibility to pathogens is a threat to its application. Diseases that cause severe damage in perennial ryegrass include ergot (*Claviceps purpurea*), leaf spot (*Drechslera* sp.), fusarium (*Fusarium* sp.), brown blight (*Drechslera siccans*), snow molds (*Typhus* sp., *Fusarium* sp., and *Sclerotinia* sp.), crown rust (*Puccinia coronata*), stem rust (*P. graminis* subsp. *graminicola*), bacterial wilt (*Xanthomonas campestris* pv. *graminis*) [1-3]. Moreover, some fungal endophytes of perennial ryegrass can cause disease in ruminants. Representative examples are ryegrass staggers and facial eczema caused by *Neotyphodium lolii* and *Pithomyces chartarum*, respectively [4-6]. Therefore, it is important to ensure the health of perennial ryegrass. Chemical pesticides have been widely used in the past decades for this purpose. However, their application causes a lot of environmental problems, including soil pollution and water pollution [7]. Thus, it is necessary to look for environmentally friendly alternatives.

Plant growth-promoting rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) are bacteria that live in the rhizosphere, a narrow region of soil influenced by root exudates [8], and are able to enhance the growth of plants [9, 10]. They were first defined by Klopfer and Schroth and have been widely reported in the past decades [11, 12]. Because of the great efficacy in plant-growth stimulation and plant disease control, they are considered to be environmentally friendly alternatives to chemical fertilizers and pesticides. A broad range of bacterial species such as *Bacillus*, *Burkholderia*, *Azospirillum*, *Azotobacter*, *Rhizobium*, and *Pseudomonas* have been reported to be PGPR, among which *Bacillus*, *Rhizobium*, and *Pseudomonas* are the most well-known species [9]. PGPR have been applied to a diverse array of plants including chickpea, maize, pea, peanut, rice, soybean, sugarcane, wheat, and sugarbeet [9, 13].

Mechanisms of PGPR

The mechanisms of PGPR have been investigated thoroughly. In general, they are classified into two different categories: direct stimulation or indirect protection. As direct mechanisms, PGPR enhance plant growth by facilitating nutrient acquisition or modulating plant hormone levels. Apart from that, PGPR produce secondary metabolites to combat plant pathogens or elicit induced systemic resistance (ISR) of plants, thus indirectly protecting the plant from pathogens (Figure 1) [9, 14]. As a reward, microbes acquire carbon from plants for metabolism (Figure 1).

Figure 1. The mechanisms of plant-microbe interaction. Biocontrol mechanisms of PGPR are classified into two different categories: direct mechanism (blue routes) and indirect mechanisms (red routes). Black routes indicate other interactions and regulations. "+", stimulation; "-", inhibition.

Direct mechanisms

Nitrogen (N) is one of the most vital elements for plant growth [15]. Even though N₂ constitutes 78% of the air, it is not directly available for plants. Biological N₂ fixation is an essential process to convert N₂ into a plant-available form. This process is catalyzed by nitrogenases that are harbored by N₂-fixing microbes [16]. The most well-known nitrogen-fixing bacteria are *Rhizobium*, *Bradyrhizobium*, and *Azospirillum* [17]. They can form nodules with legume plants such as soybean, pea, peanut, alfalfa, and are defined as symbiotic nitrogen-fixing bacteria [17]. Another type of nitrogen-fixing bacteria such as *Klebsiella pneumoniae*, *Paenibacillus polymyxa*, *Paenibacillus massiliensis*, *Bacillus megaterium*, and *Bacillus marisflavi* are free-living diazotrophs that convert N₂ into ammonia [18-20].

Phosphorus (P) is another essential element for growing plants [15]. Although P is highly abundant in the soil, the available form, i.e. soluble P, is limited. To fulfill the P requirement of crops, phosphatic fertilizers are widely used. However, this is costly and environmentally undesirable. As an alternative,

phosphate solubilizing microorganisms (PSM) provide the available form of P to the plants. The capability of phosphate solubilization has been reported in bacteria including *Azotobacter*, *Bacillus*, *Burkholderia*, and *Pseudomonas* [21-24]. The underlying mechanism is related to the production of low molecular weight organic acids and phosphatases, which solubilize inorganic phosphorus and mineralize organic phosphorus, respectively [14, 25].

Phytohormones are important signal molecules that regulate all aspects of plant growth and development [26]. It has been known for a long time that PGPR can produce phytohormones. One of the most important phytohormones produced by microorganisms is the hormone auxin (indole-3-acetic acid/indole acetic acid/IAA). It regulates important cellular processes including plant cell proliferation, root development, and photosynthesis [26]. IAA production has been reported in PGPR including *Rhizobium*, *Pseudomonas*, and *Bacillus* spp. [27-30], and its production is relevant to the amino acid tryptophan, which is commonly found in the root exudates [31]. Other phytohormones such as gibberellins and cytokinins were also reported produced by PGPR and play an important role in the plant growth regulation [32].

Other plant growth-promoting traits such as siderophore production, 1-Aminocyclopropane-1-carboxylate (ACC) deaminase production, volatile production, also stimulate the growth of plants directly. Siderophores are iron-chelating compounds produced by microorganisms that assist in the acquisition of iron by bacteria [33]. The siderophore-Fe complex can be assimilated by plants, thus making it a source of iron to plants [34]. ACC is the precursor of ethylene, which is an important plant hormone relevant to stress response [35]. Bacteria that produce ACC deaminase can take up ACC, thus decreasing the level of ethylene and enhancing the growth of plants [36]. *B. subtilis* species can also stimulate the growth of plants by producing volatile organic compounds (VOCs) such as 2,3-butanediol and acetoin [37].

Indirect mechanisms

During plant-microbe interaction, biological or chemical compounds can induce resistance of non-exposed plant parts to pathogenic microorganisms. This phenomenon is called induced systemic resistance (ISR) [38]. It is different from the systemic acquired resistance (SAR), which is triggered by an incompatible necrotizing pathogen and renders the host resistant to subsequent infection [39, 40]. PGPR such as *Pseudomonas* and *Bacillus* species are well-known ISR inducers [41, 42]. The ISR has been proven induced by cell envelope components, iron-regulated metabolites, and antibiotics [41]. Moreover, some volatile organic compounds (VOCs) produced by microorganisms were also shown to be elicitors of ISR [43]. The signal transduction of ISR is dependent on ethylene

(ET) and jasmonic acid (JA) and independent of salicylic acid and pathogenesis-related (PR) proteins [44].

Production of secondary metabolites is another important weapon of PGPR. Based on the biosynthesis pathway, bacterial secondary metabolites are classified into three categories: bacteriocins, nonribosomal peptides (NRPs), and polyketides (PKs). Bacteriocins are ribosomally synthesized and comprise three different classes: ribosomally synthesized and post-translationally modified peptides (RiPPs), unmodified bacteriocins, and large antimicrobial proteins [45, 46]. Among them, RiPPs are most well studied and have been proven to have good potential in controlling plant diseases caused by pathogenic organisms [46]. For example, plantazolicin produced by *B. velezensis* FZB42 is able to combat nematodes, which have caused serious losses to a variety of crops worldwide [47]. NRPs and PKs are synthesized in a nonribosomal way through nonribosomal peptide synthetases (NRPSs) and polyketide synthetases (PKSs), respectively. Many NRPs and PKs were shown to antagonize pathogenic organisms. For example, diffidin and bacilysin from *B. velezensis* FZB42 are active against the bacterial pathogen *Xanthomonas oryzae* [48]. Fusaricidins produced by *Paenibacillus polymyxa* can antagonize the fungal pathogen *Fusarium oxysporum* f.sp. *neovium* [49]. Locillomycin from *B. subtilis* is active against bacteria and viruses, while octapeptins from *Paenibacillus* spp. can combat bacteria, fungi, protozoa, and yeast [50, 51].

Apart from antimicrobial activity, secondary metabolites also play other roles that are relevant to biocontrol. For example, surfactins, iturins, and fengycins produced by *Bacillus* spp. are involved in ISR induction [40, 52]. Moreover, surfactins also play a crucial role in motility, biofilm formation, and quorum sensing of bacteria [53]. The motility of bacteria is important for their translocation to the plant surface, which is usually rich in nutrients. Biofilm formation of bacteria is relevant to their colonization into plants. Quorum sensing of bacteria plays an important role in the regulation of lipopeptide production [53].

Apart from the induction of ISR and the production of antimicrobials, other properties of PGPR can also contribute to the biocontrol of pathogens. For example, lactonases produced by *B. thuringiensis* can degrade the homoserine lactones (AHLs), which play a role in the synthesis of cell-wall degrading enzymes of the plant pathogen *Erwinia carotovora*, thus affecting its pathogenicity [54]. Lytic enzymes produced by some fungal *Trichoderma* species can destruct the cell wall of fungal pathogens [55]. Hydrogen cyanide (HCN) produced by *Pseudomonas* can inhibit the development of plant disease in tomato [56]. *Pseudomonas fluorescens* WCS365 can colonize the hyphae of the pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici* and likely makes it less virulent [57].

***Bacillus* and closely related species as PGPR**

Bacillus and closely related species such as *Paenibacillus* and *Brevibacillus* have certain advantages to be used as PGPR. First of all, they are great producers of antimicrobials. Around 4-5% of the genome of *Bacillus subtilis* is devoted to the biosynthesis of secondary metabolites, while this number for *Bacillus velezensis* reaches 8.5% [58, 59]. A total of 11 different secondary metabolites have been identified from the PGPR strain *B. velezensis* FZB42 [52] and up to 26 BGCs were predicted from the biocontrol strain *Brevibacillus laterosporus* MG64 [60]. Moreover, *Bacillus* and closely related species are endospore-forming bacteria. They can form endospores when encountering stresses. This property confers them a better survival ability in fluctuating environments [61] and makes them easier formulated for the development of biocontrol agents [62].

Secondary metabolites produced by *Bacillus*, *Paenibacillus*, and *Brevibacillus*

Many secondary metabolites have been identified from *Bacillus* and closely related species, in particular the *Bacillus subtilis* group, *Paenibacillus*, and *Brevibacillus* species. A total of 59 compounds (compound classes) have been reported from these species. Among them, 30 are NRPs, 2 are NRPs-PKs hybrids, 6 are PKs, 20 are bacteriocins, while the last one, rhizoctin A, is a phosphonate-containing oligopeptide (Table 1).

The secondary metabolites derived from the *B. subtilis* group, *Paenibacillus*, and *Brevibacillus* are structurally diverse (Table 1). Surfactins, iturins, fengycins, fusaricidins, locillomycin, koranimine, and marthiapeptide are circular noncationic NRPs. They contain a big ring that is formed with several amino acid residues and do not have positively charged residues. Polymyxins, octapeptins, tyrocidines, gramicidin S, lolatins, laterocidin, brevistin, relacidines, paenibacterin, and bacitracin are circular cationic NRPs. They comprise at least one positively charged amino acid residue, thus making them have a net positive charge at neutral conditions. Tridecaptins, gramicidins, edeine, spergualin, bogorols, succilins, tauramanide and tostadins form a different class of NRPs, which is linear and noncationic. Bacillibactin, bacilysin, sevadicin, and paenibactin are structurally distinct from others. They are either synthesized by iterative modules (bacillibactin and paenibactin) or simply comprise of 2-3 amino acid residues (bacilysin and sevadicin). Paenilipoheptin and paenilamicin are NRPs-PKs hybrids, which contain both amino acid residues and beta-keto moieties. Macrolactin, bacillaene, diffidin, basiliskamides, macrobrevin, and aurantinin are PKs that comprise several beta-keto functional groups. Subtilin, ericins, mersacidin, sublancin 168, subtilomycin, entianin, amyloliquecidin, lichenicidin, penisin, paenicidins, paenibacillin, and paenilan are lanthipeptides that typically contain lanthionine (Lan) and 3-methylanthionine (MeLan) residues.

Plantazolicin, subtilosin A, and paeninodin belong to linear azole-containing peptides (LAPs), sactipeptides, and lasso peptides, respectively. Amylocyclicin and pumilarin are head-to-tail cyclized peptides. Different to these ribosomally synthesized and post-translationally modified peptides (RiPPs), lichenin, laterosporulin, laterosporulin 10, and bac-GM100 are unmodified bacteriocins, which are generally small (less than 10 kDa) and heat stable.

Apart from their structural diversity, the functionality of these secondary metabolites in biocontrol is also very diverse (Table 1). Most of the compounds have been reported to exert antagonistic activity against pathogenic microorganisms, including Gram-positive bacteria, Gram-negative bacteria, fungi, yeasts, viruses, and protozoa. Moreover, surfactins, fengycins, and iturins are involved in the ISR induction of plant, thus arming the plant against phytopathogens [63-66]. Surfactins also play a key role in biofilm formation, which assists the colonization of the plants [67, 68]. Bacillibactin and paenibactin are siderophores that assist the plant in the uptake of iron from the soil environment [33, 69].

Table 1. Summary of secondary metabolites from the *Bacillus subtilis* group, *Paenibacillus*, and *Brevibacillus* species.

Types	Antimicrobials	Functions in biocontrol	Effect against	Producers	Reference
NRPs-I	Surfactins ¹	Biofilm, antagonism, ISR	Bacteria, fungi	<i>B. subtilis</i> , <i>B. velezensis</i> , <i>B. amyloliquefaciens</i> , <i>B. licheniformis</i> , <i>B. pumilus</i>	[40, 63-65, 67, 68]
NRPs-I	Iturins ²	Antagonism, ISR	Bacteria, fungi	<i>B. subtilis</i> , <i>B. amyloliquefaciens</i> , <i>B. velezensis</i> , <i>P. larvae</i>	[40, 66, 70, 71]
NRPs-I	Fengycins ³	Antagonism, ISR	Bacteria, fungi	<i>B. subtilis</i> , <i>B. velezensis</i> , <i>B. amyloliquefaciens</i> , <i>Paenibacillus</i> sp.	[40, 63, 66]
NRPs-I	Fusaricidins ⁴	Antagonism	Gram +, fungi	<i>B. subtilis</i>	[49, 72]
NRPs-I	Locillomycin	Antagonism	Gram +/-, virus	<i>B. subtilis</i>	[50, 73]
NRPs-I	Koranimine	-	-	<i>B. subtilis</i>	[74]
NRPs-I	Marthapeptide	-	Gram +	<i>Brevibacillus</i> sp.	[75]
NRPs-II	Polymyxin ⁵	-	Gram -	<i>Paenibacillus</i> sp.	[70]
NRPs-II	Octapeptins ⁶	-	Gram -, fungi, protozoa, Yeast	<i>Paenibacillus</i> sp.	[51, 70]
NRPs-II	Polypeptins ⁷	Antagonism	Gram +, fungi	<i>B. circulans</i> , <i>P. elgii</i>	[70, 76-78]
NRPs-II	Tyrocidines	Antagonism	Gram +, fungi	<i>Br. brevis</i> , <i>Br. parabrevis</i>	[79, 80]
NRPs-II	Gramicidin S	-	Gram +/-, fungi	<i>Br. brevis</i>	[79, 81]
NRPs-II	Lolotins	-	Gram +	<i>Bacillus</i> sp., <i>Br. laterosporus</i>	[79, 82]
NRPs-II	Laterocidin	-	Bacteria, fungi, protozoa	<i>Br. laterosporus</i>	[79, 83]
NRPs-II	Brevistin	-	Gram +	<i>Br. brevis</i>	[79]
NRPs-II	Relacidines ⁸	Antagonism	Gram -	<i>Br. laterosporus</i>	Chapter 3, [84]
NRPs-II	Paenibacterin	-	Gram +/-	<i>P. thiaminolyticus</i>	[85]
NRPs-II	Bacitracin	-	Gram +	<i>B. subtilis</i> , <i>B. pumilus</i> , <i>licheniformis</i> , <i>P. polymyxa</i>	[86, 87]
NRPs-III	Tridecaptins	-	Gram -	<i>B. circulans</i> , <i>P. polymyxa</i> , <i>P. terrae</i>	[70, 88]
NRPs-III	Gramicidins	-	Gram +	<i>Br. brevis</i>	[79, 89]
NRPs-III	Edeine	-	Bacteria, fungi	<i>Br. brevis</i>	[79, 90]
NRPs-III	Spergualin	-	Gram +/-	<i>Br. laterosporus</i>	[79]
NRPs-III	Bogorols ⁹	Antagonism	Gram +/-, fungi	<i>Br. laterosporus</i>	[79, 91-97]
NRPs-III	Succilins	Antagonism	Gram +/-	<i>Br. laterosporus</i>	Chapter 4
NRPs-III	Tauramide	-	Gram +	<i>Br. laterosporus</i>	[79, 98]
NRPs-III	Tostadins	-	Gram +/-	<i>Br. brevis</i>	[79, 99]
NRPs-IV	Bacillibactin	Siderophore	-	<i>B. subtilis</i> , <i>B. velezensis</i> , <i>B. amyloliquefaciens</i> , <i>B. licheniformis</i> , <i>B. pumilus</i> , etc.	[33, 69]
NRPs-IV	Bacilysin	Antagonism	Gram -	<i>B. subtilis</i> , <i>B. velezensis</i> , <i>B. pumilus</i>	[48, 100]

Types	Antimicrobials	Functions in biocontrol	Effect against	Producers	Reference
NRPs-IV	Sevadinin	-	Gram +	<i>P. larvae</i>	[101]
NRPs-IV	Paenibactin	Siderophore	-	<i>P. elgii</i>	[102]
NRPs-PKS	Paenilipoheptin	-	-	<i>P. polymyxa</i>	[103]
NRPs-PKS	Paenilamicin	Antagonism	Gram +, fungi, yeast	<i>P. larvae</i>	[104]
PKs-I	Macrolactin	Antagonism	Gram -	<i>B. velezensis</i>	[105, 106]
PKs-I	Bacillaene	Antagonism	bacteria	<i>B. subtilis</i> , <i>B. velezensis</i>	[107-109]
PKs-I	Difficidin	Antagonism	Gram -	<i>B. velezensis</i>	[48, 100]
PKs-I	Basiliskamides	-	Gram +, fungi	<i>Br. laterosporus</i>	[110, 111]
PKs-I	Macrobrevin	-	Gram +	<i>Brevibacillus</i> sp.	[75]
PKs-I	Aurantinin	-	Gram +	<i>B. subtilis</i>	[112]
Bacteriocins-I	Subtilin	-	Gram +	<i>B. subtilis</i>	[113]
Bacteriocins-I	Ericins	-	Gram +	<i>B. subtilis</i>	[113]
Bacteriocins-I	Mesacidin	-	Gram +	<i>B. subtilis</i>	[114]
Bacteriocins-I	Sublancin 168	-	Gram +	<i>B. subtilis</i>	[115]
Bacteriocins-I	Subtilomycin	-	Gram +	<i>B. subtilis</i>	[116]
Bacteriocins-I	Entianin	-	Gram +	<i>B. subtilis</i>	[117]
Bacteriocins-I	Amyloliquecidin	-	Gram +	<i>B. velezensis</i>	[118]
Bacteriocins-I	Lichenicidin	-	Gram +	<i>B. licheniformis</i>	[86]
Bacteriocins-I	Penisin	-	Gram +/-	<i>P. ehimensis</i>	[119]
Bacteriocins-I	Paenicidins	-	Gram +	<i>P. polymyxa</i> , <i>P. terrae</i>	[120, 121]
Bacteriocins-I	Paenibacillin	-	Gram +	<i>P. polymyxa</i>	[122]
Bacteriocins-I	Paenilan	-	Gram +	<i>P. polymyxa</i>	[123]
Bacteriocins-II	Plantazolicin	Antagonism	Gram +, nematode	<i>B. velezensis</i> , <i>B. pumilus</i>	[124]
Bacteriocins-III	Amylocyclicin	-	Gram +	<i>B. velezensis</i>	[125]
Bacteriocins-III	Pumilarin	-	Gram +	<i>B. pumilus</i>	[126]
Bacteriocins-IV	Subtilosin A	Antagonism	Gram +	<i>B. subtilis</i> , <i>B. atrophaeus</i>	[127, 128]
Bacteriocins-V	Paeniodin	-	-	<i>P. dendritiformis</i>	[129]
Bacteriocins-VI	Lichenin	-	Gram +	<i>B. licheniformis</i>	[130, 131]
Bacteriocins-VI	Laterosporulin	-	Gram +/-	<i>Br. laterosporus</i>	[132]
Bacteriocins-VI	Laterosporulin 10	-	Gram +	<i>Brevibacillus</i> sp.	[133]
Bacteriocins-VI	Bac-GM100	Antagonism	Gram +/-, fungi	<i>Br. brevis</i>	[134]
Others	Rhizoctcin A	Antagonism	Fungi, nematode	<i>B. subtilis</i>	[135, 136]

Note: NRPs-I, circular noncationic NRPs; NRPs-II, circular cationic NRPs; NRPs-III, linear cationic NRPs; NRPs-IV, other types of NRPs; PKs-I, synthesized by modular type I PKSs; Bacteriocins-I, lanthipeptides; Bacteriocins-II, linear azole-containing peptides (LAPs); Bacteriocins-III, head-to-tail cyclized peptides; Bacteriocins-IV, sactipeptides; Bacteriocins-V, lasso peptides; Bacteriocins-VI, unmodified bacteriocins; ¹ peptides belonging to this family: esperin, lichenysin, pumilacidin, halobacillin, isohalobacillin; ² peptides belonging to this family: bacillomycins, mycosubtilin, bacillopeptin, mixirin, subtilene, mojavensin, paenilarvins; ³ peptides belonging to this family: plipastatins; ⁴ peptides belonging to this family: gatavalin; ⁵ peptides belonging to this family: colistin, mattacin; ⁶ peptides belonging to this family: battacin; ⁷ peptides belonging to this family: pelgipeptins; ⁸ peptides belonging to this family: brevicidine, laterocidine; ⁹ peptides belonging to this family: brevivacillins, BT peptide, BL-A60

Biosynthesis of NRPs, PKs, and RiPPs

NRPs, PKs, and RiPPs are the predominant secondary metabolites reported in *Bacillus*, *Paenibacillus*, and *Brevibacillus* (Table 1). Their biosynthetic machinery is completely different from each other. NRPs are peptidic natural products assembled by large enzyme complexes termed nonribosomal synthetases (NRPSs) (Figure 2). Similarly, PKs are synthesized through multidomain enzymes, i.e. polyketide synthetases (PKSs) (Figure 3). In contrast to them, RiPPs are synthesized in a ribosomal pathway and usually involved in many post-translational modifications.

Biosynthesis of NRPs through NRPSs

NRPSs comprise different domains with different functions (Figure 2). The adenylation (A) domain, thiolation (T) domain, condensation (C) domain, and thioesterase (TE) domain/terminal reductase (R) domain are essential domains for the biosynthesis of NRPs [137]. The A domain is responsible for the activation of amino acid residues and usually has substrate specificity [137]. The T domain is also called peptide carrier protein (PCP), which is attached with 4'-phosphopantetheine and responsible for the carrying of substrates activated by the A domain [137]. The C domain catalyzes the formation of an amide bond between the thioester group and the amino group, thus elongate the peptide chain [137]. The TE domain is responsible for the hydrolysis of the polypeptide chain from the T domain. This process is usually accompanied by the formation of cyclic amides (lactams) or cyclic esters (lactones). At the end of this process, peptides are released from the assembly line [138]. The R domain is an alternative release mechanism to the TE domain. It reduces the thioester bond to terminal aldehyde or alcohol [138]. Apart from the four essential domains (A, T, C, TE/R), the epimerization (E) domain is also prevalent in NRPSs. The E domain is located right after the T domain and is responsible for the alteration of L-amino acids into D configurations [137]. These domains form different modules (typically comprised of C-A-T) that constitute an assembly line for the biosynthesis of NRPs. For the biosynthesis of lipopeptides, which have a fatty acid chain at the

N terminus, a lipinitiation is involved. The fatty acid is activated by ligating to a coenzyme A (CoA) by a fatty acid-CoA ligase (FACL), which is usually not present in the BGC. The activated fatty acid is then transferred and incorporated into the assembly line under the catalysis of a C domain, which is specifically termed starter C domain [139].

Figure 2. Surfactin-associated NRPS incorporating a fatty acid tail and seven amino acid residues. CoA, coenzyme A; FACL, fatty acid-CoA ligase; FA, fatty acid; C, condensation domain; A, adenylation domain; T, thiolation domain; E, epimerization domain; TE, thioesterase. The core biosynthetic genes are indicated in red while the additional biosynthetic gene is indicated in pink.

There are also many tailoring enzymes involved in the biosynthesis of NRPs. For example, halogenation, which adds a halogen to the Hpg13 residue of enduracidin and the Hpg17 residue of ramoplanin, is catalyzed by a flavin-dependent halogenase [140, 141]. Mannosylation of ramoplanin is mediated by a mannosyltransferase [142, 143]. N-acylation of the glucosamine moiety of teicoplanin is catalyzed by an acyltransferase [144]. Recently, a sulfotransferase was discovered from an environmental DNA library. It is involved in the production of monosulfated analogs of teicoplanin [145]. These modifications are usually important for the bioactivity of the final products.

Biosynthesis of PKs through PKSs

PKSs are classified into three different types: type I that comprises various catalytic domains (Figure 3), type II that comprises catalytic domains as well as two typical KS domains (KS_{α} and KS_{β}), and type III that typically lack multiple catalytic domains and employ an ACP independent mechanism [146]. The type I PKSs are further divided into iterative type I that utilize the domains in a cyclic fashion and modular type I that do not use the domains repetitively [146, 147].

The predominant PKs produced by bacteria are synthesized by modular type I PKSs. Acyltransferase (AT), acyl carrier protein (ACP), ketosynthase (KS) and thioesterase (TE) are essential domains in modular type I PKSs. The AT domain initiates the assembly line by selecting a specific acyl-CoA and loads it onto the ACP domain. The KS domain, which is similar to the C domain in NRPSs, is responsible for the elongation. Following the chain extension, the polyketide chain can be optionally modified by ketoreductase (KR), dehydratase (DH), and enoylreductase (ER). The final polyketide is released from the ACP domain by a TE domain [146, 147]. A characteristic of modular type I PKSs is that the number of precursors incorporated into the final product is consistent with the number of modules.

The biosynthetic domains of iterative type I PKSs are the same as the modular type I PKSs. The only difference is that the extender domains are repetitively used in the iterative type I PKSs. A representative example is the lovastatin PKS, in which one starter unit (acetyl-CoA) is condensed with eight extender units (malonyl-CoA) and S-adenosylmethionine (SAM) to produce the intermediate dihydromonacolin L. Iterative type I PKSs are previously believed only present in fungi. However, they have been reported also from many bacteria in recent years [146, 147].

Type II PKSs comprise similar catalytic domains as type I PKSs, with the exception that they also contain the typical KS_{α} and KS_{β} . KS_{α} is equivalent to the KS of type I PKSs and responsible for the elongation, while KS_{β} controls the length of the final product. The domains of type II PKSs are also iteratively used and the reduction of the β -keto group will only occur when the polyketide is fully synthesized. The anticancer drugs daunorubicin and doxorubicin are synthesized by type II PKSs [146, 147].

Type III PKSs also generate a poly- β -keto chain as other types of PKSs do. However, it is independent of the ACP, and only comprises simple homodimers of KS that catalyze the condensation of a starter unit to a series of extender units. Type III PKSs were formerly believed to be only harbored by plants, but they have also been found in many bacteria in recent years. The red pigment flaviolin and the spore germination inhibitor Germicidin A are produced by bacterial type III PKSs [146-148].

Figure 3. Macrolactin A-associated modular type I PKS, which is the predominant type of PKSs found in *Bacillus* spp. AT, Acyltransferase; KS, ketosynthase; KR, ketoreductase; ACP, acyl carrier protein; TE, thioesterase; DH, dehydratase.

Biosynthesis of RiPPs in a ribosomal pathway

The predominant bacteriocins produced by bacteria are belonging to the RiPPs family. The BGCs of RiPPs typically comprise a core biosynthetic gene and several modification-related and transport-related genes. The core biosynthetic gene encodes a precursor peptide, which usually comprises a leader peptide and a core peptide. In some special cases, a signal peptide at the N terminus or a recognition sequence at the C terminus is found. The precursor peptide is typically ~20-110 residues in length and usually does not display bioactivity. To activate the peptide, the precursor peptide has to undergo posttranslational modifications, after which the leader peptide is cleaved by enzymes and a mature peptide with bioactivity is yielded [149]. Based on the structures, which is relevant to the posttranslational modifications, RiPPs can be classified into different classes. Here, we only introduce those found in the *B. subtilis* group, *Paenibacillus*, and *Brevibacillus*.

Lanthipeptides are a class of peptides containing *meso*-lanthionine and 3-methylanthionine. The Ser and Thr residues in the precursor peptide of lanthipeptides typically undergo dehydration to form dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Lanthionines are subsequently formed between a dehydroalanine or dehydrobutyrine and a cysteine residue via a thioether linkage. Enzymes involved in the modifications include a dehydratase (LanB) and a cyclase (LanC). In some cases, dehydration and cyclization are catalyzed by a multifunctional enzyme such as LanM [149].

The most well-studied lanthipeptide is nisin, which is produced by *Lactococcus*, *Streptococcus*, and *Enterococcus* and displays a broad inhibitory spectrum against Gram-positive bacteria [150-152].

Linear azole-containing peptides (LAPs) are a group of RiPPs that typically contain oxazoles or thiazoles. The precursor of LAPs comprises abundant cysteine, serine, or threonine residues. These residues will first undergo a backbone cyclodehydration by the catalysis of cyclodehydratases. The resulting azoline heterocycles will be further processed by a dehydrogenase, thus forming azoles [149]. The plantazolicin produced by the well-known PGPR strain *B. amyloliquefaciens* FZB42 belongs to the LAPs family and displays striking antimicrobial activity to *Bacillus anthracis* [124].

Head-to-tail cyclized peptides are relatively large (35-70 residues) and typically form a peptide bond between the C and N termini. The leader sequence of head-to-tail cyclized peptides ranges from 2 to 35 residues. The leader peptide is cleaved and an amide bond is formed between two hydrophobic residues to realize a head-to-tail ligation [149]. The enzymes responsible for the cyclization are not yet identified to date. The most well-known head-to-tail cyclized peptide is enterocin AS-48, a potent antimicrobial agent produced *Enterococcus* spp. [153]. Amylocyclicin produced by *B. amyloliquefaciens* FZB42 also belongs to this class [125].

Sactipeptides are a class of peptides with crosslinks between cysteine sulfur and α -carbon. The crosslinking modification is proposed to be mediated by a radical S-adenosylmethionine (SAM) enzyme in a leader peptide-dependent manner. This modification is crucial for the bioactivity of sactipeptides [149]. The most well-understood sactipeptide is subtilisin A, which is produced by *B. subtilis* and displays good activity against Gram-positive bacteria [128]. Other sactipeptides produced by *Bacillus* spp. include the sporulation killing factor (SKF) and thuricins such as thuricin H [149].

Lasso peptides contain a characteristic lasso fold, which is formed by the N-terminal macrolactam macrocycle trapping the C-terminal tail. The lasso fold is likely formed by the catalysis of a cysteine protease (homolog) and an Asn synthetase (homolog) [149]. Microcin J25 is a lasso peptide produced by *E. coli* and inhibits RNA polymerase of bacterial pathogens [154]. Paeninodin, whose C-terminal serine residue is specifically phosphorylated by a novel kinase, also belongs to lasso peptides [129].

Mode of action of antimicrobials

Antimicrobials employ various mechanisms to antagonize other bacteria. Understanding their modes of action can help us better understand their potential in applications and is a prerequisite to stop the occurrence of antimicrobial resistance, which has become a major concern in recent years.

Cellular components including cell walls, cell membranes, and proteins have been reported to be targets of antimicrobials [155].

The bacterial cell wall is essential to maintain the structural integrity of cells. It consists of peptidoglycan, which is made from polysaccharide chains. The peptidoglycan layer constitutes as much as 95% of the Gram-positive cell wall and as little as 5-10% of the Gram-negative cell wall [156]. Some antimicrobials can inhibit the biosynthesis of peptidoglycan. For instance, bacitracin, subtilin, and mersacidin can bind to lipid II, which is a precursor of peptidoglycan, thus affecting the biosynthesis of peptidoglycan [157]. Some of the Gram-positive cell walls can be dissolved by lysozymes, an antimicrobial enzyme produced by animals [158].

The bacterial cell membrane physically separates the intracellular components from the extracellular environment. It serves as a permeability barrier for most molecules and ions. The cell membrane of Gram-negative bacteria consists of two layers: the inner membrane and the outer membrane. The inner membrane is the plasma membrane, while the outer membrane is constituted with glycerol phospholipids and lipopolysaccharides (LPS) [156]. Many antimicrobials can bind to the LPS and subsequently penetrate the cell membrane, thus depolarizing the cell membrane potential and finally killing the cells. For example, polymyxins, tyrocidines, and brevicacillins can disrupt the integrity of cell membranes by forming pores, thus causing a lethal effect on cells [159-161].

Apart from the cell wall and cell membrane, the intracellular components are also common targets of antimicrobials. Quinolones such as levofloxacin and ciprofloxacin interfere with DNA synthesis by inhibiting topoisomerase [155]. Rifampicin and fidaxomicin inhibit RNA polymerase, thus blocking the synthesis of RNA [155, 162, 163]. Several types of antibacterial agents such as chloramphenicol and tetracyclines inhibit protein synthesis by binding to subunits of the intracellular ribosomes [155]. Oligomycins bind to the F_0 subunit of ATP synthase, thus disrupting the biosynthesis of ATP [164].

Methodology to study antimicrobials

The rediscovery of known compounds has become a limit to discovering novel antimicrobials. The co-culture of microorganisms has been proven to be an effective way to overcome this limitation. It allows the microorganisms to interact and sometimes can yield novel secondary metabolites that are not observed in the pure culture. Many novel compounds have been identified using this method. For example, the co-culture of *Trichoderma harzianum* M10 and *Talaromyces pinophilus* F36CF yields harziaphilic acid [19], while phexandriols and phomesters were identified through the co-culture of *Phoma* sp. and *Armillaria* sp. [18].

The development of several new techniques also contributes to the identification of novel antimicrobials. The easy access to genomic sequences of microorganisms makes it possible to mine for antimicrobial BGCs. Many pipelines including antiSMASH and PRISM have been developed for this purpose [165]. However, genome mining cannot distinguish the active BGCs from the silent ones. Transcriptomics and comparative transcriptomics can overcome this shortage. Moreover, transcriptomics is used to study antimicrobial resistance [166]. Apart from them, more efficient techniques have been developed in recent years. Liquid extraction surface analysis (LESA) in combination with mass spectrometry (MS) allows rapid identification of secondary metabolites from the surface of bacterial colonies [167-169]. Nanospray desorption electrospray ionization (nanoDESI) MS is a similar technique for direct chemical monitoring of living microbial colonies. MS data combined with molecular networks enable one to visualize the produced molecules as familial groupings [170, 171]. Imaging mass spectrometry (IMS), in which the mass spectral data is acquired in a spatial manner and can be intuitively reconstructed as an image, is another newly developed technique to monitor the production of secondary metabolites from microbial colonies [172, 173]. These new techniques make it possible to study the antimicrobials without performing the tedious extraction process.

Scope of this thesis

In this thesis, we describe the isolation and screening of PGPR strains from perennial ryegrass for use in biocontrol of plant pathogens. We also report the identification and characterization of the antimicrobials produced by the isolated PGPR strains. In **chapter 2**, we screened seven potential PGPR strains out of 90 rhizosphere bacteria isolated from local grasslands. We further mined into their genomic sequences and discovered eleven novel secondary metabolites BGCs, including two NRPSs, four NRPS-PKS hybrids, and five bacteriocins. In **chapter 3**, we characterized a novel class of cationic circular peptide called relacidines, which selectively combat Gram-negative bacteria. Further investigation of their mode of action revealed that relacidines do not damage the cell membrane. Instead, they affect the oxidative phosphorylation process of cells and deplete the ATP. In **chapter 4**, we identified novel variants of bogorols (a class of cationic linear lipopeptides) and a novel class of peptides called succilins (succinylated bogorols). We show that bogorols are active against both Gram-positive and Gram-negative bacteria while succilins displayed limited bioactivity. We also report the special lipinitiation, the formation of valinol, the succinylation, and the mode of action (form pores in the cell membrane) of the bogorol family peptides. In **chapter 5**, we employ a comparative transcriptomic method to study the interaction of *Brevibacillus laterosporus* MG64 and different pathogens. We show that the expression of sporulation genes and secondary metabolites BGCs are regulated by the presence of pathogens. This thesis provides a comprehensive

understanding of the PGPR strains, in particular strain *B. laterosporus* MG64, as well as their antimicrobials, which are of great potential in biocontrol.

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2

Characterization of plant growth-promoting rhizobacteria from perennial ryegrass and genome mining of novel antimicrobial gene clusters

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Abstract

Background: Plant growth-promoting rhizobacteria (PGPR) are good alternatives for chemical fertilizers and pesticides, which cause severe environmental problems worldwide. Even though many studies focus on PGPR, most of them are limited in plant-microbe interaction studies and neglect the pathogens affecting ruminants that consume plants. In this study, we expand the view to the food chain of grass-ruminant-human. We aimed to find biocontrol strains that can antagonize grass pathogens and mammalian pathogens originated from grass, thus protecting this food chain. Furthermore, we deeply mined into bacterial genomes for novel biosynthetic gene clusters (BGCs) that can contribute to biocontrol.

Results: We screened 90 bacterial strains from the rhizosphere of healthy Dutch perennial ryegrass and characterized seven strains (*B. subtilis* subsp. *subtilis* MG27, *B. velezensis* MG33 and MG43, *B. pumilus* MG52 and MG84, *B. altitudinis* MG75, and *B. laterosporus* MG64) that showed a stimulatory effect on grass growth and pathogen antagonism on both phytopathogens and mammalian pathogens. Genome-mining of the seven strains discovered abundant BGCs, with some known, but also several potential novel ones. Further analysis revealed potential intact and novel BGCs, including two NRPSs, four NRPS-PKS hybrids, and five bacteriocins.

Conclusion: Abundant potential novel BGCs were discovered in functional protective isolates, especially in *B. pumilus*, *B. altitudinis* and *Brevibacillus* strains, indicating their great potential for the production of novel secondary metabolites. Our report serves as a basis to further identify and characterize these compounds and study their antagonistic effects against plant and mammalian pathogens.

Keywords: *Bacillus*, *Brevibacillus*, plant growth-promoting rhizobacteria, biosynthetic gene clusters, nonribosomal peptides, polyketides, bacteriocins.

Background

Perennial ryegrass (*Lolium perenne*) is one of the most important pasture plants in the world due to its high levels of palatability and nutritional value for ruminants [1]. The biomass and quality of perennial ryegrass are very crucial for the food chain of grass-ruminant-human since it not only produces food to ruminants but also determines the quality of meat and dairy products for human beings [2, 3]. The susceptibility of plants and potential causes of ruminant diseases by plant-originated pathogens are threats to the safety of this food chain. Although chemical fertilizers and pesticides can ensure the biomass production and the quality of the perennial ryegrass (if not vestigial), their usage may cause severe environmental problems. Thus, there is a need to find an environmentally friendly way to ensure the production of healthy grass.

Plant growth-promoting rhizobacteria (PGPR) have been widely reported to be effective in stimulating the growth of plants as well as protecting the plants from pathogens, which could be an alternative for chemical fertilizers and pesticides. *Bacillus* is one of the most famous PGPR because of its endospore-forming capability, which confers them better survival in the environment [4], and abundant plant growth-promoting traits, including nitrogen fixation, phosphorus solubilization, induced systemic resistance (ISR) induction, and most importantly antimicrobial production [5, 6].

Antimicrobials produced by *Bacillus* and closely related species are very diverse [7]. Based on their biosynthesis pathway, these antimicrobials are classified into three main groups: nonribosomal peptides (NRPs), polyketides (PKs), and bacteriocins. NRPs are synthesized in a nonribosomal pathway through nonribosomal peptide synthetases (NRPSs), which are huge enzymes constituted by different modules. Each module incorporates one amino acid residue, including non-proteinic amino acids. NRPs such as surfactin, fengycin, bacillomycin D, polymyxin, fusaricidin, etc. are very well-known antimicrobials produced by different *Bacillus* and *Paenibacillus* strains [8-10]. PKs are another class of antimicrobials synthesized with mega enzymes, which are called polyketide synthetases (PKSs). Well-known PKs produced by *Bacillus* or *Brevibacillus* include difficidin, bacillaene, macrolactin, basiliskamides, etc. [11-13]. Contrary to NRPs and PKs, bacteriocins are a class of antimicrobials synthesized in a ribosomal pathway. *Bacillus*-originated bacteriocins such as subtilisin A, plantozolicin, and subtilomycin are well studied [14-16].

Antimicrobials produced by *Bacillus* and closely related species were reported playing very important roles in biocontrol. The abolishment of surfactin production in *Bacillus subtilis* 6051 reduced its colonization to *Arabidopsis* roots and suppression of *Pseudomonas*-originated disease [17]. Iturins and fengycins produced by *B. subtilis* contribute to antagonism against *Podosphaera fusca*, a pathogen cause phyllosphere diseases in melon leaves [18]. *Bacillus velezensis* FZB42

(formerly *Bacillus amyloliquefaciens* FZB42), the Gram-positive model bacterium in biocontrol, employs diffucidin, bacilysin, and bacillaene to suppress fire blight disease of orchard trees [19]. In addition, siderophores (bacillibactin), cyclic lipopeptides (surfactin, fengycin, fusaricidin, etc.) can elicit induced systemic resistance (ISR) of plants, thus arming the plant against diseases caused by pathogens [5, 8, 20, 21]. In recent decades, volatiles such as 2,3-butanediol produced by *Bacillus* were also found to be elicitors of ISR [22].

Even though abundant studies focus on *Bacillus* and closely related PGPR, most of them are limited to the plant pathogens and neglect the mammalian pathogens that may enter the body of animals through grazing. For example, *Claviceps purpurea f. secalis*, a fungal pathogen that causes ergotism in ruminants and humans [23], is originated from forage plants. *Pithomyces chartarum*, a fungal pathogen produces sporidesmin that causes facial eczema in sheep [24], also originates from the grass. PGPR that can antagonize both phytopathogens and mammalian pathogens would ensure the safety of this food chain. Therefore, we aimed to isolate and screen *Bacillus* sp. and closely related PGPR strains from the rhizosphere of healthy perennial ryegrass and further mine into the genomes of the candidate PGPR strains to find novel biosynthetic gene clusters (BGCs) that are potentially involved in phytopathogen and plant-originated mammalian pathogen antagonism.

Results and discussion

Characterization of strains

A total of 90 Gram-positive bacterial strains were isolated from the rhizosphere of perennial ryegrass [25]. To characterize the strains, 16S rRNA genes were amplified and sequenced. A phylogenetic tree was constructed with the obtained sequences as well as 16S rRNA sequences of representative strains (Fig. 1). The strains were clustered into 4 different genera: *Bacillus* (83 strains), *Lysinibacillus* (4 strains), *Solibacillus* (2 strains), and *Brevibacillus* (1 strain). Among the dominant genus of *Bacillus*, 37 and 30 strains belong to the *B. subtilis* and *B. cereus* groups, respectively, while the rest 16 strains form an independent group that consists of *B. megaterium* and *B. simplex*. The great abundance of *Bacillus* is consistent with the study by Garbeva et al. [26], in which up to 95% of Gram-positive bacteria in the permanent grassland are *Bacillus* and related species.

Fig. 1 Phylogenetic analysis of the 90 bacterial isolates in this study. Neighbor-joining phylogenetic tree based on partial 16S rRNA sequences was constructed with MEGA7 [57]. The sequences of reference strains were retrieved from the NCBI database. The reference strains are highlighted in blue and different groups were shaded with different colors.

Antimicrobial activity

We evaluated all 90 strains for their antimicrobial activity against two pathogens: *Xanthomonas translucens* pv. *graminis* LMG587 and *Magnaporthe oryzae* Guy11. The rationale behind the selection is to cover the two major classes of pathogens (i.e. Gram-negative bacteria and fungi) in perennial ryegrass. *X. translucens* pv. *graminis* is a Gram-negative phytopathogen that causes bacterial wilt in perennial ryegrass and leads to great loss in temperate grassland regions [27]. *M.*

oryzae is a fungal pathogen that causes severe blast disease in many *Poaceae* plants, including perennial ryegrass [28, 29]. In total 30 strains showed inhibition against *X. translucens* pv. *graminis* LMG587 and 23 strains against *M. oryzae* Guy11. Of all strains, 16 showed antagonistic activity against both pathogens and 15 of them belong to the *B. subtilis* group, while the last one belongs to the genus of *Brevibacillus* (Additional file 1: Table S1). A total of 7 most inhibitory strains (i.e. MG27, MG33, MG43, MG52, MG64, MG75, and MG84) were selected to extend their antimicrobial spectrum and for further evaluation of their plant growth promotion potential.

Soil-borne plant pathogens and animal pathogens widely exist and cause serious diseases in higher organisms [30, 31]. Some animal pathogens can first adapt to the plant host and finally transmit to animals [32]. Plant pathogens and plant-originated mammalian pathogens that are a potential threat to perennial ryegrass and mammals in the food chain of grass-ruminant-human, were selected as indicators for the antimicrobial activity test. As indicated in Table 1, MG27, MG33, MG43, and MG64 showed very broad inhibitory spectra. They can antagonize Gram-positive bacteria, Gram-negative bacteria, fungi, and oomycetes (Table 1). In contrast to that, MG52, MG75, and MG84 showed better activity on bacteria than on fungi and oomycetes (Table 1). It is worth to note that some of the selected strains displayed antimicrobial activity against the fungal mammalian pathogens. MG27, MG33, MG43, and MG64 can antagonize *C. purpurea* f. *secalis* and *P. chartarum*, while MG75 and MG84 showed activity against *C. purpurea* f. *secalis*. This result suggests the possibility of using PGPR to control animal pathogens, thus safeguarding the food chain of grass-ruminant-human. However, we also realize the current knowledge gaps in this area. The mechanisms underlying the interaction of animal pathogens, plants, and PGPR are largely unknown. How to employ PGPR to control animal pathogens in the natural environment is a big question need to be answered. More in-depth studies will be needed before its application.

Plant growth-promoting effect of the selected strains

The plant growth-promotion effect of the candidate strains was tested with two different methods: 1) by inoculating onto the root tips of perennial ryegrass seedlings to test direct plant growth-promoting effect of the isolates; 2) via two-compartment petri dishes, where bacteria can only stimulate plant growth through volatile organic compounds (VOCs). When inoculated to root tips, MG27 and MG33 showed more than a 2.5-fold increase on shoot biomass and an approximately two-fold increase on root biomass compared to control. MG43 showed more than a two-fold increase in shoot biomass and no increase in root biomass. Other strains tested did not show any significant plant growth-promotion effect (Additional file 1: Figure S1). When the bacteria were inoculated with two-compartment petri dishes, all tested strains showed significant increases in shoot and root biomass of the perennial ryegrass with variations among strains. MG64 showed an approximately two-fold

increase of both shoot and root biomass, while other strains showed more than a 2.5-fold increase of both shoot and root biomass (Additional file 1: Figure S1).

Table 1 Antimicrobial activity of the seven selected bacterial strains from perennial ryegrass

Pathogen types	Species or strains	MG27	MG33	MG43	MG52	MG64	MG75	MG84
Gram-negative bacteria	<i>Erwinia carotovora</i> subsp. <i>brasiliensis</i> LMG21371	+	+	+	-	-	-	-
	<i>Pectobacterium carotovorum</i> LMG05863	+	+	+	-	+	-	-
	<i>Pseudomonas syringae</i> pv. <i>antirrhini</i> LMG02131	+	+	+	++	+	+	++
	<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	+	+	+	++	+	+	++
	<i>Ralstonia syzygii</i> subsp. <i>syzygii</i> LMG06969	+	+	+	-	+	-	-
	<i>Xanthomonas campestris</i> pv. <i>campestris</i> NCCB92058	+	++	++	++	+	+	++
	<i>Xanthomonas translucens</i> pv. <i>graminis</i> LMG587*	+	++	++	++	++	++	++
Gram-positive bacteria	<i>Streptomyces scabies</i> ISP5078	+	+	+	++	+	-	++
Fungi	<i>Botrytis cinerea</i>	●	●	●	○	●	○	○
	<i>Fusarium culmorum</i>	●	●	●	○	●	○	○
	<i>Fusarium oxysporum</i>	●	●	●	○	●	○	○
	<i>Rhizoctonia solani</i>	●	●	●	○	●	●	●
	<i>Magnaporthe oryzae</i> Guy 11*	●	●	●	○	●	○	○
	<i>Pithomyces chartarum</i> CBS485.71#	●	●	●	○	●	○	○
	<i>Claviceps purpurea</i> f. <i>secalis</i> CBS112.45#	●	●	●	○	●	●	●
Oomycetes	<i>Pythium aphanidermatum</i>	●	●	●	○	●	○	○
	<i>Pythium ultimum</i>	●	●	●	○	●	○	○

In the antibacterial assay, no inhibition (-), inhibitory zone < 5 mm (+), inhibitory zone ≥ 5 mm (++). In the antifungal/oomycetal assay, no inhibition (○), clear inhibition (●).

* Pathogens used to screen the isolated strains.

Mammalian pathogens

Genome sequencing of the selected strains and phylogenetic analysis

The genomes of the seven selected strains were sequenced and their DNA sequences were described previously [25]. Phylogenetic analysis using whole-genome sequences was conducted with Gegenees [33] and a phylogenetic tree was built with SplitTree [34]. As presented in Fig. 2, MG27, MG33, and MG43 fall into the *B. subtilis* subgroup, while MG52, MG75, and MG84 belong to the *B. pumilus* subgroup, which is different from 16S rRNA phylogenetic analysis that these two

subgroups did not clearly separate (Fig. 1). There are no strains belonging to the group of *B. cereus*, which is in accordance with the 16S rRNA phylogenetic tree (Fig. 1). MG64 was clustered to the genus of *Brevibacillus*, which is far away from the *Bacillus* genus phylogenetically (Fig. 2). The species names of the seven strains were designated as their most closely related strains, namely *B. subtilis* subsp. *subtilis* MG27, *B. velezensis* MG33 and MG43, *B. pumilus* MG52 and MG84, *B. altitudinis* MG75, and *B. laterosporus* MG64.

Fig. 2 Phylogenetic analysis of the selected strains and their reference strains based on the genomic sequences. The comparison was conducted with Gegenees using a default setting [33]. The dendrogram was constructed in SplitTree [34]. Different groups of bacteria were indicated with different colors of shading. The seven strains isolated in this study were indicated with red font.

Genome mining for BGCs

Bacillus and closely related species form a great reservoir of antimicrobials [7]. In order to evaluate the biosynthetic potential of the selected strains, their genomic sequences were analyzed by antiSMASH 5.0 [35] for mining of nonribosomal peptide synthetase (NRPS), polyketide synthetase (PKS), NRPS-PKS hybrid, and terpene BGCs, and by BAGEL4 [36] for mining of bacteriocin BGCs. Among the genus of *Bacillus*, the *B. subtilis* subgroup members (*B. subtilis* subsp. *subtilis* MG27, *B. velezensis* MG33, and *B. velezensis* MG43) harbor abundant NRPSs and PKSs (Fig. 3a) and the majority of the BGCs are assigned to known products (Fig. 3b, Additional file 1: Table S2). The remaining-unknown BGCs from this subgroup are terpene and PKS (Fig. 3c, Additional file 1: Figure

S2), including type III PKS, a homodimeric iterative polyketide synthase recently found present in microorganisms [37]. The total size of the BGCs in *B. subtilis subsp. subtilis* MG27 is approximately 176 kb and accounts for 4.2% of the genome size (Fig. 3d). This percentage is in line with the estimation of other *B. subtilis* strains, which is 4-5% on average [38]. *B. velezensis* MG33 and MG43 devote around 8.9% and 8.4% of their genomes to synthesis antimicrobial metabolites, respectively (Fig. 3d). This result is similar to the estimation of *Bacillus velezensis* FZB42, which is 8.5% [39]. The *B. pumilus* subgroup members (*B. pumilus* MG52, *B. pumilus* MG84, and *B. altitudinis* MG75) possess 8 to 11 BGCs (Fig. 3a). The abundance of terpene is an outstanding characteristic of this subgroup (Fig. 3a). Most of the BGCs from this group remain unknown, especially bacteriocins and terpenes (Fig. 3c, Additional file 1: Figure S2, Figure S3). The *B. pumilus* subgroup members devote 2.9% to 4.2% of their genomes to BGCs.

B. laterosporus has drawn increasing attention in recent years because of its outstanding ability of antimicrobial production. Borogols [40], brevibacillins [41], tauramamide [42], brevicidine [43], laterocidine [43], etc. are antimicrobials reported in the past two decades. Genome mining reveals that *B. laterosporus* MG64 harbors the most abundant gene clusters among the seven strains, which reach a total number of 26 (Fig. 3a). Five NRPSs were assigned to brevicidine, auriprocine, tyrocidine, petrobactin, bogorol, respectively (Fig. 3b, Additional file 1: Table S2). Up to 21 BGCs in *B. laterosporus* MG64 remained unknown and the majority of them are NRPS, NRPS-PKS hybrid, and bacteriocin (Fig. 3c, Additional file 1: Figure S2, Figure S3). The total size of the BGCs is approximately 500 kb, which accounts for 9.7% of the genome (Fig. 3d). This percentage is higher than *Bacillus velezensis* (8.5%) and *Streptomyces avermitilis* (6.4%), which are well-known antimicrobial producing strains [39, 44]. This result suggests the great value of *B. laterosporus* MG64 in biocontrol and pharmaceutical application, for some of its natural products might have the potential to be antibiotics.

Fig. 3 Numbers of BGCs harbored by the strains and the percentage of the total size of BGCs in the genomes. (a) total number of BGCs in the strains. (b) number of reported BGCs in the genomes of strains. (c) number of unknown BGCs found in the strains. BGCs that have different numbers of genes or show less than 70% protein identity to the reported ones were regarded as novel. (d) the percentage of BGCs sizes in the genomes.

Potential novel modular BGCs

NRPS, PKS, and NRPS-PKS hybrid are modular enzymes that synthesize secondary metabolites, some of which are well-known weapons for plant disease control [8]. Modular BGCs found in the selected strains with all essential modules (starting module, elongation module, termination module) were listed in Fig. 4. Despite the abundantly identified BGCs in *B. velezensis* MG33, one modular gene cluster showing no similarity to known BGCs was found (Fig. 4a). This BGC consists of 9 genes and has a total size of 40 kb. The cooccurrence of NRPS domains and PKS domains indicates it is a hybrid of both. The NRPS modules incorporate six amino acid residues while PKS modules likely incorporate and modify one polyketide moiety. It is difficult to predict the potential activity of its

final product because the antimicrobials (surfactin, fengycin, bacillomycin D, bacilysin, diffidicin, etc.) produced by *B. velezensis* MG33 are well-known for killing different kinds of pathogens [8, 19].

An interesting BGC discovered in *B. pumilus* MG52, *B. pumilus* MG84, and *B. altitudinis* MG75 is partially identical to lichenysin (Fig. 4b), which is a surfactin family lipopeptide biosurfactant produced by *Bacillus licheniformis*. Lichenysin is not only involved in direct pathogen antagonism but also affects the colonization of bacteria to plant, thus regarded as important in biocontrol [8]. This lichenysin-like BGC contains six genes, and four of them showed more than 50% sequence identity to the lichenysin BGC. Furthermore, the amino acid residues incorporated by these four genes are identical to lichenysin as well. However, there are two additional genes in between *lchAC* and *lchAD* (Fig. 4b). They encode four modules, which are responsible for the incorporation of four residues (Fig. 4b). Whether the additional genes are functional or not remains unclear. On the one hand, a thioesterase (TE) domain was encoded by *lchAC*, indicating that the biosynthesis of the lichenysin is likely not affected. On the other hand, other *B. pumilus* strains also showed this interesting phenomenon (data not shown), which suggests this is likely to be an evolutionary horizontal gene transfer. Experimental proofs are needed to answer this interesting question. Another unique NRPS-PKS hybrid BGC in *B. pumilus* MG52 and MG84 contains thirteen genes and encodes ten modules (Fig. 4c). This gene cluster showed 21% similarity to paenilamicin, an antibacterial and antifungal NRPs-PKs hybrid produced by *Paenibacillus larvae* [45]. However, neither the order of genes nor the predicted amino acid composition shows similarity to paenilamicin, indicating the putative novelty of the final product. *B. pumilus* MG52 and MG84 displayed potent activity against bacterial pathogens (Table 1). However, well-known antibacterial compounds were not found by the genome mining (Additional file 1: Table S2). This suggests the potential functionality of these novel BGCs.

Three potential intact and novel BGCs were discovered in *B. laterosporus* MG64 (Fig. 4). The first one is a 32-kb NRPS-PKS hybrid BGC (Fig. 4d). This BGC contains seven genes and encodes six NRPS modules and two PKS modules. This gene cluster does not show any similarity to the reported BGCs, indicating its great novelty. The second one is a 25-kb NRPS that contains nine genes (Fig. 4e). Six modules are encoded by the three core biosynthetic genes, indicates the incorporation of six amino acids. This BGC showed an 11% similarity to zwittermycin A, an NRPs-PKs hybrid produced by *B. cereus* [46]. However, the type of BGCs and the gene numbers are different from zwittermycin A, suggesting the putative novelty of the final product. The third one is also identified to be an NRPS (Fig. 4f). This BGC is around 27 kb in size and is constituted with two large core biosynthetic genes, which encode seven modules. The structure of this peptide remained unclear owing to the diverse function of a TE domain [47]. Bogorol and brevicidine that are identified by the genome mining were reported to have antibacterial activity [40, 43]. They are likely responsible for

the antibacterial activity of *B. laterosporus* MG64. However, the antimicrobials responsible for its antifungal and antioomycetal activity (Table 1) are not yet clear. Therefore, the three BGCs identified here are potentially functional.

Fig. 4 Potential intact and novel BGCs discovered in the genomes of selected strains. (a) an NRPS-PKS hybrid discovered in *B. velezensis* MG33. (b) a lichenysin-like NRPS-PKS hybrid present in the strains from the *B. pumilus* subgroup. (c) an unknown NRPS-PKS hybrid found in both *B. pumilus* MG52 and MG84. (d) a potential novel NRPS-PKS hybrid harbored by *B. laterosporus* MG64. (e-f) two potential novel NRPSs present in *B. laterosporus* MG64. Amino acid residues predicted by antiSMASH was indicated inside the A domains. Cluster number in the brackets corresponding to those in Figure S2. Genes with different functions are shown in different colors: orange, additional biosynthetic genes; wine-red, core biosynthetic genes; grey, unknown-function genes.

Potential novel bacteriocin BGCs

Bacteriocins are ribosomally synthesized antimicrobial peptides that mainly kill bacteria closely related to producers. They are classified into three main classes: class I small ribosomally produced and posttranslationally modified peptides (RiPPs), class II unmodified peptides, and class III large antimicrobial peptides (>10 kDa) [7, 48]. Among them, RiPPs (including lanthipeptides, circular

bacteriocins, sactipeptides, linear azole-containing peptides, thiopeptides, glycocins, and lasso peptides) are the most well-studied, wide-distributed, and active peptides [7, 49]. Potential novel RiPPs BGCs with predicted precursors discovered in the selected strains are listed in Fig. 5.

One circular bacteriocin BGC was harbored by both *B. pumilus* MG52 and MG84 (Fig. 5a). The gene cluster contains six genes. The precursor peptide contains 96 amino acids and the C terminus (from I33 to F96) showed 39% identity to amylocyclicin, which is a 6381-Da circular bacteriocin produced by *B. velezensis* FZB42 and showed to be active against closely related Gram-positive bacteria [50]. However, the rest of the genes show very low similarity to amylocyclicin BGC (data not shown), which indicates putative novel modification patterns. The potent activity of *B. pumilus* MG52 and MG84 against the Gram-positive bacterium *S. scabies* (Table 1) and the lack of known antimicrobials in their genomes (Additional file 1: Table S1) suggest the potential activity of this circular bacteriocin.

B. altitudinis MG75 harbors two circular bacteriocin BGCs (Fig. 5). The first one constitutes six genes (Fig. 5b). Its precursor peptide contains 108 amino acids and showed 67% protein identity to enterocin AS-48, which is a model circular bacteriocin produced by *Enterococcus* [51]. The second circular bacteriocin from *B. altitudinis* MG75 potentially contains 10 genes (Fig. 5b). The precursor peptide comprises 118 amino acids and the C terminus (from L58 to W118) showed 52% identity to enterocin NKR-5-3B, which is a broad-spectrum antimicrobial produced by *Enterococcus faecium* NKR-5-3 [52]. *B. altitudinis* MG75 did not antagonize the Gram-positive bacterium *S. scabies* (Table1). Therefore, the functionality of these circular bacteriocins remains unclear.

B. laterosporus MG64 harbors one circular bacteriocin and one lanthipeptide (Fig. 5). The BGC of circular bacteriocin contains seven genes (Fig. 5d). The core biosynthetic gene encodes a 58-amino acid peptide, which showed 62% protein identity to laterosporulin and 52% to laterosporulin10, both of which are produced by *B. laterosporus* and display antimicrobial activity against several bacterial pathogens [53, 54]. The lanthipeptide BGC from *B. laterosporus* MG64 (Fig. 5e) contains five genes. The second, fourth, and fifth genes were annotated as *lanB* (dehydratase), *lanC* (cyclase), and *lanD* (decarboxylase), respectively, which are commonly found in lanthipeptide BGCs. The core biosynthetic gene encodes a 43-amino acid peptide, which showed 39% similarity to both gallidermin and epidermin, two cationic lanthipeptides produced by *Staphylococcus* and displayed activity against a wide range of Gram-positive bacteria [55]. The antibacterial activity of *B. laterosporus* MG64 is likely conferred by bogorol and brevicidine [40, 43]. Therefore, we cannot elaborate on the putative functions of these bacteriocins.

Fig. 5 Potential novel bacteriocins with predicted precursor peptides. The BGCs were predicted by BAGEL4 [36]. (a) a circular bacteriocin found in both *B. pumilus* MG52 and MG84. (b-c) two circular bacteriocins discovered in *B. altitudinis* MG75. (d) a circular bacteriocin and (e) a lanthipeptide harbored by *B. laterosporus* MG64. The potential core peptides are indicated in red. The potential cleavage sites are indicated with arrows. The numbers indicate the position of amino acid residues.

Conclusions

In this study, we identified 7 potential PGPR strains, out of 90 strains screened, that can antagonize both phytopathogens and plant-originated mammalian pathogens, thus showing the possibility to employ PGPR to protect the food chain of grass-ruminant-human. Further mining into the genomes of the potential PGPR strains reveals a great number of BGCs, including known and potential novel ones. We show the great potential of *B. pumilus* subgroup strains in bacteriocin and terpene production and the great values of *B. laterosporus* MG64 in the production of natural products, which may also have pharmaceutical potential. Furthermore, eleven potential intact and novel BGCs were analyzed in detail, including two NRPSs, four NRPS-PKS hybrids, and five bacteriocins (four circular

bacteriocins and one lanthipeptide). Further efforts will be directed to identify these interesting secondary metabolites as well as their contribution to biocontrol.

Methods

Plant material, Bacterial isolation, and Strains

Perennial ryegrass seeds (cultivar Barsprinter) used in this study were provided by the company Barenbrug in Nijmegen, the Netherlands. Bacterial isolation from rhizosphere soil samples has been described previously and the genomic sequences of the most promising strains (MG27, MG33, MG43, MG52, MG64, MG75, and MG84) were placed in GenBank under accession no. of QJJA00000000, QJJB00000000, QJJC00000000, QJIZ00000000, QJJD00000000, QIMF00000000, and QJJE00000000, respectively [25]. The seven promising strains were also deposited in the NCCB collection (the Netherlands) under the accession numbers from NCCB100736 to NCCB100742.

Identification of bacterial strains

The cells of each bacterial isolate were collected by centrifuging at 10,000 rpm for 1 min and suspending in Mili-Q water. The suspension was heated at 100 °C for 10 min and centrifuged at 10,000 rpm for 1 min after cooling down to room temperature. The supernatant was used as template DNA in a PCR to amplify 16S rRNA for characterization. PCR amplifications were conducted with bacterial-specific 16S rRNA primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTGTGTACGACTT-3') as well as the high fidelity Phusion polymerase (Thermo Fisher Scientific). PCR products were purified with a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and sequenced at Macrogen Inc. The resulted partial sequences of 16S rRNA were aligned with relevant type strains with Muscle [56] in MEGA7 [57]. A neighbor-joining consensus tree [58] was constructed based on the alignment. The main parameters used were as follows: Bootstrap method and 1000 bootstrap replications for phylogeny test [59], Tamura-Nei model for nucleotide substitution [60], Gamma distribution for rate variation among sites, and complete deletion for treatment of gaps. The resulted phylogenetic tree was visualized and modified in iTOL [61].

In vitro antagonistic assay

Bacterial pathogens were streaked on LB plates and incubated at 28 °C overnight. The colonies were suspended in LB broth and mixed with melted LB medium (cool down to 45 °C) at a final concentration of 1×10^{-6} CFU/ml before pouring plates. After solidification, 5 µl bacterial solution ($OD_{600} = 1.0$) made with isolates was inoculated onto the plate. The plates were incubated at 28 °C for 48 h before measuring the diameters of inhibition halos. Fungal and oomycetal pathogens were inoculated on TSA plates (TSB solidified with 1.5% agar) and incubated at 28 °C for 5 days. An agar

plug (5 mm diameter) with fungal hyphae or oomycete spore was cut and inoculated onto the center of a new TSA plate. A 5 μ L sample of each bacterial solution at an OD₆₀₀ of 1.0 was spotted 2 cm away from the plug symmetrically. Plates were double sealed with parafilm and incubated at 28 °C for another 5 days. The antagonistic activities were then documented.

Plant growth-promoting assay

For surface sterilization, the ryegrass seeds were pre-treated with 0.3 M HCl for 6 h, followed by submerging in 2% sodium hypochlorite for 5 min and then washed with sterile water for 10 times to remove HCl and sodium hypochlorite completely. The seeds were germinated on wet sterile filter paper in a large petri-dish which was sealed with parafilm and then incubated at 25 °C without light. After germination for 5 days, the ryegrass seedlings were then transferred to fresh ½ MS (Duchefa Biochemie) plates solidified with 0.8% plant agar (Duchefa Biochemie). After 2 days growing in ½ MS plates, 5 μ L bacterial solution in 10 mM MgSO₄ with an OD₆₀₀ of 1.0 was inoculated, while the same amount of 10 mM MgSO₄ solution was used as control. For root tip inoculation assays, the bacterial solution was inoculated to the root tip of each seedling. To study the volatile effect, the bacterial solution was inoculated onto the center of a small LB agar plate that forms a physically separated compartment inside the ½ MS medium plate. After co-culture for 7 days. The ryegrass seedlings were harvested and the biomass of shoot and root were measured separately. A one-way ANOVA analysis using a Tukey post-hoc test was conducted with SPSS ($P < 0.05$) to evaluate the significance.

Genome sequencing and phylogenetic analysis

The genome sequences of the selected strains were determined as described previously [25]. Genome-scale comparison of the seven bacterial strains and other relevant strains were conducted with Gegenees [33] based on a fragmented nucleotides alignment with a setting of 200/100. Based on the alignment, a dendrogram was constructed in SplitTree 4.14.4 [34] with a neighbor-joining method and visualized in iTOL [61].

Genome mining for BGCs

The genome mining for biosynthetic gene clusters of antimicrobial compounds including NRPs, PKs, NRPs-PKs hybrids, bacteriocins, and terpenes was conducted with antiSMASH 5.0 [35] and BAGEL4 [36]. Each draft genome was assembled into a pseudomolecule using a closely related strain as a reference before applying to the pipelines. The genes predicted from both pipelines were further confirmed with protein BLAST. BGCs that have different numbers of genes or show less than 70% protein identity to the reported ones were regarded as novel.

Abbreviations

PGPR: Plant growth-promoting rhizobacteria; BGCs: Biosynthetic gene clusters; NRPs: Nonribosomal peptides; PKs: Polyketides; NRPSs: Nonribosomal peptide synthetases; PKSs: Polyketide synthetases; ISR: Induced systemic resistance; VOCs: Volatile organic compounds; RiPPs: Ribosomally produced and posttranslationally modified peptides. TE: Thioesterase; A: Adenylation; PCP: Peptidyl carrier protein; C: Condensation; E: Epimerization; AT: Acyltransferase; KS: Keto-synthase; Atd: Trans-acyltransferase docking; DH: Dehydratase; KR: Keto-reductase; CAL: Co-enzyme A ligase domain; ACP: Acyl-carrier protein

Availability of data and material

The genomic sequences of the seven selected strains are publicly available in DDBJ/EMBL/GenBank. Their accession numbers are QJJA000000000, QJJB000000000, QJJC000000000, QJIZ000000000, QJJD000000000, QIMF000000000, and QJJE000000000. The seven selected strains were deposited in the NCCB collection (the Netherlands) under the accession numbers from NCCB100736 to NCCB100742.

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Authors' contributions

Conceived and designed the experiments: ZL, CS, YY, and OK. Execution of experimental work and interpretation of the data: ZL and CS. Manuscript writing: ZL. Corrected the manuscript: ZL, CS, YY, and OK.

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Supplementary data

Table S1. Screening of bacterial isolates for antagonistic strains

Genus	Group	Strain	Activity		Strain	Activity		Strain	Activity	
			Xtg	Mo		Xtg	Mo		Xtg	Mo
<i>Bacillus</i>	<i>B. subtilis</i> group	MG18	-	+	MG55	+	+	MG69	-	-
		MG27	+	+	MG56	+	+	MG73	+	-
		MG28	+	+	MG57	+	+	MG74	+	+
		MG32	+	+	MG58	+	-	MG75	+	-
		MG33	+	+	MG59	+	-	MG77	+	-
		MG37	+	+	MG60	-	-	MG79	+	-
		MG39	+	+	MG61	-	+	MG82	+	+
		MG42	+	+	MG62	-	+	MG84	+	-
		MG43	+	+	MG63	-	+	MG85	-	+
		MG44	-	-	MG65	+	+	MG89	-	-
		MG51	+	+	MG66	-	-	MG90	-	+
		MG52	+	-	MG67	+	+			
		MG53	+	-	MG68	-	-			
	<i>B. cereus</i> group	MG4	-	-	MG22	-	-	MG70	-	-
		MG10	-	-	MG23	-	-	MG71	-	-
		MG11	-	+	MG25	+	-	MG72	+	-
		MG14	-	-	MG26	-	-	MG78	-	-
		MG15	-	-	MG30	-	-	MG80	-	-
		MG16	-	-	MG31	-	-	MG81	+	-
		MG17	-	-	MG34	-	-	MG83	-	-
		MG19	-	-	MG38	-	-	MG86	-	-
		MG20	-	-	MG46	-	-	MG87	-	-
		MG21	-	-	MG48	-	-	MG88	-	-
	<i>B. megaterium</i> group	MG1	-	-	MG9	-	-	MG41	-	-
		MG2	-	-	MG13	-	-	MG45	-	-
		MG3	-	-	MG29	-	-	MG47	-	-
		MG6	-	-	MG35	-	-	MG49	+	-
		MG7	-	-	MG36	-	-			
		MG8	-	-	MG40	-	-			
<i>Lysinibacillus</i>	-	MG5	-	-	MG24	-	-			
<i>Solibacillus</i>	-	MG12	-	-	MG50	-	-			
<i>Brevibacillus</i>	-	MG54	-	-	MG76	-	-			
		MG64	+	+						

Xtg is short for *X. translucens* pv. *graminis*. Mo stands for *M. oryzae*. The selected strains are indicated in red. '+' means clear inhibition halo was observed while '-' indicates no activity.

Table S2. Known antimicrobial BGCs found in the genomes of selected strains

Strain	Locus tag in NCBI	Size (bp)	Type	Antimicrobial compound
<i>B. subtilis</i> MG27	HS3_01760 - HS3_01767	7284	NRPS	Bacilysin
	HS3_01800 - HS3_01807	6939	Bacteriocin	Subtilisin A
	HS3_02375 - HS3_02387	19594	NRPS	Bacillibactin
	HS3_02542 - HS3_02557	77097	PKS	Bacillaene
	HS3_03507 - HS3_03511	8075	Bacteriocin	Subtilomycin
	HS3_03670 - HS3_03673	26146	NRPS	Surfactin
	HS3_04320 - HS3_04321	7516	NRPS	Fengycin (partial)
<i>B. velezensis</i> MG33	HS9_00027 - HS9_00030	37245	NRPS	Bacillomycin D (Iturin)
	HS9_00297 - HS9_00308	70109	PKS	Bacillaene
	HS9_00628 - HS9_00636	53268	PKS	Macrolactin
	HS9_02290 - HS9_02296	7298	NRPS	Bacilysin
	HS9_03348 - HS9_03351	26159	NRPS	Surfactin
	HS9_03453 - HS9_03455	9288	Bacteriocin	Amyloliquecidin
	HS9_03812 - HS9_03824	19408	NRPS	Bacillibactin
	HS9_03966 - HS9_03972	72103	PKS	Difficidin
	HS9_03835 - HS9_03839	3934	Bacteriocin	Amylocyclicin
	HS9_02779 - HS9_02780	11419	NRPS	Fengycin (partial)
	HS9_02781	3672	NRPS	Fengycin (partial)
	HS9_00002 - HS9_00003	7536	NRPS	Fengycin (partial)
<i>B. velezensis</i> MG43	C2W63_00620 - C2W63_00629	54848	PKS	Macrolactin
	C2W63_00883 - C2W63_00894	70106	PKS	Bacillaene
	C2W63_01016 - C2W63_01019	37286	NRPS	Bacillomycin D (Iturin)
	C2W63_01638 - C2W63_01649	66186	PKS	Difficidin
	C2W63_02562 - C2W63_02568	7298	NRPS	Bacilysin
	C2W63_03182 - C2W63_03187	4173	Bacteriocin	Amylocyclicin
	C2W63_03197 - C2W63_03209	19410	NRPS	Bacillibactin
	C2W63_03554 - C2W63_03556	26159	NRPS	Surfactin
	C2W63_01267 - C2W63_01268	11908	NRPS	Fengycin (partial)
	C2W63_01269	3789	NRPS	Fengycin (partial)
	C2W63_01043 - C2W63_01044	8025	NRPS	Fengycin (partial)
<i>B. altitudinis</i> MG75	US8_00456 - US8_00461	6103	NRPS	Bacilysin
<i>B. pumilus</i> MG84	C2W59_01112 - C2W59_01118	12865	NRPS	Bacillibactin
<i>B. laterosporus</i> MG64	C2W64_00357 - C2W64_00362	55318	NRPS	Brevicidine
	C2W64_03725 - C2W64_03728	41606	NRPS	Auriprocine
	C2W64_03774 - C2W64_03778	37314	NRPS	Tyrocidine
	C2W64_04196 - C2W64_04201	7104	NRPS	Petrobactin
	C2W64_00401 - C2W64_00410	54323	NRPS	Bogorol

Known BGCs were not found for the strain of *B. pumilus* MG52.

* only these genes can be found in the NCBI database, but the whole operon was found in the pseudomolecule that is assembly with all contigs using close related strains as a template.

Figure S1. Plant growth-promotion effects of the selected strains on perennial ryegrass. **(a)** plant growth-promotion effect of root-tip inoculation of bacteria. **(b)** effect of VOCs produced by candidate strains on the growth of perennial ryegrass. Sterile water was used as a control. Three replicates were used for each treatment. Different letters indicate significant differences between treatments (One-way ANOVA, Tukey post-hoc test, $P < 0.05$).

Figure S3. Potential novel bacteriocin BGCs mined from BAGEL4. Each draft genome was assembled into a pseudomolecule using a closely related strain as a reference before applying to the pipeline. BGCs that have different numbers of genes or show less than 70% protein identity to the reported ones were regarded as novel.

3

Characterization of two relacidines belonging to a novel class of circular lipopeptides that act against Gram-negative bacterial pathogens

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Originality-Significance statement

We report a new class of cationic circular peptides, which display outstanding bioactivity against Gram-negative bacteria. We also report the mode of action of these relacidines, which is affecting the oxidative phosphorylation process of cells. Our study provides a comprehensive understanding of this new class of nonribosomal peptides and is a solid base for their application in biocontrol and pharmacy.

Summary

The development of sustainable agriculture and the increasing antibiotic resistance of human pathogens call for novel antimicrobial compounds. Here, we describe the extraction and characterization of a class of cationic circular lipopeptides, for which we propose the name relacidines, from the soil bacterium *Brevibacillus laterosporus* MG64. Relacidines are composed of a fatty acid side chain (4-methylhexanoic acid) and 13 amino acid residues. A lactone ring is formed by the last five amino acid residues and three positively charged ornithines are located in the linear fragment. Relacidines selectively combat Gram-negative pathogens, including phytopathogens and human pathogens. Further investigation of the mode of action revealed that relacidine B binds to the lipopolysaccharides (LPS) but does not form pores in the cell membrane. We also provide proof to show that relacidine B does not affect the biosynthesis of the cell wall and RNA. Instead, it affects the oxidative phosphorylation process of cells and diminishes the biosynthesis of ATP. Transcription of relacidines is induced by plant pathogens, which strengthens the potential of *B. laterosporus* MG64 to be used as a biocontrol agent. Thus, we identified a new group of potent antibiotic compounds for combating Gram-negative pathogens of plants or animals.

Keywords: relacidines, antimicrobials, cationic circular lipopeptides, mode of action, *Brevibacillus laterosporus*, biocontrol

Introduction

Antimicrobials are substances that kill or inhibit the growth of microorganisms. They are of great value in different applications. Some of them are being used as weapons for plant disease biocontrol [1], while others are used as antibiotics for preventing and curing bacterial infections in animals, including humans [2]. The discovery of novel antimicrobials is of paramount importance not only for the development of sustainable agriculture but also to overcome antibiotic resistance, which has become one of the biggest threats to human health in recent decades.

Soil is an ecosystem that hosts a large and diverse population of microorganisms [3]. Microbes develop strategies to adapt to fluctuating soil environments and to survive in the competition with other organisms. Production of antimicrobials is one of the most potent strategies for this adaptation [4]. Thus, soil microorganisms form a natural reservoir of antimicrobials [2]. Screening of soil microorganisms that harbor novel biosynthetic gene clusters (BGCs) against pathogens is a traditional but efficient way to discover novel antimicrobials.

In a previous study, we isolated a rhizosphere bacterium, *Brevibacillus laterosporus* MG64, which displayed potent activity against plant pathogens and mammalian pathogens [5]. *Brevibacillus* is a genus of bacteria reclassified from *Bacillus* based on the 16S rRNA sequence analysis [6]. It is a rich resource for antimicrobials and many compounds have been isolated and characterized [7]. For instance, gramicidin S, lolatins, tyrocidines, etc. were discovered from *Brevibacillus brevis* [8-10], while tauramamide, bogorols, laterosporulin, etc. were isolated from *B. laterosporus* [11-14]. The current study was initiated to unveil novel antimicrobials produced by *B. laterosporus* MG64, which harbors abundant novel BGCs [5]. The bioactivity against different kinds of pathogens and the underlying mode of actions of the bioactive compounds were further investigated and dissected in order to evaluate their potential in applications.

Results

Purification and identification of relacidines

We previously isolated *B. laterosporus* MG64 from an agricultural rhizosphere sample and showed that this organism has several biosynthetic gene clusters encoding potential antimicrobials, and secretes compounds that inhibit the growth of bacterial and fungal pathogens [5]. To characterize these antimicrobials, supernatant from a *B. laterosporus* MG64 culture was precipitated using ammonium sulfate and applied to HPLC for purification. Each peak that eluted from the HPLC was collected for an *in vitro* activity test using *X. campestris* pv. *campestris* as an indicator. Two peaks (Figure S1) that displayed the most potent activity against the indicator were subjected to LC-MS to

determine their molecular masses. The first peak showed m/z values of 1549.82 and 1571.80, corresponding to a singly protonated compound $[M + H]^+$ and its sodium-cationized ion $[M + Na]^+$, respectively. The second peak showed m/z values of $[M + H]^+$ 1563.83 and $[M + Na]^+$ 1585.82 (Figure S2). The similar elution time and molecular mass suggested that they belong to the same class of compounds. We designated them as relacidine A and relacidine B, respectively. Relacidines represent some of the major secondary metabolites produced by *B. laterosporus* MG64. With the method used, around 0.2 mg of relacidine A and 0.5 mg of relacidine B can be obtained from one liter of culture.

To further characterize the compounds, the contents of both peaks were applied to LC-MS/MS analysis. As shown in Figure S3, both compounds were identified to contain a fragment of Tyr-Trp-Orn-Orn-Gly-Orn-Trp. With this information and the prediction by antiSMASH [15], we successfully identified the gene cluster of relacidines (Figure 1a). This gene cluster contains five genes, including two ATP-binding cassette transporter genes (*rlcA* and *rlcB*), two large core biosynthetic genes (*rlcC* and *rlcD*), and one drug resistance transporter gene (*rlcE*). The core biosynthetic genes encode 13 modules, among which the module 4 to module 7 incorporating the identified fragment of Orn-Orn-Gly-Orn (Figure 1a, Figure S3). According to the prediction, the final product should contain 13 amino acid residues. However, the signals from the C terminus were largely missing in the LC-MS/MS data, suggesting the potential existence of a cyclic structure, which is prevalent in natural products. To confirm this hypothesis, each compound was hydrolyzed with 2 M NaOH, desalted, and subjected to LC-MS/MS analysis. The signals at the C termini were found after hydrolysis, indicating the presence of a ring in the original compounds (Figure 1b and 1c). The ring of relacidine A was determined to be constituted of Thr-Ile-Gly-Ser-Gly, which is in perfect accordance with the prediction from antiSMASH (Figure 1a). Relacidine B has the same b ions as relacidine A, but the y ions show a difference of 14 Da, which indicates their difference at the last two amino acid residues of the C terminal region (Figure 1b-c). Residue Ser-12 (the number indicates the position of the amino acid residue, the same for the rest below) was predicted with high confidence by antiSMASH, and therefore we speculate the last amino acid residue of relacidine B to be Ala. The fatty acid tail and the first amino acid residue are predicted to be $C_7H_{13}O_1$ and Ser, respectively, which is supported by the tandem MS data (Figure 1b and 1c).

Figure 1. Characterization of biosynthetic gene cluster and structure of relacidines. (a) Gene cluster of relacidines predicted by antiSMASH. ABC transporter genes are indicated in red; core biosynthetic genes are shown in green; drug resistance related gene is labeled with blue. The letters in the adenylation domains represent the predicted amino acid residues. Tandem MS analysis of hydrolyzed relacidine A (b) and relacidine B (c). Peptides were hydrolyzed with 2 M NaOH and desalted before applying to LC-MS/MS. (d) ^1H - ^1H TOCSY and ^1H - ^1H NOESY NMR crosspeaks of relacidine B.

The structure of relacidine B was further confirmed by 1D and 2D NMR spectroscopy, where ^1H NMR, ^1H - ^1H -TOCSY NMR, ^1H - ^1H -COSY NMR, and ^{13}C - ^1H -HSQC NMR techniques were used to

identify the ^1H and ^{13}C signals of the different amino acid residues. All chemical shifts found were consistent with the MS/MS results. The structure of the fatty acid side chain was found to be 4-methylhexanoic acid (Figure 1d). Cross-peaks in the ^1H - ^1H -NOESY NMR verified the amino acid sequence found for this peptide in MS/MS (Figure 1d). Moreover, NOESY cross-peaks of both Ile10 and Ala-13 with Thr-9 confirmed the presence of the lactone macrocycle (Figure 1d). The chemical shift assignments are summarized in Table S1.

Taken together, relacidines are identified as novel lipopeptides that are constituted with a fatty acid tail (4-methylhexanoic acid) and 13 amino acid residues. Moreover, the last amino acid residue was linked to Thr-9 with an ester bond, thus forming a lactone ring (Figure 1d). Relacidines contain three positively charged ornithine residues, resulting in a net positive charge at neutral pH conditions, and therefore are classified as cationic peptides, which are considered to be attractive therapeutic candidates to combat Gram-negative pathogens [16, 17].

Antibacterial activity and mechanism of action

The relacidines were assessed for antibacterial activity. Phytopathogens (*Xanthomonas* species, *Pseudomonas syringae*, *Pectobacterium carotovorum*, and *Ralstonia syzygii*), food pathogen (*Bacillus cereus*), and human pathogens (*E. coli* ET8, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecium*) were tested in order to evaluate the potential of relacidines in different applications. As shown in Table 1, the relacidines displayed potent activity against all the Gram-negative bacteria tested. The minimum inhibitory concentration (MIC) values are in the range of 0.25-2 $\mu\text{g/ml}$ (0.16-1.29 μM), which is comparable to that of polymyxin B, a cationic peptide antibiotic commonly used to treat infections caused by multiple drug-resistant pathogens [18]. The addition of exogenous lipopolysaccharides (LPS) from *E. coli* increase the MIC values of both relacidines and polymyxin B, suggesting their binding to LPS. Relacidines did not affect the growth of the tested Gram-positive bacteria (i.e. *Staphylococcus aureus* subsp. *aureus* 533 R4, *Bacillus cereus* ATCC 14579, and *Enterococcus faecium* LMG16003) at a concentration up to 32 $\mu\text{g/ml}$. A similar phenomenon was observed for polymyxin B, where activity against Gram-positive bacteria was not observed.

The plant pathogen *X. campestris* pv. *campestris* was used to study the mode of action of relacidine B because of its great sensitivity. A growth curve was first determined to understand the efficacy of relacidines. Growth inhibition is defined as a drop in the increase of the optical density of a culture compared to the control. As shown in Figure 2a, the control cells are continuously growing, while the cells treated with a relacidine or polymyxin B displayed slower growth after a defined period of time, followed by an absence of growth. The effective time of relacidines is around 2.5 h when applied at $1 \times \text{MIC}$ and a faster effect was observed when the concentrations used are higher. Polymyxin B acts faster than relacidines when applied at the same MIC level. A time-kill assay was conducted to determine if relacidines have a bactericidal effect. The result showed that cell viability

was significantly reduced after 30 min of exposure to relacidines at ten times the MIC (Figure 2b), which confirms the bactericidal effect of relacidines.

Table 1 MIC values of relacidines against selected pathogenic bacteria.

Type of pathogen	Pathogen	MIC (µg/mL)		
		Relacidine A	Relacidine B	Polymyxin B
Gram-negative	<i>Xanthomonas campestris</i> pv. <i>campestris</i> NCCB92058	0.5	0.25-0.5	≤0.06
	<i>X. campestris</i> pv. <i>campestris</i> NCCB92058 + LPS (100 µg/mL)	4	4	8
	<i>Xanthomonas translucens</i> pv. <i>graminis</i> LMG587	0.25	0.25	≤0.06
	<i>Pseudomonas syringae</i> pv. <i>antirrhini</i> LMG2131	0.5	0.5	0.12
	<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	0.5	0.5	0.12
	<i>Pectobacterium carotovorum</i> LMG5863	2	0.5	0.25
	<i>Ralstonia syzygii</i> subsp. <i>syzygii</i> LMG6969	2	1	0.25
	<i>Escherichia coli</i> TOP10	2	2	0.25
	<i>E. coli</i> TOP10 + LPS (100 µg/mL)	8	8	8
	<i>E. coli</i> ET8	2	2	0.25
	<i>Klebsiella pneumoniae</i> LMG20218	2	2	0.25
	<i>Pseudomonas aeruginosa</i> PAO1	2	2	0.5
Gram-positive	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> 533 R4	>32	>32	>32
	<i>Bacillus cereus</i> ATCC 14579	>32	>32	>32
	<i>Enterococcus faecium</i> LMG16003	>32	>32	>32

A common mechanism of action employed by a lot of antibiotics, including cationic peptides, is interfering with membrane integrity [17]. We analysed whether relacidine B can permeabilize the cell membranes of *X. campestris* pv. *campestris* using a combination of two fluorescent DNA dye, one (SYTO9) green and membrane permeable, and the other (Propidium Iodide) red and membrane impermeable. Cells treated with polymyxin B, a cationic peptide antibiotic known to disrupt cellular membranes [19], were stained red, indicating that membranes were damaged. In contrast, cell membranes remain intact after treating with relacidine B and DMSO (control) for up to 7 h, indicating that relacidine B does not form holes in the cell membrane (Figure 2c).

Next, we investigated the membrane potential of cells treated with relacidine B using the potential sensitive membrane dye DiSC(3)-5 [20]. Considering that cationic peptides may affect the fluorescence signal, blanks without cells were also involved. As shown in Figure 2d, the control

treatment did not affect the signal regardless of the presence of cells. In contrast, the cationic peptide polymyxin B caused an increase in the blank, but the relative intensity of the signal is higher when cells were present. This is in accordance with its pore-formation in the cell membrane. Relacidine B, which is identified to be a cationic peptide in this study, is compatible with the dye and the fluorescence signal was decreased when the cells were present. This suggests the hyperpolarizing effect of relacidine B on cell membranes and further proved the integrity of cell membranes.

Figure 2. Growing curve, killing curve, membrane permeability, and membrane potential effect of relacidines. (a) Growing curve of *X. campestris* pv. *campestris* exposed to relacidines. The overnight culture was diluted with fresh LB to an OD₆₀₀ of 0.05 and dispensed into a 96-well plate (100 µL each well). Compounds were added at different concentrations when the cells reach the early stationary phase. (b) Killing curve of relacidines to *X. campestris* pv. *campestris*. All compounds were added at a concentration of 2.5 µg/mL. (c) Membrane permeability of relacidine B. *X. campestris* pv. *campestris* cells (OD₆₀₀ = 0.2) were treated with different compounds (DMSO and 0.5 µg/mL relacidine B for up to 7 h, and 1 µg/mL polymyxin B for 5 min). Cells were washed with PBS buffer and stained with SYTO9 and PI before inspecting with a microscope. (d) Membrane potential effect of relacidine B. The membrane potential dye DiSC(3)-5 was added at a final concentration of 6 µM. Relacidine B and polymyxin B were added at final concentrations of 0.5 µg/mL and 1 µg/mL, respectively. The fluorescence signal intensity is relative to the stabilized signal after adding DiSC(3)-5 (0 min). Con, control (Milli-Q water), RelB, relacidine B; PlyB, polymyxin B. ns, no significant difference; *, significant difference (T-test, $p < 0.05$), **, very significant difference (T-test, $p < 0.01$).

Inhibition of macromolecule (such as peptidoglycan, RNA, etc.) biosynthesis is another common mode of action employed by antibiotics. To investigate whether peptidoglycan biosynthesis is

affected by relacidine B, incorporation of the fluorescent D-amino acid analog HADA (hydroxycoumarin-carboxylic acid-amino-D-alanine) was followed. The incorporation of HADA in the cells treated with relacidine B or DMSO (negative control) was not affected, while that in the cells exposed to ceftriaxone (positive control) was significantly reduced (Figure S4). This result suggests that relacidine B does not abolish the biosynthesis of peptidoglycan. A competition experiment in which lipid II was added to relacidine before treatment confirmed that the peptidoglycan precursor lipid II, which is the target for many membrane-active peptides, is not the target for relacidine (Figure S5). The slow effect of relacidine B is similar to that of rifampicin, an antibiotic that inhibits RNA biosynthesis. To investigate RNA synthesis during relacidine treatment, incorporation of [^3H] uridine in RNA was followed over time. As shown in Figure S6, rifampicin blocks incorporation of [^3H] uridine, whereas relacidine had no effect and was comparable to the control. This result indicates that relacidine B does not affect the biosynthesis of RNA.

Next, the effects of relacidine B on cellular metabolism were studied. The addition of relacidine B caused a drop in the intracellular ATP concentration of cells compared to DMSO-treated cells (negative control, Figure 3a). The positive control, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a compound known to inhibit the oxidative phosphorylation of cells, caused a more drastic drop in intracellular ATP. Notably, cells treated with relacidine B for up to 2 h did not affect the growth curve (Figure 2a) and the integrity of the cell membrane (Figure 2c), suggesting that the ATP drop is caused by the cellular metabolism but not the cell death. A possible explanation for the decrease in intracellular ATP would be that ATP synthetase is targeted by relacidine B. This is unlikely however, as *E. coli* cells, in which enhanced substrate-level phosphorylation [21] can compensate for the absence of ATP synthetase, are also killed by relacidine B. A comparison of the sensitivity of wild type and *atp* (ATP synthetase gene) mutants of *E. coli* to relacidine B confirmed this, showing similar MIC values for all strains (Table S2).

To further investigate the cause of the decrease of ATP levels in bacteria treated with relacidine B, the NADH level of cells was determined using resazurin, which is reduced to resorufin by NADH in the presence of NADH dehydrogenase [22-26]. The NADH level of cells treated with relacidine B was higher than that of the control, although both of them showed the same trends of increase, whereas polymyxin B and rifampicin blocked the reduction after 30 min and 250 min, respectively (Figure 3b). This result indicates that relacidine B increases NADH of cells. The increase of the NADH level and the decrease of the ATP level suggest that the oxidative phosphorylation process of cells is affected by relacidine B (Figure S7). More precisely, either the electron transport chain is disrupted or ATP synthase is uncoupled from the electron transport chain (ETC).

Figure 3. Relacidine B affects the ATP and NADH level of cells. (a) ATP concentration of *X. campestris* pv. *campestris* cells treated with compounds. Relacidine B was added at a concentration of 0.25 $\mu\text{g/mL}$. CCCP (40 $\mu\text{g/mL}$) was used as a positive control and DMSO is used as a negative control. (b) NADH level of *X. campestris* pv. *campestris* cells treated with antimicrobials. Resazurin was added to the culture at a final concentration of 0.1 mg/mL. Relacidine B, rifampicin, and polymyxin B were added at final concentrations of 0.25 $\mu\text{g/mL}$, 0.125 $\mu\text{g/mL}$, and 1 $\mu\text{g/mL}$, respectively. DMSO was used as a negative control. RelB, relacidine B; Rif, rifampicin; PlyB, polymyxin B.

Expression of relacidines during the interaction with pathogens

Various strains of *B. laterosporus* have been reported to have good biocontrol potential [27-30]. A *B. laterosporus* strain isolated from honeybees was even reported to have a probiotic effect on the host [31]. Notably, all of these biocontrol strains (if the genomic sequence is available) were found to harbor an unidentified BGC that showed high similarity to relacidines (Figure S8). Given the effectiveness of relacidines in combating pathogens, the potential of their newly isolated producer, *B. laterosporus* MG64, to be used as a biocontrol agent or probiotic culture was evaluated. The expression and production of relacidines during the interaction with two plant pathogens (*X. campestris* pv. *campestris* and *P. syringae* pv. *tomato*) and two human pathogens (*K. pneumoniae* and *E. coli*) was investigated. As shown in Figure 4, *B. laterosporus* MG64 displayed a clear

inhibition activity against *X. campestris* pv. *campestris* and *P. syringae* pv. *tomato* grown at 28 °C. Both plant pathogens clearly induced the expression of the core biosynthetic gene (*rlcC*) of the BGC. The relacinine peptides were also discovered in the cell extracts, however, the presence of the pathogens did not seem to result in higher amounts of relacinine in the cell extracts. No inhibition was observed when *B. laterosporus* MG64 was cocultured with *K. pneumoniae* and *E. coli* at 37 °C, even though its secondary metabolites, relacinines, displayed potent activity against these two pathogens. Further inspection of the transcripts revealed that *rlcC* was expressed when growing alone, but down-regulated when interacting with the two human pathogens and that no relacinine peptides were detected in the cell extracts, regardless of the presence of the human pathogens. Together, these results suggest *B. laterosporus* MG64 can be a good biocontrol strain for plants, but it is likely not suitable to be used as probiotic.

Figure 4. Investigation of expression of relacinines during the interaction with two plant pathogens (left panel) and two human pathogens (right panel) at transcriptional and metabolic levels. The expression of a relacinine biosynthetic gene (*rlcC*) was tested with real-time PCR using three replicates. Different lowercase letters indicate a significant difference (ANOVA, $p < 0.001$) between treatments. The production of relacinines was analyzed by HPLC. Controls are producers growing on LB plates without pathogens. Xcc, *X. campestris* pv. *campestris* NCCB92058; Pst, *P. syringae* pv. *tomato* DC3000; Kp, *K. pneumoniae* LMG20218; Ec, *E. coli* ET8. Relacinine A and relacinine B in the HPLC chromatogram are indicated as A and B, respectively.

Discussion

Here, we identified two relacidines as members of a novel class of cationic cyclic lipopeptides. They have both similarities and differences compared to brevicidine and laterocidine, which are also antimicrobials produced by *Brevibacillus laterosporus* [32]. Brevicidine is different from relacidines in the first amino acid residue (Ser-1 in relacidines is replaced by Asn-1 in brevicidine) and the lactone ring (the Gly-13 in relacidines is absent in brevicidine) (Figure S9). Laterocidine is different from relacidines in the fatty acid side chain and the lactone ring: $C_7H_{13}O_1$ and Gly-Ser-Gly/Ala in relacidines are replaced by $C_9H_{17}O_1$ and Asn-Gly-Gly in laterocidine, respectively (Figure S9). Apart from the differences, brevicidine, laterocidine, and relacidines share outstanding characteristics. All of them contain a linear cationic chain and a hydrophobic lactone ring (Figure S9), both of which were considered crucial for the antibacterial activity [32].

The structure of relacidines, as well as of the recently reported brevicidine and laterocidine, is novel compared to other cationic cyclic lipopeptides such as tyrocidines, gramicidin S, and polymyxins (Figure S10), some of which are considered antibiotics of the last resort for combating multidrug-resistant bacteria [33]. First of all, they contain a cationic linear fragment constituted with a fatty acid tail and eight amino acid residues. Gramicidin S and tyrocidines do not possess a linear fragment and polymyxins contain a shorter cationic linear fragment constituted with a fatty acid tail and three amino acid residues (Figure S10). Also, the ring of relacidines is formed by an ester bond, in contrast to the peptide bond found in gramicidin S, tyrocidines, and polymyxins (Figure S10). Moreover, the ring of relacidines is constituted with 5 amino acid residues while that of gramicidin S, tyrocidines, and polymyxins contain 10, 10, and 7 amino acid residues, respectively (Figure S10). Lastly, the ring of relacidines is uncharged, unlike gramicidin S, tyrocidines, and polymyxins have 2, 1, and 4 positive charges, respectively (Figure S10).

Relacidines selectively combat Gram-negative pathogens, which is similar to the structurally related peptides brevicidine and laterocidine [32]. Another class of cationic peptides, polymyxins, also displays potent activity against Gram-negative bacteria but not against Gram-positive bacteria. The similarity of the inhibitory spectra of these peptides is believed to be related to their structures. Both relacidines and polymyxins possess a cationic linear fragment, which is proven to be important for binding to the anionic lipopolysaccharide (LPS) of Gram-negative cells [17]. Apart from the inhibitory spectra, the antimicrobial potency of relacidines is comparable to that of polymyxins, which are used in the treatment of infections caused by multi-drug resistant Gram-negative bacteria. Moreover, research on brevicidine and laterocidine showed that they have a low risk of resistance development in *E. coli* [32]. This is of paramount importance since the multidrug-resistant bacteria have become

a major concern in recent decades. All these factors show the great potential of relacidines, brevicidine, laterocidine for pharmaceutical applications.

Likely because of the structural uniqueness, relacidines, as well as brevicidine and laterocidine, display a different mode of action compared to other cationic cyclic peptides, which disrupt the cellular membranes and resulting in cell death. Our results show that relacidines bind to LPS but do not form pores in the cell membrane (Figure 2c). This is in line with an investigation on brevicidine and laterocidine [32]. We also provide evidence to show that relacidines do not affect the biosynthesis of peptidoglycan and RNA (Figure S4-S6). Instead, they affect the oxidative phosphorylation of cells, thus disrupting the biosynthesis of ATP (Figure 3a), which supplies energy for metabolisms. There are two possible mechanisms employed by relacidines to affect the oxidative phosphorylation. First of all, it can be an uncoupler of ATP synthase and the ETC. The ETC is a process of electron transfer from electron donors to electron acceptors. Meanwhile, it couples the transfer of protons across the cellular membrane, thus creating a proton-motive force. An uncoupler can disrupt the proton gradient and affect the biosynthesis of ATP. CCCP is a typical uncoupler that carries protons across the membrane and disrupts the biosynthesis of ATP [34, 35]. Additionally, relacidine B can also be an inhibitor of the protein complexes that constitute the electron transport chain, thus disrupting proton export. Examples of such compounds are rotenone and antimycin A that bind to complex I and complex III, respectively [36-41]. Both mechanisms result in a higher intracellular proton concentration, thus explaining the hyperpolarization of the cellular membrane (Figure 2d). Future studies will be directed to reveal which exact mechanism is employed by relacidines.

Our previous study showed that *B. laterosporus* MG64 can inhibit a broad range of pathogens [5]. In this study, by inspecting the production of the potent antimicrobial relacidines during the interaction with plant pathogens, we show that *B. laterosporus* MG64 can be applied as a biocontrol agent. The application of *B. laterosporus* in biocontrol of pathogenic microorganisms, insects, and nematodes has been reported previously [27-29, 42-44]. Some *B. laterosporus* strains were even used as probiotics [31, 45]. However, from the perspective of relacidines production during interaction with pathogens, *B. laterosporus* MG64 seems not suitable to be used as probiotics in animals, including humans. The abolishment of relacidine production is more likely a result of unfavorable growing temperatures (37 °C) than inhibition by pathogens (Figure 4).

Experimental Procedures

Extraction and purification of antimicrobial compounds

The producing strain, *B. laterosporus* MG64, was grown in Lennox broth (LB) overnight. The overnight culture was then diluted 100 times in fresh LB broth and incubated at 28 °C with agitation for 18 h. The supernatant was collected by centrifuging at 10,000 × g for 10 min and the secondary metabolites were precipitated with ammonium sulfate to 40% saturation. The precipitates were then dissolved in Milli-Q water and filtered through a 0.45 µm cellulose acetate membrane to achieve crude extracts. The crude extracts were applied to a reverse high-performance liquid chromatography (HPLC) for purification. An analytical C-18 column was used and 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). The compounds were eluted with a linear gradient of solvent B (from 15 % to 45%) in 30 min at a flow rate of 1.0 ml/min. A UV-detector set at a wavelength of 280 nm was used to monitor the effluents. All the peaks were collected separately and their antibacterial activities were tested against a plant pathogen *Xanthomonas campestris* pv. *campestris*.

LC-MS/MS analysis

The active compounds were characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS). An Ultimate 3000 UHPLC system coupled with a Q-Exactive Orbitrap™-based mass spectrometer (Thermo Scientific, San Jose, CA, USA) was used. The UHPLC system was equipped with a kinetex WVO-C18 column (2.6 µm particles, 100 × 2.1 mm, Phenomenex) and the mass spectrometer was equipped with a HESI-II electrospray source. The mobile phases of the LC were water with 0.1% formic acid and acetonitrile with 0.1% formic acid. In each run, a 10 µL sample was injected and separated with mobile phases at a flow rate of 500 µl min⁻¹. The MS/MS data was acquired with a spray voltage of 3.5 kV (positive mode) and a capillary temperature of 275 °C. The m/z range was set to 300 to 2000 and the resolution was 70,000. MS/MS data were recorded in targeted MSMS using PRM mode. In order to investigate the amino acid composition of the lactone ring, peptides were hydrolyzed in 2 M NaOH and desalted with an open column filled with C18 silica gel spherical (Sigma, United States) before applying to LC-MS/MS.

NMR spectroscopy

Prior to NMR analysis, the isolated peptide was purified further by RP-HPLC (XBridge C8 250 mm analytical column, solvent A: 0.1 % FA in ACN, solvent B: 0.1 % FA in ddH₂O, gradient 90 % B to 30 % B over 40 minutes, flow: 0.5 mL/min.) and the pure fractions were pooled and lyophilized. Around 1 mg of compound was dissolved in 0.5 mL DMSO-*d*₆. A Brüker Ascend 600 MHz spectrometer was used to record ¹H NMR, ¹H-¹H-COSY NMR, ¹H-¹H-TOCSY NMR, and ¹H-¹H-

NOESY NMR spectra. Chemical shifts in ^1H NMR spectra were internally referenced to solvent signals (DMSO- d_6 at $\delta\text{H} = 2.50$ ppm, $\delta\text{C} = 39.51$ ppm).

Minimal inhibitory concentration (MIC) test

The MICs of relacidine A and B were tested with the standard broth dilution method (Wiegand et al., 2008). Polymyxin B was used as a reference. MHB medium was used for all the bacteria tested. Peptides were diluted from 32 $\mu\text{g/mL}$ to 0.06 $\mu\text{g/mL}$ in a two-fold serial dilution and cells were adjusted to a concentration of 5.0×10^5 CFU/mL. The 96-well plate was incubated at 28 °C for 36 h and the OD₆₀₀ was measured with a Tecan Infinite F200 Pro Luminometer. The lowest concentration that causes invisible growth of bacteria was defined as the MIC values. The experiment was done in quadruplicate for each peptide and each strain.

Bacterial growth kinetics and time-kill assay

X. campestris pv. *campestris* was first inoculated in LB broth and incubated at 28 °C overnight. The overnight culture was then diluted with fresh LB to an OD₆₀₀ of 0.03 and distributed in a 96-well plate. The plate was incubated in a microplate spectrophotometer (1000 rpm, 28 °C) until OD₆₀₀ reached 0.05. Relacidines was added at concentrations of 0.25 $\mu\text{g/mL}$ (1 $\times$ MIC) and 2.5 $\mu\text{g/mL}$ (10 $\times$ MIC). Polymyxin B was added at a concentration of 0.625 $\mu\text{g/mL}$ (10 $\times$ MIC, lower concentration of polymyxin B does not affect the growth of cells although its MIC value is ≤ 0.06 $\mu\text{g/mL}$). The same amount of DMSO was added as a control. The plate was incubated at the same condition for 20 h and the growth kinetics were monitored. Each treatment was done in quadruplicate. For the time-kill assay, all compounds were added at a concentration of 2.5 $\mu\text{g/mL}$ when the OD₆₀₀ reached 0.1. At each time point (0, 30, 60, and 90 min), 10 μL of culture was tenfold serially diluted and plated on LB agar plates. After incubation at 28 °C for 36 h, colonies were counted and the number of CFU per mL was calculated.

Membrane permeability assay

A commercial LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) was used to test the integrity of the cell membrane of *X. campestris* pv. *campestris* after treatment with peptides. Cells were grown in LB overnight and diluted to an OD₆₀₀ of 0.2. Relacidine B was added at a concentration of 0.25 $\mu\text{g/mL}$ (1 $\times$ MIC), while polymyxin B was added at a concentration of 1 $\mu\text{g/mL}$. The same amount of DMSO was added to the control. Cells were treated at room temperature for 30 min before harvest. The harvested cells were washed and resuspended in 200 μL 0.9% saline (NaCl) solution. Two different dyes (3.34 mM SYTO9 and 20 mM propidium iodide) were added at a ratio of 1:1 (v/v). Cells were stained in the dark for 15 min and a 5 μL sample was mounted on a 1% agarose pad

before being imaged using a Nikon Ti-E microscope (Nikon Instruments, Tokyo, Japan) equipped with a Hamamatsu Orca Flash 4.0 camera.

Membrane potential assay

Fresh *X. campestris* pv. *campestris* cells were inoculated in MHB medium and grown until OD₆₀₀ reached 0.4. The culture was two times diluted with fresh MHB medium and dispensed in a 96 well plate. The membrane potential dye DiSC(3)-5 was added to a final concentration of 6 μ M. The fluorescence signal (excitation 633 nm, emission 660 nm) was allowed to stabilize for 20 min in a microplate spectrophotometer (BioTek Synergy Mx). After stabilization, relacidine B and polymyxin B were added at final concentrations of 0.5 μ g/mL and 1 μ g/mL, respectively. Milli-Q water was used as a negative control. Blanks (without cells) were used to show that the signal change is not caused by the interaction of the dye and compounds. The fluorescence signal was then monitored for 60 min at an interval of 5 min with a microplate spectrophotometer. Three replicates were used for each treatment.

Hydroxycoumarin-carboxylic acid-Amino-D-Alanine (HADA) incorporation assay

D-amino acids are involved in peptidoglycan biosynthesis of diverse bacteria (Lam et al., 2009). HADA is a fluorescent D-amino acid analogue that can be used for monitoring the peptidoglycan synthesis activity of bacteria (Kuru et al., 2015). Cells were grown in LB broth until OD₆₀₀ reached 0.2. Relacidine B was added at a concentration of 0.25 μ g/mL and cells were kept growing for another 2, 4, and 5 h. DMSO was used as a negative control while ceftriaxone (4 μ g/mL) was used as a positive control. At each time point, cells were treated with 500 μ M HADA for 24 min. After treatment, cells were washed twice with PBS buffer. Incorporation of HADA was inspected with a Nikon Ti-E microscope (Tokyo, Japan) equipped with a Hamamatsu Orca Flash 4.0 camera.

Lipid II binding assay

Generally, lipid II from Gram-negative bacteria has *meso*-diaminopimelic acid (mDAP) on residue 3, while that from Gram-positive bacteria has lysine [48]. Both types were used in the lipid II binding assay. An overnight culture of the indicator strain (*X. campestris* pv. *campestris*) was added to melted LB agar at a final concentration of 0.2% (v/v) before pouring plates. Lipid II and relacidine B were mixed at different ratios (0.5, 1, and 2) before spotting onto the plate. The plate was incubated at 28 °C overnight. The binding of lipid II and relacidine B is indicated by the decrease of halo size.

Intracellular ATP concentration assay

The BacTiter-Glo™ Microbial cell viability assay kit (Promega) was used to determine the intracellular ATP concentration of *X. campestris* pv. *campestris*. Cells were grown in LB broth until OD₆₀₀ reached 0.2. Relacidine B was added at a concentration of 0.25 µg/mL (1 × MIC) and the DMSO was used as a solvent control. Carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), an uncoupler of oxidative phosphorylation, was used as a positive control (40 µg/mL). Cells were treated at 28 °C for 0, 1, 2, 3 h. At each time point, 100 µL cell culture was added to 100 µL BacTiter-Glo™ reagent and incubated at room temperature for 5 min. Luminescence was with a Tecan Infinite F200 Pro luminometer and the intracellular ATP concentration was calculated with a standard curve made with a commercial ATP solution.

Resazurin assay

Nonfluorescent resazurin can be reduced to fluorescent resorufin in a NAD(P)H dependent manner in the presence of NAD(P)H dehydrogenase [22-26]. Therefore, the resazurin/NAD(P)H dehydrogenase/NAD(P)H system can be used to detect the NAD(P)H level of cells. *X. campestris* pv. *campestris* cells were grown in LB broth until OD₆₀₀ reached 0.1. Resazurin was added to the culture at a final concentration of 0.1 mg/ml. Relacidine B, rifampicin, and polymyxin B were added at final concentrations of 0.25 µg/mL, 0.125 µg/mL, and 1 µg/mL, respectively. DMSO was used as a negative control. Blanks (without cells) revealed that the signal change is not caused by the interaction of resazurin and compounds. Fluorescence was recorded at a wavelength of 560 nm every 20 min over a period of 4 h with a BioTek Synergy Mx 96-well plate reader.

Determining relacidine expression during the interaction with different pathogens

A fresh culture of each pathogen was mixed with melted agar (around 45 °C) before preparing plates. *B. laterosporus* MG64 was inoculated (10 µL inoculum with an OD₆₀₀ of 1.0) at the center of each plate containing pathogens. The plates were incubated at 28 °C or 37 °C based on the growth temperature of the indicator strain. After 2 days, the inhibition against pathogens was documented with photos and *B. laterosporus* MG64 cells were collected for further analysis. The total RNA was extracted using the High Pure RNA isolation kit (Roche Diagnostics Nederland BV) and 500 µg of each RNA sample was used for reverse transcription using the SuperScript™ III Reverse Transcriptase (Invitrogen) following the instruction manual. The cDNAs were 5-times diluted to make templates. The primers used in the qPCR are as follows: Housekeeping gene: *rpoB*-forward, CCAAGACATTTGCGCAATCC; *rpoB*-reverse, CGTTCCTTCGACTCGTCTAC. Target gene: *rlcC*-forward, TCGACAGTACGATGCCTTTC; *rlcC*-reverse, GAGCTTCGCCATCAACAC. The qPCR mixture contained with 1 µL template, 0.6 µL forward primer, 0.6 µL reverse primer, 7.8 µL distilled water, and 10 µL SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad). The procedure is

95 °C 3 min, 95 °C 30 s, 57 °C 30 s, 72 °C 30 s, 72 °C 5 min and 40 cycles were used. The relative expression of *relC* is calculated with the $2^{-\Delta\Delta CT}$ method. The metabolites were extracted from the cells with 50% acetonitrile and lyophilized. The compounds were redissolved in Milli-Q water and filtered through a 0.45 µm cellulose acetate membrane before applying to HPLC and MALDI-TOF analysis.

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Supplementary data

Figure S1. HPLC chromatogram of bioactive compounds extracted from *B. laterosporus* MG64. The two peaks showed the most potent activity toward *X. campestris* pv. *campestris* were labeled as A and B, respectively.

Figure S2. LC-MS analysis of the two bioactive compounds isolated from *B. laterosporus* MG64. The ions at m/z ($z = 1$) 1549.82 and 1563.83 represent the singly protonated $[M + H]^+$ of relacidine A-B, respectively. Their sodium-cationized $[M + Na]^+$ are 1571.80 and 1585.82, respectively.

Figure S3. Fragmentation of relacidines by tandem MS to generate *b* and *y* ions. *b* ions are indicated in blue while *y* ions are indicated in red. (A) Fragmentation of relacidine A. (B) Fragmentation of relacidine B.

Table S1. Chemical shift (ppm) assignments of relacidine B (d_6 -DMSO).

Amino acid	Position	$\delta^{13}\text{C}$ NMR	$\delta^1\text{H}$ NMR	Amino acid	Position	$\delta^{13}\text{C}$ NMR	$\delta^1\text{H}$ NMR
Ser1	1-NH		7.95	Trp8	28-NH		8.28
	1	54.6	4.29		28	52.9	4.68
	2	61.4	3.50		29	27.1	3.11 / 2.96
Tyr2	3-NH		8.04		30	123.8	7.11
	3	54.5	4.32		31		10.84
	4	35.9	2.81 / 2.64		32	111.0	7.31
	5	129.8	6.88		33	120.6	7.04
Trp3	6	114.6	6.56		34	118.0	6.96
	7-NH		8.14		35	118.2	7.57
	7	53.3	4.51	Thr9	36-NH		7.98
	8	27.1	3.14 / 2.93		36	53.2	4.64
	9	123.6	7.14		37	69.4	5.00
	10		10.81		38	14.1	0.98
	11	111.0	7.31	Ile10	39-NH		7.73
	12	120.6	7.04		39	56.5	4.15
	13	118.0	6.96		40	35.4	1.64
	14	118.2	7.57		41	24.7	1.53 / 1.10
Orn4	15-NH		8.04		42	10.5	0.86
	15	51.5	4.32		43	14.8	0.85
	16	29.0	1.72 / 1.57	Gly11	44-NH		9.20
	17	24.4	1.51		44	43.5	3.90 / 3.35
Orn5	18	38.6	2.73	Ser12	45-NH		8.47
	19-NH		8.23		45	56.3	4.10
	19	51.6	4.34		46	60.4	3.80 / 3.68
	20	29.0	1.72 / 1.58	Ala13	47-NH		7.74
	21	24.4	1.50		47	48.0	4.39
Gly6	22	38.6	2.69		48	16.9	1.41
	23-NH		8.32	FA	F-1	32.6	2.11
	23	41.7	3.77 / 3.72		F-2	31.5	1.50 / 1.27
Orn7	24-NH		8.05		F-3	33.3	1.26
	24	51.5	4.32		F-4	28.4	1.28 / 1.09
	25	29.2	1.42 / 1.29		F-5	10.9	0.81
	26	24.4	1.27		F-6	18.6	0.81
	27	overlap	2.51

^1H NMR of relacidine B (d_6 -DMSO)

^1H - ^1H -TOCSY-NMR of relacidine B (d_6 -DMSO) ^1H - ^1H -COSY-NMR of relacidine B (d_6 -DMSO)

^{13}C - ^1H -HSQC NMR of relacidine B (d_6 -DMSO) ^1H - ^1H -NOESY NMR of relacidine B (d_6 -DMSO)

Figure S4. The effects of peptides on HADA incorporation during cell wall biosynthesis. DMSO was used as a negative control and ceftriaxone (4 $\mu\text{g}/\text{mL}$) was used as a positive control. *X. campestris* pv. *campestris* cells were treated with compounds (up to 5 h for DMSO and relacidine B and 30 min for ceftriaxone) and pulsed with HADA for 30 min. The white bar represents 5 μm . The signal indicates the incorporation of HADA, which is abundantly present in DMSO and relacidine B treatments and barely present in ceftriaxone treatment.

Figure S5. Lipid II was mixed with compounds at different ratios before spotting onto the plate, which contains the indicator strain *X. campestris* pv. *campestris*. Nisin was used as a negative control. D or L in the bracket means a Gram-negative or Gram-positive type of lipid II was used. The plate was incubated at 28 $^{\circ}\text{C}$ overnight before recording the result.

Figure S6. RNA biosynthesis of *X. campestris* pv. *campestris* after treating with compounds. [^3H] uridine was used as a precursor for RNA biosynthesis. Radioactivity was recorded by liquid scintillation counting. DMSO is a negative control while rifampicin is a positive control. Both rifampicin and relacidine B were added at a final concentration of $2\times\text{MIC}$. Two replicates were used for each treatment and the mean values are shown.

Table S2. MICs relacidine B against *E. coli* MG1655 wild type strain and its *atp* knock out mutants.

Strain	MIC ($\mu\text{g/mL}$)	Strain	MIC ($\mu\text{g/mL}$)
<i>E. coli</i> MG1655 (WT)	0.5	$\Delta atpE$	1.0
$\Delta atpA$	0.5	$\Delta atpF$	1.0
$\Delta atpB$	0.5	$\Delta atpG$	1.0
$\Delta atpC$	1.0	$\Delta atpH$	0.5
$\Delta atpD$	1.0	$\Delta atpI$	1.0

Note: *atpA-H* are ATP synthetase biosynthetic genes.

Figure S7. Schematic presentation of the effect of relacidine B on the oxidative phosphorylation of cells. Complex I, NADH dehydrogenase; complex II, succinate dehydrogenase; complex III, cytochrome c reductase; complex IV, cytochrome c oxidase; Q, quinone. The electron transport chain is indicated by a green curve. The change of NADH and ATP concentration after treatment with relacidine B are indicated with yellow arrows (up, increase; down, decrease).

B. laterosporus BMP3

B. laterosporus BGSP7

B. laterosporus BGSP9

B. laterosporus BGSP9

Figure S8. BGCs harbored by biocontrol strains of *B. laterosporus* that show high similarity to the relacidines BGC. Genomic sequences of the biocontrol strains were analyzed with antiSMASH.

Relacidines

Brevicidine

Laterocidine

Figure S9. Structure comparison of relacidines, brevicidine, and laterocidine. The parts of brevicidine and laterocidine that are different from relacidines were indicated in red. The thirteenth amino acid residues in relacidines were indicated in blue.

Relacidine B **Polymyxin B** **Gramicidin S** **Tyrocidine A**

Figure S10. Structure comparison of relacidines, polymyxins, gramicidin S, and tyrocidines. The positive-charged residues are indicated in red. The ester bond in relacidine B is indicated in green.

4

Novel modifications of nonribosomal peptides from *Brevibacillus laterosporus* MG64 and investigation of their mode of action

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Abstract

Nonribosomal peptides (NRPs) form a class of secondary metabolites that are of paramount importance in different applications including biocontrol and pharmacy. In this study, we characterize four novel bogorol variants (bogorol I-L) and four succilins (succilin I-L, containing a succinyl group that is attached to the Orn₃/Lys₃ in bogorol I-L) from the biocontrol strain *B. laterosporus* MG64. We show that these peptides employ an adenylation pathway for lipoinitiation, which is different from the usual pattern of lipopeptides. Moreover, the formation of valinol was proven to be mediated by a terminal reductase domain and a reductase encoded by the gene *bogl*. Furthermore, succinylation, which is a novel type of modification in NRPs, was discovered. We show that bogorols combat both Gram-positive and Gram-negative bacteria by forming pores in the cell membranes. Our study provides a comprehensive understanding of bogorols and succilins and extends the knowledge of the biosynthetic machinery of lipopeptides.

Keywords

Bogorol variants, succilins, *Brevibacillus laterosporus*, biosynthesis, lipoinitiation, reduction, succinylation, mode of action, synergy

Introduction

Nonribosomal peptides (NRPs) are a class of secondary metabolites synthesized by megaenzymes named nonribosomal peptide synthetases (NRPSs). NRPs display diverse properties, some of which can help the producer to survive in natural environments. Surfactins and viscosins are essential for the motility of *Bacillus subtilis* and *Pseudomonas putida*, respectively [1-4]. Massetolide A and rhamnolipid are involved in biofilm formation of different *Pseudomonas* species [5-7]. Bacillibactin and petrobactin are siderophores that can chelate iron and making it available for *Bacillus* species [8, 9]. Many NRPs display antimicrobial activity against a broad range of microorganisms, including bacteria, fungi, oomycetes, viruses, etc. Because of this important property, some NRPs such as surfactins and iturins are used as biocontrol agents to defend against plant diseases [10], while others such as daptomycin and polymyxin are used as antibiotics to treat animal and human diseases caused by pathogenic bacteria [11, 12].

Brevibacillus is a genus belonging to the Firmicutes, one of the phyla with a great abundance of NRPS enzymes [13]. *Brevibacillus* spp. form a rich source of NRPs, which has been known for many decades. Tyrocidine and Gramicidin S, two cyclic lipopeptides with potent antibacterial activity, were discovered from *Brevibacillus brevis* in the 1940s [14, 15]. A class of linear cationic lipopeptides including bogorols, BT peptide, BL-A60, and brevibacillins, was discovered from *Brevibacillus laterosporus* in the past two decades [16-20]. Brevicidine, laterocidine, and relacidines, a class of cationic lipopeptides that selectively combat Gram-negative bacteria, were characterized from *B. laterosporus* in the past two years [21]. Even so, we believe that the biosynthetic potential of *Brevibacillus* spp., especially *B. laterosporus*, has been underestimated. On the one hand, the genome mining of *B. laterosporus* MG64, a plant growth-promoting rhizobacterium from perennial ryegrass, revealed that around 80% of the biosynthetic gene clusters (BGCs) has not been assigned to known products yet [22]. On the other hand, some structurally similar NRPs produced by *Brevibacillus* spp. were overlooked [23]. For example, bogorols, BT peptide, and brevibacillins share similar structures but have different amino acid residues in the second, fourth, fifth or ninth positions. A minor difference of the amino acid residues has been shown to affect the biological properties of the bogorols: a replacement of isoleucine by valine at the fourth position decreases the antibacterial activity severely [17]. Therefore, more attention should be paid to the characterization of the rich variety of NRPs from *Brevibacillus* spp.

In this study, we aimed to identify and characterize novel NRPs produced by the biocontrol strain *B. laterosporus* MG64, which showed very good activity against a variety of pathogens, including plant pathogens and mammalian pathogens [22]. Furthermore, we investigate the biosynthesis and the mode of action of the compounds to have a better understanding of their potential for application.

Results

Purification and characterization of bioactive peptides from *B. laterosporus* MG64

We previously isolated a biocontrol strain *B. laterosporus* MG64 that can inhibit a wide range of plant pathogens and mammalian pathogens [22]. To identify and characterize the bioactive compounds, *B. laterosporus* MG64 was inoculated in LB broth for 24 h and the supernatant was used to extract the metabolites with a column filled with C18 silica gel. The crude extract was further purified with HPLC and the different peaks that were collected were screened for bioactivity against *X. campestris* pv. *campestris*, a Gram-negative pathogen that causes bacterial wilt in perennial ryegrass, and *Bacillus cereus*, a Gram-positive bacterium that causes food spoilage. The major peaks (labeled peak 1-3 in Figure S1) showed bioactivity against both strains and were selected for further characterization.

Four different compounds were characterized by LC-MS/MS (Figure S2). Compounds **1** and **2** have the same mass of 1555.06 Da (m/z 778.53 [$M+2H$]²⁺). The clear signals of y and b ions indicated the presence of Val (5), Orn (1), Ile (1), Lys (2), Tyr (1) (Figure S2a). The difference between compounds **1** and **2** are the amino acid residues at positions 4 and 5, where compound **1** has Ile₄-Val₅ and compound **2** has these inverted. The total mass and the amino acid composition of both compounds are similar to bogorols, which form a class of linear peptides that are constituted with a fatty acid tail (2-hydroxy-3-methylvaleric acid) and 13 amino acid residues (including a valinol at the last position) [16, 17]. The bogorol biosynthetic gene cluster identified from antiSMASH also supports this hypothesis (Figure 1a). With the reference of bogorol, we successfully identified the components at both termini, namely C₆H₁₁O₂-Dhb for N-terminus (b_2 = 198.11, Figure S2a) and Leu-Valinol for C-terminus (y_2 = 217.19, Figure S2a). However, Leu₉ in the reported bogorols is replaced by Val₉ in compounds **1** and **2**. Therefore, we propose them to be novel variants of bogorol. Similarly, compounds **3** (m/z 785.54 [$M+2H$]²⁺) and **4** (m/z 792.54 [$M+2H$]²⁺) were identified to be novel variants of bogorol as well. Compared to compounds **1** and **2**, compound **3** has a 14-Da increase in mass, which is a result of two consecutive Ile residues at positions 4 and 5 (Figure S2b). Compound **4** has Lys instead of Orn at position 3, which is the only difference from compound **3** (Figure S2c). Compounds **1-4** were therefore nominated as bogorol I-L, respectively (Figure 1b).

The structures of bogorol K and bogorol L were further confirmed with ¹H NMR, ¹H-¹H TOCSY NMR, and ¹H-¹H NOESY NMR (Figure S3). Their chemical shift (ppm) assignment (partial) are shown in Table S1 and Table S2, respectively. A Marfey-type analysis [24] was also conducted to detect the configuration of amino acids in bogorol K and bogorol L. Both compounds have an L-configuration of all the Val, Leu, Ile, and Valinol residues, and a D-configuration of Tyr in position 11 (Table S3).

The Orn in position 3 of bogorol K is in D-configuration, which suggests the Lys in bogorol L should also be a D-configuration. The other two Lys residues in positions 7 and 10 of both compounds displayed either D- or L-configuration, which could not be distinguished from each another. This result is consistent with the prediction from antiSMASH, where epimerization domains were predicted at the 3rd, 7th, and 11th modules (Figure 1a).

Fortuiously, we found another group of peptides (**5-8**) that have masses of 1655.10 (**5** and **6**), 1669.11 (**7**), and 1683.10 Da (**8**), which is 100 Da more than those of bogorol I-L, respectively. They are eluted from HPLC in the same order as bogorol I-L, but with a longer elution time (Figure S1, peak 4-6). Further analysis in tandem MS data revealed that these peptides are likely products of a succinyl group attached to the Orn₃ or Lys₃ of bogorol I-L with a peptide bond (Figure 1b, Figure S4). In the NMR analysis, the succinylation at Orn₃ in peptide **7** was supported by the appearance of 2 methylene signals at 2.28 and 2.36 ppm which shared a strong correlation in the ¹H-¹H TOCSY NMR (Figure 1c, Figure S5). Both signals showed NOESY cross-peaks with an amide N-H signal at 7.79 ppm, which in turn showed a correlation with the signal of the δ -CH₂ of Orn₃ at 2.98 ppm in both TOCSY and NOESY NMR (Figure 1c, Figure S6 and S7). The downfield shift of this δ -H from 2.76 (in bogorol K) to 2.98 ppm is consistent with the acetylation of the Orn₃ amine resulting in a more electron-withdrawing amide (Table S4). This result confirms the attachment of a succinyl group at the end of the side chain of the Orn₃. Therefore, we nominate compounds **5-8** as succilin I-L, which are probably modification products arising from bogorol I-L (Figure 1b).

Figure 1. Bogorols and succilins produced by *B. laterosporus* MG64. (a) Gene cluster of bogorols harbored by *B. laterosporus* MG64. Green, core biosynthetic gene; blue, transport-related gene; gray, unknown gene; red, additional biosynthetic gene. (b) structures of bogorols and succilins characterized by LC-MS/MS and NMR. (c) 2D NMR correlations (red: ^1H - ^1H -TOCSY, blue: ^1H - ^1H -NOESY) confirming succinylation at the third amino acid residue (Lys) of succilin K (Figure S5-S7).

Adenylation domain-mediated lipoinitiation

The bogorol family of peptides contain a fatty acid tail at the N terminus, which is a representative characteristic of lipopeptides. The lipid tails of lipopeptides are usually incorporated into the peptides through a starter condensation (C) domain [25]. However, such a domain was not found in the gene cluster of bogorols (Figure 1a), suggesting a potentially novel lipidation of bogorols. Inspection on the biosynthetic modules reveals an additional module that is encoded by the gene *bogJ*. The knockout mutant of *bogJ* in *B. laterosporus* LMG15441, a transformable bogorol-producer, impaired the production of bogorols completely (Figure 2a, Figure S8), suggesting that gene *bogJ* is essential for the biosynthesis of bogorols. To investigate the potential substrate activation by this module, a phylogenetic tree involving the 14th adenylation (A) domain and the other A domains from the bogorol gene cluster, as well as other A domains that are known to activate alpha-hydroxy acid, was constructed. The result revealed that A14 is phylogenetically far away from other A domains in the bogorol gene cluster. Instead, it was clustered together with A domains that activate alpha-hydroxy acid (Figure 2b). Further inspection revealed that such A domains are normally followed by a ketoreductase (KR) domain. They form a special module together with a peptidyl carrier protein (PCP) domain. A similar module was found in another gene cluster from *B. laterosporus* MG64 (Figure S9a). This gene cluster was identified to be auriprocine, a lipopeptide originally identified from *B. laterosporus* PE36 (Figure S9a) [26]. Interestingly, the fatty acid tail of auriprocine, which is incorporated by the A-KR-PCP module, is the same as that of bogorols (Figure S9b). All these results suggest that the lipoinitiation of bogorols is mediated by the module encoded by *bogJ*. This lipoinitiation is different from the usual pattern employed by most of the NRPs, in which the fatty acid is activated by ligating to a coenzyme A in the presence of a fatty acid coenzyme ligase and the activated fatty acid is then incorporated into the assembly line by a starter C domain (Figure 2c) [25]. In the case of bogorols, a 3-methyl-2-oxopentanoic acid was first activated by the A domain, which usually activates amino acid residues. After activation, the α -keto acid was reduced into 2-hydroxy-3-methylvaleric acid by the KR domain and entered the next module under the guidance of a condensation (C) domain [27]

Figure 2. Adenylation domain-mediated lipoinitiation of bogorol peptides. (a) Production of bogorols by the wild type and $\Delta bogJ$ mutant of *B. laterosporus* LMG15441. Strains were grown in MEM broth for 48 h and extracted with a C-18 column filled with silica gel. The extracted products were subsequently subjected to HPLC. (b) A phylogenetic tree of A domains from the bogorol gene cluster harbored by *B. laterosporus* MG64 and reference A domains that incorporate hydroxy acid. Auriporcine is retrieved from *B. laterosporus* PE36 while cereulide and valinomycin are retrieved from the miBIG database. The A domains activating α -hydroxy acids are indicated in red. (c) The usual pattern of fatty acid incorporation in NRPs. CoA: coenzyme A, FAcL: fatty acid coenzyme ligase; (d) The pattern of bogorols incorporating fatty acid.

The formation of valinol is mediated by two reductases

An outstanding characteristic of bogorol family peptides is the presence of a valinol (an alcohol form of valine) at the C-terminus. Its formation is mediated by two possible mechanisms: 1) valinol is incorporated by the corresponding module; 2) valine is incorporated and then reduced into a valinol. The first mechanism was considered more straightforward and energy-saving. However, analysis of adenylation (A) domains indicates that A13 shows very high similarity to other A domains that incorporate valine (Figure 2b). Therefore, the second mechanism is more likely to be employed. Investigation of the gene cluster led us to the discovery of two reductases: terminal reductase domain (TD) in BogF, and an aldo/keto reductase BogI. TD is responsible for the release of the peptide chain from the PCP domain in a reduction pathway [28] while the function of BogI was unknown. To investigate the latter, the gene *bogI* in *B. laterosporus* LMG15441 was knocked out, resulting in the formation of a new group of compounds. These new peptides have masses that are 2 Da less than those of the wild type (Figure 3a and 3b, Figure S10). This mass reduction corresponds to the change of the alcohol form of valine to the aldehyde form, which indicates that

valine was first reduced to the aldehyde form when released by the TD domain, then further reduced to the alcohol form by BogI (Figure 3c). A comparison of the antibacterial activity of bogorols in the two different forms revealed that the alcohol form has a better activity, underlining the importance of the reduction for biological activity (Figure 3d).

Figure 3. The formation of valinol in bogorol peptides. Bogorols and their intermediates (aldehyde form) produced by wild type strain (a) and $\Delta bogI$ mutant (b) of *B. laterosporus* LMG15441, respectively. The masses (Da, based on MALDI-TOF analysis) of the three major compounds produced by each strain are indicated. (c) The two-step formation of alcohol form amino acids in bogorol family peptides. (d) Spot on lawn assay of aldehyde form and alcohol form bogorols. *X. campestris* pv. *campestris* NCCB92058 was used as an indicator. Each compound was added at the same amount, namely 10 μ L of a 200 μ g/mL stock.

Succinylation occurring in lipopeptides

In this study, a novel class of peptides with an additional succinyl group attached to the Orn₃/Lys₃ residue of bogorols were identified as succilins. This modification is not observed in *B. laterosporus* LMG15441 (Figure S11), suggesting that succilins are not always formed when bogorols are produced. A comparison of the flanking genes of the bogorol BGC reveals some specific genes in each strain (Figure S12a). However, the annotation of these genes did not result in a potential candidate, which was expected to be a transferase based on the similar modification, mannosylation, of ramoplanin [29, 30]. Further investigation of the gene cluster revealed two transferases, *bogN* and *bogR*, which are present in both strains (Figure S12a). However, the expression of *bogN* and *bogR* from *B. laterosporus* MG64 in *B. laterosporus* LMG15441 did not result in the production of succilins, suggesting they are not responsible for succinylation (Figure S12b, Figure S12c). Interestingly, we found that bogorols were detected from *B. laterosporus* MG64 after 14-h incubation in our experiment, whereas succilins were only detected with MALDI-TOF after 16 h and with HPLC after 18 h (Figure 4, Figure S13). This suggests that succinylation may require a high substrate

concentration, and is likely mediated by enzymes. However, the responsible gene is still unclear and further research using transcriptomics and/or heterologous expression is needed.

Figure 4. The production of bogorols and succilins by *B. laterosporus* MG64 at different time points.

Fresh cells (10 μ L culture with an OD600 of 1.0) were inoculated in 50 mL MEM broth and grown at 28°C for 14 h, 16 h, 18 h, 20h, respectively. The compounds were extracted from the supernatant at each time point and analyzed with HPLC and MALDI-TOF.

Antibacterial activity and mechanism of action

To investigate the potential of the newly identified bogorols and succilins for different applications, the antibacterial activity of bogorol K, bogorol L, and succilin K was assessed using plant pathogens (*Xanthomonas* species, *Pseudomonas* species, *Pectobacterium carotovorum*, and *Ralstonia syzygii*), a food pathogen (*Bacillus cereus*), and human pathogens (*P. aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus*) as indicators. As shown in Table 1, bogorol K and bogorol L displayed potent activity against the Gram-positive pathogens tested (MIC values range from 1 to 4 μ g/mL). Bogorol K and L were also active against various Gram-negative pathogens, except for the human pathogen *P. aeruginosa* and plant pathogen *P. carotovorum* and *R. syzygii*, which are not inhibited by both compounds until their concentrations reach 32 μ g/mL. Their antibacterial potency is similar to that of brevivacillin, a structurally similar lipopeptide. Succilin K, which is derived from bogorol K by succinylation at Orn₃, only showed weak activity against *Xanthomonas* spp. and the Gram-positive pathogens tested, and the MIC values are at least 4 times higher than that of bogorol K.

Table 1 MIC values of the identified compounds against different bacteria.

Pathogen	MIC (µg/mL)			
	Bogorol K (3)	Bogorol L (4)	Succilin K (7)	Brevibacillin
<i>Xanthomonas campestris</i> pv. <i>campestris</i> NCCB92058	4	2	32	2
<i>Xanthomonas translucens</i> pv. <i>graminis</i> LMG587	8	4	16	2
<i>Pseudomonas syringae</i> pv. <i>antirrhini</i> LMG2131	16	8	>32	8
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	16	8	>32	16
<i>Pseudomonas aeruginosa</i> PAO1	32	32	>32	32
<i>Klebsiella pneumoniae</i> LMG20218	16	32	>32	16
<i>Escherichia coli</i> ET8	8	8	>32	8
<i>E. coli</i> MG1665	16	16	>32	16
<i>Pectobacterium carotovorum</i> LMG5863	>32	>32	>32	ND
<i>Ralstonia syzygii</i> subsp. <i>syzygii</i> LMG6969	>32	32	>32	ND
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> 533 R4	2	2	16	1-2
<i>Bacillus cereus</i> ATCC 14579	2	2	32	2
<i>Enterococcus faecium</i> LMG16003	4	2	16	1-2

ND: not determined.

The Gram-positive human pathogen *S. aureus* subsp. *aureus* 533 R4 and the Gram-negative plant pathogen *X. campestris* pv. *campestris* NCCB92058 were used to investigate the mode of action of bogorol K. As shown in Figure 5a, bogorol K inhibited the growth of *Staphylococcus* within 15 min, which is comparable to the positive control nisin. The addition of lipoteichoic acid (LTA), an important constituent of the cell wall of gram-positive bacteria, did not affect the activity of bogorol K against *Staphylococcus*, suggesting that bogorol K does not bind to LTA, in contrast to nisin (Figure 5a). Moreover, the addition of lipid II originating from Gram-positive bacteria did not decrease the inhibition zone of bogorol K towards *Staphylococcus* (Figure S14a), suggesting that bogorol K does not bind to Gram-positive type lipid II. However, the membrane permeability assay showed that bogorol K forms pores in the cell membrane of *Staphylococcus*, similar to the positive control nisin (Figure 5b). Bogorol K inhibited the growth of the Gram-negative bacterium *X. campestris* within 15 min and showed a weaker binding to the lipopolysaccharide (LPS) compared to polymyxin B (Figure 5c). Besides, bogorol K does not bind to lipid II originating from *E. coli* (Figure S14b). Membrane

permeability assays revealed that bogorol K disrupts the cellular membrane of *X. campestris* as it does to that of Gram-positive bacteria (Figure 5d). Collectively, the results show that bogorol K employs the same mechanism for killing Gram-negative as for Gram-positive bacteria by forming holes in the cellular membrane.

Figure 5 Mode of action employed by bogorol family of peptides. (a) Killing curve and LTA binding assay of bogorol K on *S. aureus*. (b) Permeabilization of bogorol K to the cell membrane of *S. aureus* (c) Killing curve and LPS binding assay of bogorol K on *X. campestris*. (d) Permeabilization of bogorol K to the cell membrane of *X. campestris*. Permeabilization of bogorol K and succilin K to the outer membrane (e) and inner membrane (f) of *X. campestris*, respectively. The permeabilization of the outer membrane is indicated by the staining of NPN to the hydrophobic part, while the PI indicates the inner membrane permeabilization. The MIC values of bogorol K and succilin K are around 2.5 μM and 20 μM, respectively. Con: control, BogK: bogorol K, PlyB: polymyxin B, SuckK: succilin K.

We also investigated the cause of the difference in antibacterial activity between bogorol K and succilin K using two different dyes, 1-N-phenyl-naphthylamine (NPN) and propidium iodide (PI). The hydrophobic NPN cannot penetrate the intact outer membrane of Gram-negative bacteria. However, once this membrane is damaged, NPN will bind to the phospholipid layer and become fluorescent. PI binds to DNA and emits fluorescence only when the inner membrane is damaged. Consequently, a combination of both dyes can be used to show the difference between bogorol K and succilin K in penetration of the different layers of the cell membrane. It was observed that bogorol K can penetrate

both the outer membrane and inner membrane of *X. campestris* when applied above 2.5 mM (Figure 5e and 5f). In contrast, succilin K barely showed penetration to neither the outer membrane nor the inner membrane at the same concentration as bogorol K (Figure 5e and 5f). This result suggests the outer membrane is the first barrier to succilins, and that the Orn₃ in bogorols plays an important role in penetrating the outer membrane.

Synergistic effect of relacidine and bogorol

Apart from discovering novel antibiotics, combining existing antibiotics is another strategy to combat the increasing number of drug-resistant bacteria [31]. Here we investigated the synergistic effect of relacidine, a compound that affects the oxidative phosphorylation in cells (unpublished data), and bogorol, a compound that was proven to cause damage to the cell membranes in this study. Both compounds are produced by *B. laterosporus* MG64 but employ different modes of action. A study of their synergistic effect could lead to a better understanding of the antibacterial mechanism of *B. laterosporus* MG64 as well as its potential in different applications. As shown in Table 2, relacidine B and bogorol K displayed a synergistic effect on all the pathogens tested, including Gram-negative plant pathogen *X. campestris*, *P. syringae* pv. *antirrhin*, *P. syringae* pv. *tomato* and Gram-positive human pathogen *S. aureus* subsp. *aureus*. This result suggests that *B. laterosporus* MG64 may employ a combination of compounds to combat other bacteria, thus making it better capable of occupying desirable niches in the natural environment.

Tabel 2 Synergistic effect of relacidine B and bogorol K

Pathogen	MICa	MICb	MICab	MICba	FICI
<i>Xanthomonas campestris</i> pv. <i>campestris</i> NCCB92058	4	0.5	0.5	0.125	0.375
<i>Pseudomonas syringae</i> pv. <i>antirrhin</i> LMG2131	16	0.5	2	0.0625	0.25
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	16	0.5	2	0.0312	0.1875
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> 533 R4	2	64*	0.5	2	0.2812

Note: The unit of MIC values is µg/mL. MICa, MIC value of bogorol K alone; MICab, MIC value of bogorol K when relacidine B is present; MICb, MIC value of relacidine B alone; MICba, MIC value of relacidine B when bogorol K is present; FICI value below 0.5 indicate a synergistic effect of the two peptides.

* the MIC is >32 µg/mL, here we use 64 µg/mL for calculation.

Discussion

In this study, we characterized four novel bogorol variants (bogorol I-L) from the biocontrol strain *B. laterosporus* MG64. The bogorol variants show at least one amino acid residue in differences to the structurally similar peptides (Table S5) [16-20]. Leu₉ in bogorol B is replaced by Val₉ in bogorol I, while Leu₂ in brevibacillin is replaced by Val₂ in bogorol K. Bogorol J has different amino acids at positions 5 and 9 compared to bogorol C, while bogorol L is different at positions 2 and 3 compared to brevibacillin. Nevertheless, the newly characterized peptides share many characteristic features with the reported ones. First of all, they are linear lipopeptides containing three positively charged amino acid residues (Orn or Lys), which contribute to the hydrophilicity of the peptides. Moreover, the bogorol family peptides have highly conserved fragments, and residues at position 1, positions 6-8, and positions 10-13 show no difference between variants. Moreover, the C-terminus of all the peptides contain a valinol, the alcohol form of valine.

We also characterized four novel succilins (succilin I-L) from the same strain, *B. laterosporus* MG64. Succilins are similar to bogorols, except that an additional succinyl group was attached to the side chain of Orn₃/Lys₃. This modification alters the chemical properties of succilins. First of all, the masses of succilins are 100-Da larger than their corresponding bogorols. Moreover, the polarity of the peptides is decreased. As a result, they are eluted later than bogorols (Figure S1) and have a lower solubility in water (data not shown). Besides, succilins only retain one positive charge, compared to the three in bogorols. These changes, especially the polarity and charges, result in a negative effect on the bioactivity of peptides (Table 1). This is considered reasonable because i) the phospholipid of the cell membrane in bacteria is negatively charged and has an electrostatic attraction to the cationic peptides [32]. A decrease of charges, therefore, causes a weaker penetration to the membrane (Figure 5e and 5f). ii) the amphipathic property of peptides is important for bioactivity. Too high or too low hydrophobicity could dramatically decrease antimicrobial activity [33]. To the best of our knowledge, this is the first report on succinylation of NRPs. Similar modified peptides were not detected in *B. laterosporus* LMG15441 (Figure S11), which also produce bogorols. The biological function of succilins remains unknown, but a higher production of succilins on agar plates using bogorols as a reference (Figure S15) suggests that they are possibly involved in motility or biofilm formation, which needs further investigation.

Although several variants of bogorol have been reported [16-20], their biosynthesis was unclear to date. In this study, we successfully unraveled the biosynthesis process of bogorols and succilins with both *in silico* analysis and experimental methods (Figure S16). We reveal that the lipoinitiation of bogorols does not follow the usual pattern observed in other lipopeptides, which is mediated by FACL, coenzyme A, and a starter C domain [25]. Instead, the fatty acid of bogorols is incorporated

by a special module A-KR-PCP. Such modules are reported to incorporate α -keto acids and are normally found in depsipeptide, a group of compounds containing both hydroxy acid residues and amino acid residues [27]. Modules that incorporate α -keto acid are found in natural products including cereulide [34, 35], valinomycin [36, 37], cryptophycin [38], kutzneride [39], and hectochlorin [40]. However, these modules were located in between other modules that incorporate amino acids, which suggests that they are not responsible for lipoinitiation. In contrast, such modules in bogorols are encoded by an independent gene *bogJ*. A C domain following the A-KR-PCP module and a TD domain at the end of the last module encoded by *bogEF* suggests that *bogJ* is responsible for the lipoinitiation (Figure S16). To the best of our knowledge, only bogorols and auriprocine employ such a novel lipoinitiation pathway. This expands our knowledge of lipoinitiation in natural products and provides a possible pathway for the engineering of lipid tail in NRPs.

We also reveal the pathway leading to the formation of valinol in bogorols. The aldehyde form of bogorols is released from the PCP domain by the TD and further reduced to the alcohol form by a reductase encoded by gene *bogI* (Figure S16). A similar process in gramicidin A was reported to happen in the presence of a TD, the aldoreductase LgrE, and the electron donor NADPH *in vitro* [41]. A similar reduction in myxochelin A was reported to be mediated by the TD alone [42], which is different from the two-step process found in bogorols and gramicidin A. A previous study on BT peptides revealed that the valinol at the C-terminus confers the peptides with protease resistance [20]. Our results also reveal that the aldehyde form of bogorols is less active to pathogens than the alcohol form. Therefore, the formation of valinol is of vital importance for the biological activity of these peptides.

Succinylation is commonly found in proteins of diverse organisms including bacteria, yeasts, and animals [43, 44]. Studies have shown that succinylation is possibly relevant to numerous human diseases associated with mitochondria [45]. However, the reports on the succinylation of secondary metabolites is highly uncommon. The only report is on the ribosomal peptides subtilin and entianin, which are produced by *Bacillus subtilis* [46]. The succinylation of subtilin and entianin occurs at a tryptophan residue at position 1 and is affected by the concentration of glucose in the growth medium as well as the transition state regulator AbrB [46]. In this study, we report the first succinylation occurring in NRPs. Compared to other modifications such as halogenation, glycosylation, sulfation, etc., in NRPs [47], succinylation makes a more profound difference in the chemical properties of peptides and its occurrence likely demands a high concentration of substrate (Figure 4). The mechanism of succinylation in succilins remained unknown and further research will be directed to reveal the responsible enzymes.

The bogorol family of peptides was initially reported to have potent bioactivity against Gram-positive bacteria and weak bioactivity towards Gram-negative bacteria [16-19]. However, we show that bogorol K and bogorol L also have potent activity to Gram-negative bacteria, especially *Xanthomonas* species. We also show that bogorol K targets the cell membrane of both Gram-positive and Gram-negative bacteria. This result is similar to the study on the mode of action of brevivacillin on Gram-positive bacteria [48]. The membrane-targeting mechanism makes bogorol a promising candidate for studies on the synergistic effect of antimicrobials. Bogorols damage the cell membranes and remove the barrier to other compounds that target intracellular components. This is supported by the synergistic effect of bogorols and relacidines (Table 2), a class of compounds affecting the oxidative phosphorylation of cells (data unpublished). Further research will be directed to further evaluate its potential in different applications.

Material and methods

Extraction and purification of antimicrobial compounds from *B. laterosporus* MG64

A fresh colony of *B. laterosporus* MG64 was inoculated in 3 ml Lennox broth (LB) and incubated at 28 °C overnight with shaking. The overnight culture was then diluted with fresh LB to an OD₆₀₀ of 1.0 as inoculum. One milliliter of inoculum was inoculated into 100 ml of fresh LB broth and incubated at 28 °C with shaking of 220 rpm for 24 h. After centrifugation at 10,000 × g for 10 min, the supernatant was collected and applied to a column filled with 10 g C18 silica gel (Sigma-Aldrich, 97727-U). After washing with 20 ml of 20 % acetonitrile with 0.1% trifluoroacetic acid (TFA), the crude extract was eluted with 20 ml 95% acetonitrile with 0.1% TFA. The crude extract was lyophilized and re-dissolved in Milli-Q water. After filtering with a 0.45 µm cellulose acetate membrane, an aliquot of crude extract was applied to high-performance liquid chromatography (HPLC) equipped with an analytical C-18 column (3.6 µm particles, 250 × 4.6 mm, Phenomenex) for purification. A linear gradient of water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B) was used to separate compounds. In each run, solvent B was linearly increased from 15% to 60% within 45 min at a flow rate of 0.5 ml/min. The effluent was monitored with a UV-detector at a wavelength of 214 nm. Every single peak was collected and concentrated with lyophilization before testing activity against *Xanthomonas campestris* pv. *campestris* (Xcc).

Structure elucidation

LC-MS/MS. The antimicrobial compounds were applied to liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the amino acid sequences. An Ultimate 3000 UHPLC system equipped with a Kinetex WVO-C18 (2.6 µm particles, 100 × 2.1 mm) column (Phenomenex) was used. A Q-Exactive Orbitrap™-based mass spectrometer (Thermo Scientific, San Jose, CA,

USA) equipped with a HESI-II electrospray source was coupled to the UHPLC system. Each sample (10 μ L) was injected into LC and separated with water (with 0.1% formic acid) and acetonitrile (with 0.1% formic acid) at a flow rate of 500 μ L min⁻¹. A spray voltage of 3.5 kV (positive mode) and a capillary temperature of 275 °C were used. The samples were measured in positive mode from *m/z* 300 to 2000 at a resolution of 70,000. MS/MS data were recorded either in DDA-top10 setup or targeted MSMS using PRM mode at a resolution of 17500.

NMR. Prior to NMR analysis, the peptides were purified by RP-HPLC as described above and the pure fractions were pooled and lyophilized. The peptides were obtained as white solids (0.5 - 1 mg) and were dissolved in 0.5 mL DMSO-*d*₆. ¹H NMR, ¹H-¹H-TOCSY NMR, and ¹H-¹H-NOESY NMR spectra were recorded on a Brüker Ascend 600 MHz spectrometer. Chemical shifts in ¹H NMR spectra were internally referenced to solvent signals (DMSO-*d*₆ at δ H = 2.50 ppm).

Genome editing with the CRISPR-Cas9 system

To knock out the *bogl* or *bogJ* genes in *B. laterosporus* LMG15441, vector pJOE8999 harboring the clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) system was employed [49]. Single-guided RNA (sgRNA) fragments were ligated into Eco31I sites of pJOE8999 after annealing. Short fragments located up-stream and down-stream of the sgRNA were PCR amplified with the primers listed in Table S6. The template for homologous repair was achieved with overlap PCR of the up-stream and down-stream fragments and ligated into the SfiI sites of the pJOE8999 to make the final construct.

To prepare competent cells, a single colony of *B. laterosporus* LMG15441 was inoculated in LBS medium (LB medium with 0.5 M sorbitol) and incubated at 37 °C with shaking (220 rpm) overnight. The overnight culture was diluted 100 times into fresh LBS medium and grown until OD₆₀₀ reached 0.8-0.9. Cells (100 mL) were cooled on ice for 5 min and centrifuged at 5,000 g at 4 °C for 5 min to collect the cells. The pellet was washed four times with electroporation buffer (0.5 M sorbitol, 0.5 M mannitol, and 10% glycerol) and finally resuspended in the same buffer. One microgram of the reconstructed vector was mixed with 100 μ L competent cells in an electroporation cuvette (1 mm) and shock with 2.0 kV voltage, 25 μ F capacitance, 200 Ω resistance. One milliliter of recovery medium (LM medium with 0.5 M sorbitol and 0.38 M mannitol) was added immediately and the cells were incubated at 28 °C with 150 rpm shaking for 3-5 h. The cells were plated on LB plates with 30 μ g/ml kanamycin and 0.2% mannose for inducing the expression of Cas9 protein. The plate was incubated at 28 °C for 24 h and colonies were checked with colony PCR.

Inspection of bogorol production in mutants

The mutants and wild type strain of *B. laterosporus* LMG15441 were grown in MEM broth at 28 °C overnight (16 h). The overnight culture was diluted with MEM broth to an OD₆₀₀ of 1.0. Each 20 µL diluted culture was inoculated in 50 mL MEM broth and the cells were incubated at 28 °C for 48 h. The supernatant was collected by centrifuging at 10,000 g for 2 min. The compounds were extracted as described above and dissolved in Milli-Q water before filtering through a 0.45 µm cellulose membrane. The bogorol peptides were purified with HPLC as described above and identified with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

Production time of bogorols and succilins

To investigate the production time of bogorols and succilins, the producer was grown in MEM broth overnight. The overnight culture was diluted to an OD₆₀₀ of 1.0. For each 50 mL MEM broth, 10 µL diluted bacterial culture was added. The cells were grown at 28 °C for 14 h, 16 h, 18 h, 20h, respectively. At each time point, the supernatant was collected and the compounds were extracted as described above. The extracts were lyophilized and dissolved in Milli-Q water before applying to HPLC and MALDI-TOF for analyzation.

Antibacterial assays

The MICs of the peptides were determined with the broth dilution method [50]. MHB medium was used for all the indicator bacteria. The concentration of bacteria was adjusted to 5.0×10^5 CFU/mL in each well. The compounds (32 µg/mL) were two-fold serial diluted in the 96-well plate. OD₆₀₀ was measured using the Tecan Infinite F200 Pro Luminometer after incubating at 28 °C for 36 h and the MIC values were defined as the lowest concentration that completely inhibit the growth of indicator bacteria.

Killing dynamics and LPS/LTA binding assay

Overnight cultures of *X. campestris* pv. *campestris* and *Staphylococcus aureus* subsp. *aureus* were diluted to an OD₆₀₀ of 0.05 in a 96-well plate and incubated in a microplate spectrophotometer (1000 rpm, 28 °C). When the OD₆₀₀ reached 0.1, bogorol K (dissolved in DMSO) were added to each well at a final concentration of 8 µg/mL (2×MIC) and the same volume of DMSO was used as a negative control. Polymyxin B (1 µg/mL) was used as a positive control for *X. campestris* pv. *campestris* while nisin (2 µg/mL) was used for *S. aureus* subsp. *aureus*. Exogenous lipopolysaccharide (LPS, 0.1 mg/mL) or lipoteichoic acid (LTA, 0.1 mg/mL) was added to investigate the association with peptides. The growth curve in the following 3 h was recorded with the same microplate spectrophotometer at the same condition and four replicates were used for each treatment. The release of growing pressure indicates the binding of peptides to LPS or LTA.

Membrane permeability assay

Membrane integrity of *X. campestris pv. campestris* and *S. aureus* subsp. *aureus* after treatment with bogorol was tested with the commercial LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen). Briefly, the overnight culture of each strain was diluted to an OD₆₀₀ of 0.2. Bogorol K (dissolved in DMSO) was added to a concentration of 8 µg/mL (2×MIC) and the same volume of DMSO was added to the negative control. Polymyxin B was used as a positive control for *X. campestris pv. campestris* while nisin was used for *S. aureus* subsp. *aureus*. After treating at room temperature for 15 min, cells were harvested (10,000 g for 2 min) and washed with 0.9% saline solution. Cells were finally suspended in 200 µl 0.9% saline solution and two different dyes (3.34 mM SYTO9 and 20 mM propidium iodide) were added at a ratio of 1:1 (v/v). After staining at dark for 15 min, cells were mounted on 1% agarose pads and analyzed with a Nikon Ti-E microscope (Japan).

NPN and PI staining assay

X. campestris pv. campestris cells were grown in LB broth at 28 °C overnight. The overnight culture was diluted 100 times with fresh LB broth and grown until OD₆₀₀ reached 1.0. The cells were collected (5,000 g for 3 min) and washed with HEPES buffer twice. The cells were resuspended in HEPES buffer and the OD₆₀₀ was adjusted to 1.0. Both NPN and PI were added to the cells at a final concentration of 30 µM. The mixture was distributed to a 96-well plate, which DMSO, bogorol K, and succilin K were added before. The concentration range of each compound was 1.25 mM, 2.5 mM, 5 mM, and 10 mM. The fluorescent signal at 350/420 was recorded every 10 min during a period of 1 h and normalized with OD₆₀₀. Three replicates were used in each treatment.

Synergy test

The synergistic effect of bogorol K and relacidine B was tested using a checkerboard method [51]. Briefly, the bogorol K was two-fold series diluted in each column while relacidine B was diluted with the same method in each row in a 96-well plate. The fresh cells were added to each well at a final concentration of 5×10^5 CFU/mL. The plate was incubated at 28 °C for 24 h and the OD₆₀₀ was recorded. The fractional inhibitory concentration index (FICI) was calculated using the following formula $FICI = MIC_{ab}/MIC_a + MIC_{ba}/MIC_b$. MIC_a and MIC_b are the MIC values of bogorol K and relacidine B alone, respectively. MIC_{ab} is the MIC value of relacidine B in the presence of bogorol K while MIC_{ba} is the other way around. The FICI value suggests a synergistic (≤ 0.5), additive ($> 0.5 - 1$), no interaction ($1 - 4$), and antagonism (> 4) effect of the two peptides [51].

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Author contributions

ZL and OPK designed the study. ZL performed most of the experiments and prepared the manuscript. RHV performed the NMR analysis. PC and XZ contributed to the mode of action assays. All authors were involved in analyzing and discussing the results and revising the manuscript.

Competing interests

The authors declare they have no conflict of interest.

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Supplementary data

Figure S1. HPLC chromatogram of compounds extracted from *B. laterosporus* MG64. Three different peaks display potent activity toward *X. campestris* pv. *campestris* and *B. cereus* are labeled as peak 1, 2, and 3, respectively. They were identified to be bogorol variants. Peak 4-6 were identified to be succilins. Both peak 1 and peak 4 were identified to be a combination of two different compounds (compound **1** and **2** for peak 1, compound **5** and **6** for peak 4).

Figure S2. Fragmentation of bogorol I and J (compound **1** and **2**, panel a), bogorol K (compound **3**, panel b), and bogorol L (compound **4**, panel c) by tandem MS to generate *b* and *y* ions. The *b* ions were indicated in blue, while the *y* ions were indicated in red. The different amino acids of bogorol I and bogorol J are indicated in green.

a Bogorol K (**3**) b Bogorol L (**4**) Figure S3. ^1H - ^1H TOCSY and ^1H - ^1H NOESY NMR correlations of bogorol K (**3**) and bogorol L (**4**).

Table S1. Partial chemical shift (ppm) assignments of bogorol K (d_6 -DMSO).

Residue	NH	$^1\text{H}\alpha$	$^1\text{H}\beta$	Others
FA		3.79	$\text{OH}^{\beta 1}$, 5.61; $\text{CH}^{\beta 2}$, 1.71	$\text{CH}_2^{\gamma 1}$, 1.38/1.12; $\text{CH}_3^{\gamma 2}$, 0.88; $\text{CH}_3^{\delta 1}$, 0.80
Dhb-1	9.15		5.93	$\text{CH}_3^{\gamma 1}$, 1.78
Val-2	7.81	4.24	2.11	CH_3^{γ} , 0.87
Orn-3	8.08	4.42	1.68	CH_2^{γ} , 1.55; CH_2^{δ} , 2.76; NH_2^{ϵ} , 7.63
Ile-4	7.85	4.28	1.70	$\text{CH}_2^{\gamma 1}$, 1.35/1.02*
Ile-5	8.00	4.17	1.69	$\text{CH}_2^{\gamma 1}$, 1.43/1.04
Val-6	7.81	4.18	1.93	
Lys-7	7.93	4.38	1.63	CH_2^{γ} , 1.26; CH_2^{δ} , 1.48; CH_2^{ϵ} , 2.70; NH_2^{ζ} , 7.64
Val-8	7.86	4.22	1.91	
Val-9	7.88	4.11	1.94	
Lys-10	7.78	4.24	1.43/1.32*	CH_2^{γ} , 1.05; CH_2^{δ} , 1.42; CH_2^{ϵ} , 2.65; NH_2^{ζ} , 7.65
Tyr-11	8.09	4.47	2.84/2.60	CH^{δ} , 6.99; CH^{ϵ} , 6.62
Leu-12	8.08	4.24	1.38	$\text{CH}_3^{\delta 1}$, 0.82; $\text{CH}_3^{\delta 2}$, 0.76*
Valinol-13	7.40	3.53	$\text{CH}_2^{\beta 1}$, 3.34; $\text{CH}^{\beta 2}$, 1.80	CH_3^{γ} , 0.81

*Tentative assignment due to overlap, but in accordance with the literature [18].

 ^1H NMR of bogorol K (3)

^1H - ^1H -TOCSY NMR of bogorol K (**3**) ^1H - ^1H -NOESY NMR of bogorol K (**3**)

Table S2. Partial chemical shift (ppm) assignments of bogorol L (d_6 -DMSO).

Residue	NH	$^1\text{H}\alpha$	$^1\text{H}\beta$	Others
FA		3.79	$\text{OH}^{\beta 1}$, 5.61*; $\text{CH}^{\beta 2}$, 1.72	$\text{CH}_2^{\gamma 1}$, 1.38/1.13; $\text{CH}_3^{\gamma 2}$, 0.88; $\text{CH}_3^{\delta 1}$, 0.80
Dhb-1	9.11		5.97	$\text{CH}_3^{\gamma 1}$, 1.79
Val-2	7.79	4.24	2.11	CH_3^{γ} , 0.87
Lys-3	8.08	4.34	1.63/1.55	CH_2^{γ} , 1.28; CH_2^{δ} , 1.48; CH_2^{ϵ} , 2.70;
Ile-4	7.79	4.24	1.70	
Ile-5	7.99	4.17	1.71	$\text{CH}_2^{\gamma 1}$, 1.41/1.05
Val-6	7.78	4.17	1.94	
Lys-7	7.94	4.37	1.63	CH_2^{γ} , 1.27; CH_2^{δ} , 1.48; CH_2^{ϵ} , 2.70;
Val-8	7.86	4.21	1.93	
Val-9	7.88	4.11	1.94	
Lys-10	7.78	4.24	1.42/1.31*	CH_2^{γ} , 1.05; CH_2^{δ} , 1.42; CH_2^{ϵ} , 2.65;
Tyr-11	8.09	4.47	2.84/2.61	CH^{δ} , 6.99; CH^{ϵ} , 6.62
Leu-12	8.09	4.24	1.39	CH^{γ} , 1.35*; $\text{CH}_3^{\delta 1}$, 0.81; $\text{CH}_3^{\delta 2}$, 0.76
Valinol-13	7.40	3.54	$\text{CH}_2^{\beta 1}$, 3.34; $\text{CH}^{\beta 2}$, 1.81	CH_3^{γ} , 0.81

*Tentative assignment due to overlap, but in accordance with the literature [47].

^1H NMR of bogorol L (**4**)

^1H - ^1H -TOCSY NMR of bogorol L (4) ^1H - ^1H -NOESY NMR of bogorol L (4)

Table S3. Marfey analysis of bogorol K (3) and bogorol L (4).

Standard	Retention Time (min.)	bogorol K (3)	bogorol L (4)
L-Val	10.30	10.25	10.21
D-Val	12.05	-	-
L-Leu	12.06	12.02	11.98
D-Leu	13.76	-	-
L-Ile	11.76	11.71	11.68
D-Ile	13.60	-	-
L-Phe (standard)	11.98	11.93	11.89
L-Tyr	6.59 / 9.14	-	-
D-Tyr	6.58 / 9.77	6.44 / 9.69	6.41 / 9.65
L-Orn	4.55 / 4.91	-	-
D-Orn	4.04 / 4.89	3.80 / 4.69	-
L-Lys	5.14 / 5.61	4.84 / 5.39	4.85 / 5.38
D-Lys	5.12 / 4.78	4.78 / 4.50	4.79 / 4.51
L-(S)Vol	9.94	9.87	9.84
D-(R)Vol	12.05	-	-

Figure S4. Fragmentation of succilin I and J (compound 5 and 6, panel a), succilin K (compound 7, panel b), and succilin L (compound 8, panel c) by tandem MS to generate *b* and *y* ions. The *b* ions were indicated in blue, while the *y* ions were indicated in red. The different amino acids of succilin I and succilin J are indicated in green.

^1H - ^1H TOCSY and ^1H - ^1H NOESY NMR correlations of succilin K (**7**).

^1H NMR of succilin K (**7**)

^1H - ^1H -TOCSY NMR of succilin K (7) ^1H - ^1H -NOESY NMR of succilin K (7)

Figure S5. ^1H - ^1H -TOCSY NMR correlation of the methylene signals of the succinyl group of succilin K (7).

Figure S6. ^1H - ^1H -TOCSY NMR correlation of the delta methylene of Orn₃ with the amide NH of the succinyl group of succilin K (7).

Figure S7. ^1H - ^1H -NOESY NMR correlations of the succinyl methylenes with the amide NH of the succinyl group of succilin K (**7**).

Table S4. Partial chemical shift (ppm) assignments of succilin K (d_6 -DMSO).

Residue	NH	$^1\text{H}\alpha$	$^1\text{H}\beta$	Others
FA		3.77	OH $^{\beta 1}$, 5.61; CH $^{\beta 2}$, 1.72	CH $_2^{\gamma 1}$, 1.37/1.12; CH $_3^{\gamma 2}$, 0.88; CH $_3^{\delta 1}$, 0.79
Dhb-1	9.08		5.99	CH $_3^{\gamma 1}$, 1.79
Val-2	7.76	4.25	2.10	CH $_3^{\gamma}$, 0.86
Orn-3 (Suc)	8.12	4.31	1.63/1.52	CH $_2^{\gamma}$, 1.41/1.33; CH $_2^{\delta}$, 2.98; NH $^{\epsilon}$, 7.79; CH$_2^{\text{suc}1}$, 2.28; CH$_2^{\text{suc}2}$, 2.36
Ile-4	7.78	4.21	1.71	
Ile-5	8.01	4.16	1.72	CH $_2^{\gamma 1}$, 1.43/1.05
Val-6	7.84	4.16	1.94	
Lys-7	8.01	4.35	1.63	CH $_2^{\gamma}$, 1.27; CH $_2^{\delta}$, 1.48; CH $_2^{\epsilon}$, 2.70;
Val-8	7.89	4.20	1.93	
Val-9	7.89	4.10	1.94	
Lys-10	7.82	4.24	1.41/1.32*	CH $_2^{\gamma}$, 1.04; CH $_2^{\delta}$, 1.41; CH $_2^{\epsilon}$, 2.65;
Tyr-11	8.11	4.47	2.84/2.61	CH $^{\delta}$, 6.99; CH $^{\epsilon}$, 6.62
Leu-12	8.10	4.24	1.39	CH $^{\gamma}$, 1.35*; CH $_3^{\delta 1}$, 0.82; CH $_3^{\delta 2}$, 0.76
Valinol-13	7.40	3.53	CH $_2^{\beta 1}$, 3.34; CH $^{\beta 2}$, 1.80	CH $_3^{\gamma 1}$, 0.82; CH $_3^{\gamma 2}$, 0.80

*Tentative assignment due to overlap, but in accordance with the literature [18]

Figure S8. MALDI-TOF analysis of extracts from wild type and $\Delta bogJ$ mutant of *B. laterosporus* LMG15441. The $[M + H]^+$ of representative compounds were indicated.

Figure S9. (a) Biosynthetic gene cluster of bogorol and auriporcine. The modules responsible for fatty acid incorporation are indicated with purple shading. The unfunctional modules in auriporcine BGC are indicated in grey shading. (b) The structures of bogorol and auriporcine. The fatty acids of both compounds are indicated with red shading.

Figure S10. MALDI-TOF analysis of extracts from wild type and $\Delta bogl$ mutant of *B. laterosporus* LMG15441. The $[M + H]^+$ of representative compounds were indicated.

Figure S11. LC-MS of extracts from *B. laterosporus* MG64 (up) and LMG15441 (down). Bogorols are found in both strains, while succilins are only found in *B. laterosporus* MG64. z in the labels indicate the charges of the ions.

Figure S12. (a) Flanking genes of the bogorol BGC in *B. laterosporus* MG64 and LMG15441. Blue, present in both strains; green, only present in *B. laterosporus* MG64; red, only present in *B. laterosporus* LMG15441; purple, transferase. The table presents the annotation of some of the genes. (b) MALDI-TOF of extracts from *B. laterosporus* LMG15441 with heterologous expression of the gene *bogN* (C2W64_00415, from *B. laterosporus* MG64). (c) MALDI-TOF of extracts from *B. laterosporus* LMG15441 with heterologous expression of the gene *bogR* (C2W64_00421, from *B. laterosporus* MG64).

Figure S13, MALDI-TOF analysis of extracts from *B. laterosporus* MG64 at after 14-h (top) and 16-h (down) incubation. The Figure shows that succilins are found at 16 h but not at 14 h.

Figure S14. Lipid II binding assay. Lipid II was mixed with compounds at different ratios before spotting onto the plate, which contains an indicator strain. Nisin was used as a control. D or L in the bracket means a Gram-negative or Gram-positive type of lipid II was used. The plate was incubated at 28 °C overnight before recording the result.

Table S5. Comparison of bogorol and succilin peptides

Position	FA	1	2	3	4	5	6	7	8	9	10	11	12	13
Bogorol A	C ₆ H ₁₁ O ₂	Dhb	Leu	Orn	Ile	Val	Val	Lys	Val	Leu	Lys	Tyr	Leu	Valinol
Bogorol B	C ₆ H ₁₁ O ₂	Dhb	Val	Orn	Ile	Val	Val	Lys	Val	Leu	Lys	Tyr	Leu	Valinol
Bogorol C	C ₆ H ₁₁ O ₂	Dhb	Val	Orn	Val	Val	Val	Lys	Val	Leu	Lys	Tyr	Leu	Valinol
Bogorol D	C ₆ H ₁₁ O ₂	Dhb	Met	Orn	Ile	Val	Val	Lys	Val	Leu	Lys	Tyr	Leu	Valinol
Bogorol E	C ₆ H ₁₁ O ₂	Dhb	Met-O	Orn	Ile	Val	Val	Lys	Val	Leu	Lys	Tyr	Leu	Valinol
Brevibacillin	C ₆ H ₁₁ O ₂	Dhb	Leu	Orn	Ile	Ile	Val	Lys	Val	Val	Lys	Tyr	Leu	Valinol
Brevibacillin V	C ₆ H ₁₁ O ₂	Dhb	Leu	Orn	Ile	Val	Val	Lys	Val	Val	Lys	Tyr	Leu	Valinol
BT peptide	C ₁₁ H ₂₀ O ₂ N ₁	Dhb	Leu	Orn	Ile	Val	Val	Lys	Val	Leu	Lys	Tyr	Leu	Valinol
Bogorol I (1)	C ₆ H ₁₁ O ₂	Dhb	Val	Orn	Ile	Val	Val	Lys	Val	Val	Lys	Tyr	Leu	Valinol
Bogorol J (2)	C ₆ H ₁₁ O ₂	Dhb	Val	Orn	Val	Ile	Val	Lys	Val	Val	Lys	Tyr	Leu	Valinol
Bogorol K (3)	C ₆ H ₁₁ O ₂	Dhb	Val	Orn	Ile	Ile	Val	Lys	Val	Val	Lys	Tyr	Leu	Valinol
Bogorol L (4)	C ₆ H ₁₁ O ₂	Dhb	Val	Lys	Ile	Ile	Val	Lys	Val	Val	Lys	Tyr	Leu	Valinol
Succilin I (5)	C ₆ H ₁₁ O ₂	Dhb	Val	Orn-S	Ile	Val	Val	Lys	Val	Val	Lys	Tyr	Leu	Valinol
Succilin J (6)	C ₆ H ₁₁ O ₂	Dhb	Val	Orn-S	Val	Ile	Val	Lys	Val	Val	Lys	Tyr	Leu	Valinol
Succilin K (7)	C ₆ H ₁₁ O ₂	Dhb	Val	Orn-S	Ile	Ile	Val	Lys	Val	Val	Lys	Tyr	Leu	Valinol
Succilin L (8)	C ₆ H ₁₁ O ₂	Dhb	Val	Lys-S	Ile	Ile	Val	Lys	Val	Val	Lys	Tyr	Leu	Valinol

Note: The position number of conserved residues are indicated in black. The newly identified peptides and their closest peptides are indicated in the same background color, and the different residues in the characterized peptides are indicated in red font. FA: fatty acid.

Figure S15. LC-MS analysis of extracts from *B. laterosporus* MG64 grown on LB agar plates. Cells were grown on LB plates for 2 days, and the compounds were extracted with 50% acetonitrile.

Figure S16. The biosynthetic process of bogorols and succilins. A, adenylation domain; KR, ketoreductase domain; T, thiolation domain; C, condensation domain; E, epimerization domain; TD, terminal reductase domain; TE, thioesterase domain.

Table S6. Primers used in this study.

Name	Sequence	Purpose
bogJ-sgRNA-F	TACGATTAGTAGAAAGTGTTTACG	Knock out of <i>bogJ</i>
bogJ-sgRNA-R	AAACCGTAAACACTTTCTACTAAT	Knock out of <i>bogJ</i>
bogJ-up-Sfil-F	aaGGCCaacgaGGCCGTAGCTCCGCTTCACAGTAG	Knock out of <i>bogJ</i>
bogJ-up-R	GGCGTTCTTCCTTGTCTCCTCCTGCTCTATG	Knock out of <i>bogJ</i>
bogJ-down-F	GGAGGAGACAAGGAAGAACGCCCTATTTCTG	Knock out of <i>bogJ</i>
bogJ-down-Sfil-R	aaGGCCttattGGCCGCTTGTAACGCTTGCAATG	Knock out of <i>bogJ</i>
bogI-sgRNA-F	TACGGAAGCCGTCTCAGTTAGACG	Knock out of <i>bogI</i>
bogI-sgRNA-R	AAACCGTCTAACTGAGACGGCTTC	Knock out of <i>bogI</i>
bogI-up-Sfil-F	aaGGCCaacgaGGCCTATGTAGCGGCTGTAAAGGG	Knock out of <i>bogI</i>
bogI-up-R	AGTAGGTGGCCACGGGAGCACTTTAACTCAG	Knock out of <i>bogI</i>
bogI-down-F	AGTGCTCCCGTGGCCACCTACTAAGGAAGAG	Knock out of <i>bogI</i>
bogI-down-Sfil-R	aaGGCCttattGGCCTAACCCGATCTTGAGGCTTG	Knock out of <i>bogI</i>
pJOE8999-check-F	Cgtgtgatgcgaattcttgac	Knock out of <i>bogI</i>
pJOE8999-check-R	Gatgccactcttatccatcaatcc	Knock out of <i>bogI</i>

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Transcriptional profiling of microbe-microbe interactions reveals the response of *Brevibacillus laterosporus* MG64 to different pathogens

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Abstract

Brevibacillus is a genus of Gram-positive bacteria that is prevalent in various environments. It has been reported to have good biocontrol potential. However, in-depth studies on the interaction of *Brevibacillus* and other bacteria are rare. In this study, we conducted a transcriptomic analysis of the biocontrol strain *B. laterosporus* MG64 during interaction with two different pathogens, i.e. plant pathogen *Xanthomonas campestris* and animal pathogen *Pithomyces chartarum*. We reveal that the altered expression of sporulation genes and secondary metabolite biosynthetic BGCs is likely a response pattern of *B. laterosporus* MG64 to other microorganisms. Sporulation genes are downregulated by other microbes likely because of higher demand for secondary metabolites, which are produced by vegetative cells. Depending on the competitor, *B. laterosporus* MG64 adjusts the expression of secondary metabolite biosynthetic BGCs and retains those that can antagonize the specific competitor. Our study leads us to a better understanding of the interaction of *B. laterosporus* MG64 and different pathogens and sheds light on three potentially active BGCs that have not been assigned to any known products. Future studies will be directed to the identification and characterization of these compounds.

Keywords

Transcriptomics, microbe-microbe interaction, *Brevibacillus laterosporus*, sporulation, secondary metabolites, biosynthetic gene cluster

Introduction

Brevibacillus is a genus of rod-shaped Gram-positive bacteria that are found in diverse environments including soil and water [1]. Many *Brevibacillus* strains have been reported to display biocontrol potential [1]. For example, a *Brevibacillus brevis* strain isolated from the rhizosphere of cotton effectively promotes the growth of cotton [2]. *Brevibacillus laterosporus* Lak1210 originating from mangrove marsh soil can produce chitinase that inhibits fungal pathogens and insects [3]. Another *B. laterosporus* strain isolated from the digestive tract of honeybees was able to stimulate the productivity of honeybees' colonies [4]. *Brevibacillus* spp. are also strong producers of antimicrobials [5]. Linear lipopeptides such as bogorols [6, 7] and brevibacillins [8, 9], cyclic nonribosomal peptides including Gramicidin [10], loloatins [11], brevicidine [12], and bacteriocins like laterosporulin [13] and laterosporulin 10 [14] are produced by *Brevibacillus* spp. These antimicrobials have either been used as biocontrol weapons for controlling plant diseases or as antibiotics for the treatment of animal diseases [5].

In a previous study, we isolated the biocontrol strain *B. laterosporus* MG64 that displayed potent bioactivity against various plant pathogens and animal pathogens [15]. Some of the antimicrobial compounds produced by *B. laterosporus* MG64 were also identified. However, most of the biosynthetic gene clusters (BGCs) remain unknown and cannot be identified with the traditional methods. The co-culture of microorganisms has been reported to offer some great advantages (such as yield enhancement, de novo compound induction, etc.) on the identification of natural products, compared to monoculture growth [16, 17]. Many novel compounds were identified through the co-culturing of microorganisms. For example, phexandiol and phomesters were identified through the co-culture of *Phoma* sp. and *Armillaria* sp. [18]. Harziaphilic acid is isolated from the co-culture of *Trichoderma harzianum* M10 and *Talaromyces pinophilus* F36CF [19]. Therefore, the co-culture method is considered very suitable for the identification of novel compounds produced by *B. laterosporus* MG64.

Transcriptomics in combination with the co-culturing of microorganisms can provide a better understanding of microbe-microbe interactions. It can also help to distinguish the active BGCs (those that show altered expression during the interaction are considered to be likely active) from the silent ones. For example, a transcriptomic study on *Serratia plymuthica* revealed that pyrrolnitrin BGC is induced when co-cultured with *Rhizoctonia solani* [20]. However, studies on direct microbe-microbe interactions are relatively rare, especially on the interaction of *Brevibacillus* and other microbes. In this study, we aimed to get a better understanding of the interaction of the biocontrol strain *B. laterosporus* MG64 and two different pathogens, i.e. the Gram-negative plant pathogen *Xanthomonas campestris* that causes bacterial wilt in the grass and the animal pathogen

Pithomyces chartarum that originates from grass and causes facial eczema in ruminants. We were in particular interested in its secondary metabolites biosynthetic genes, and their expression in response to pathogens.

Results and discussion

Global transcriptional profiles of *B. laterosporus* MG64 in response to pathogens

B. laterosporus MG64 is a plant growth-promoting rhizobacterium (PGPR) isolated from the rhizosphere of perennial ryegrass. It displays a broad inhibitory spectrum towards plant pathogens and animal pathogens [15, 21]. In order to study the response of *B. laterosporus* MG64 to different pathogens, a transcriptomic study was conducted using the Gram-negative phytopathogen *Xanthomonas campestris* pv. *campestris* and the fungal animal pathogen *Pithomyces chartarum* as indicators. More than 5 million reads were generated by RNA-seq for each sample. The qualify reads (after quality check with FastQC [22] and trimmed with Trimmomatic [23]) were mapped to the genome of *B. laterosporus* MG64 to deduce the RPKM values of genes, which were used to the compare the transcriptomes of *B. laterosporus* MG64 in response to different pathogens. The statistics of the differentially expressed genes (DEGs) were analyzed with T-REx [24]. As shown in Figure 1, during the interaction with *X. campestris* pv. *campestris*, a total of 170 genes were significantly changed, 70 genes of which were upregulated and 100 genes were downregulated. In response to *P. chartarum*, 60 genes were upregulated while 43 genes were downregulated.

DEGs showing the same trend or different trends

Only 10 genes showed the same trends in response to different pathogens, of which 4 genes upregulated and 6 genes downregulated (Figure 1e, Table 1). Among the upregulated genes, C2W64_00473 is annotated as a formate C-acetyltransferase, which participates in pyruvate metabolism of bacteria [25]. C2W64_02128 encodes a protein containing a PKD domain, which was found conserved and important for many bacterial collagenases and proteases [26, 27]. C2W64_00998 is identified to be a membrane-associated protease, which is important for a wide range of physiological processes of bacteria [28]. The last one upregulated gene encodes a hypothetical protein. Among the downregulated genes, C2W64_00668 encodes a YhfH family protein, which is repressed by the stress response regulation gene *MgsR* [29]. C2W64_01829 and C2W64_01902 are annotated as anti-sigma F factor and stage IV sporulation protein A (spoIVA), respectively. Both proteins are involved in the regulation of sporulation in Firmicutes [30]. C2W64_00103 is identified to be an aldehyde dehydrogenase, which catalyzes the oxidation of

aldehyde [31]. The functions of the rest two downregulated genes (C2W64_04305 and C2W64_03144) encodes proteins containing unknown function domains. These results suggest a similar alteration on the membrane protein associated genes and sporulation relevant genes. Further research is needed to determine how these genes exactly regulate the adaptation of *B. laterosporus* MG64 to different pathogens.

Figure 1. Analysis of differentially expressed genes (DEGs) of *B. laterosporus* MG64 in response to pathogens. Volcano plots of DEGs of *B. laterosporus* MG64 to *X. campestris* (a) and *P. chartarum* (b). The number of up and down regulated DEGs of *B. laterosporus* MG64 to *X. campestris* (c) and *P. chartarum* (d). Venn diagram (e) showing the numbers of overlapped and non-overlapped DEGs.

Table 1. Altered gene showing the same trend to different pathogens

	Gene	Annotation
Upregulated by both pathogens	C2W64_00473	formate C-acetyltransferase
	C2W64_00435	hypothetical protein
	C2W64_02128	PKD domain-containing protein
	C2W64_00998	membrane-associated protease 1
Downregulated by both pathogens	C2W64_04305	DUF3870 domain-containing protein
	C2W64_00668	YhfH family protein
	C2W64_01829	anti-sigma F factor
	C2W64_00103	aldehyde dehydrogenase
	C2W64_03144	DUF4367 domain-containing protein
	C2W64_01902	stage IV sporulation protein A

The response of *B. laterosporus* MG64 to *X. campestris*

The response of *B. laterosporus* MG64 to the Gram-negative plant pathogen *X. campestris* was investigated carefully. Three stress response genes are upregulated by *X. campestris* (Table 2). Among them, C2W64_04147 and C2W64_04149 belong to the TerD family protein, which is a part of the stress response system related to metal sensing [32] while C2W64_02315 is identified to be a universal stress protein, which is commonly induced by various stresses including nutrient starvation, antibiotics presence, and metal presence [33]. This result suggests that the presence of *X. campestris* likely creates a metal-dependent stressful condition for *B. laterosporus* MG64. However, this stress did not induce sporulation, which is a process of microorganism forming spores to protect themselves from stress. On the contrary, we observed a total of 25 sporulation relevant genes downregulated from 2.6 to 19.5 fold during the interaction with *X. campestris* (Table 2). These genes are members of the SigF, SigE, SigG, and SigK regulons, which primarily control the sporulation of firmicutes at the transcriptional level [34, 35]. C2W64_01830 encodes the RNA polymerase sigma factor sigF, which is activated when the spoIIAA (C2W64_01828) is dephosphorylated and antagonizes the anti-sigma factor spoIIAB (C2W64_01829) [36-38]. The activated SigF regulon then activates the SigE (C2W64_01528), a sigma factor crucial for the forespore engulfment and coat proteins synthesis, in the mother cell [30, 39-41]. C2W64_03288, C2W64_01744, and C2W64_01745 encode SpoIIQ, SpoIIIAA, and SpoIIAB, respectively, which

bridge the forespore and the mother cell [42-44]. This bridging is crucial for the activation of SigG (C2W64_01527), which is possibly involved in the engulfment [42-45]. The spoIIID (C2W64_03287) and SigE jointly activate SigK, which is essential for the cortex and coat formation [46, 47]. The downregulation of these genes implies that the presence of *X. campestris* likely delays the sporulation of *B. laterosporus* MG64, thus focusing on combating other bacteria.

Regulation of secondary metabolite production is one of the most effective strategies to combat other bacteria. Indeed, a total of 9 genes are altered during the interaction with *X. campestris*, of which 8 upregulated and 1 downregulated. These genes are from three different BGCs including two nonribosomal synthetases (NRPSs) and one bacteriocin (Figure 2). C2W64_00360 is a core biosynthetic gene (*rlcC*) of relacidines, a class of novel cationic cyclic lipopeptides that combat Gram-negative bacteria (unpublished data) (Figure 2a). The upregulation of *rlcC* is consistent with our previous real-time PCR results (unpublished data) and suggest that relacidines are important for antagonizing *X. campestris*, which was also confirmed experimentally (unpublished data). The other seven upregulated genes are from the same NRPS (Figure 2b). C2W64_02020 and C2W64_02021 encode seven NRPS modules and has been described by us previously [15]. The other five genes are potential modification genes (Figure 2b). However, the function of these genes remains unclear, thus making the final product of this gene cluster difficult to predict.

The downregulated bacteriocin is a Linear azole-containing peptide (LAP) type lanthipeptide (Figure 2c). The open reading frame (ORF) encoding the precursor peptide was predicted by neither BAGEL4 nor antiSMASH 5.0 [48, 49]. Here, we manually found this ORF upstream of the three modification genes (Figure 2c). The precursor peptide encoded by this ORF contains 118 amino acid residues with abundant Cys and Ser residues (Figure 2c), which is an outstanding characteristic of LAPs [50, 51]. The last 62 residues are predicted to be the core peptide. C2W64_03088 and C2W64_03089 are identified to be cyclodehydratases while C2W64_03090 is a dehydrogenase. Catalyzed by these enzymes, most of the Ser and Cys will undergo cyclodehydration and dehydrogenation and form azoles [50, 52]. The downregulation of this LAP suggests that it is not active against *X. campestris*. This is considered reasonable because LAPs are only found active against Gram-positive bacteria, especially those closely related to the producers [51]. Its downregulation is a possible strategy to save energy for the production of other active compounds.

Taken together, as a response to *X. campestris*, genes encoding stress response proteins are upregulated while sporulation genes in *sigF*, *sigE*, *sigG*, and *sigK* operons are downregulated. Besides that, *B. laterosporus* MG64 was found to adjust its strategy on secondary metabolite production when encountered *X. campestris*, namely upregulating two NRPSs and downregulating one LAP type bacteriocin.

Table 2 Downregulated sporulation genes in response to *X. campestris* pv. *campestris*

Genes	Annotation	Fold change
Stress relevant genes		
C2W64_04147	TerD family protein	2.7
C2W64_02315	universal stress protein	2.1
C2W64_04149	TerD family protein	2.1
Sporulation genes		
C2W64_01830	RNA polymerase sporulation sigma factor SigF	-2.6
C2W64_01829	anti-sigma F factor	-2.8
C2W64_04439	spore germination protein	-3.3
C2W64_01745	stage III sporulation protein AB	-3.4
C2W64_01528	RNA polymerase sporulation sigma factor SigE	-3.6
C2W64_01769	SpoIVB peptidase	-3.7
C2W64_03145	gamma-type small acid-soluble spore protein	-3.8
C2W64_01828	anti-sigma F factor antagonist	-4
C2W64_01457	stage V sporulation protein SpoVM	-4.7
C2W64_01902	stage IV sporulation protein A	-4.8
C2W64_01751	SpoIIAH-like family protein	-4.9
C2W64_02158	alpha/beta-type small acid-soluble spore protein	-5.3
C2W64_01256	stage V sporulation protein E	-5.4
C2W64_04294	alpha/beta-type small acid-soluble spore protein	-5.6
C2W64_01744	stage III sporulation protein AA	-5.7
C2W64_01527	RNA polymerase sporulation sigma factor SigG	-5.8
C2W64_03030	sporulation protein	-6.2
C2W64_01309	sporulation protein	-6.6
C2W64_04050	sporulation protein YhbH	-7.1
C2W64_03737	spore coat protein CotJC	-7.1
C2W64_03287	sporulation transcriptional regulator SpoIIID	-7.6
C2W64_04291	sporulation protein YtfJ	-8.1
C2W64_01074	stage V sporulation protein T	-8.6
C2W64_04777	spore coat protein	-19.5
Secondary metabolites		
C2W64_02018	DJ-1/Pfpl family protein	3.6
C2W64_02015	alpha/beta hydrolase	3.6
C2W64_02016	ThiF family adenylyltransferase	3.6
C2W64_02017	molybdopterin biosynthesis protein MoeB	3.5
C2W64_02014	2-isopropylmalate synthase	3.1
C2W64_02021	non-ribosomal peptide synthase/polyketide synthase	2.3
C2W64_00360	non-ribosomal peptide synthetase	2.3
C2W64_02020	amino acid adenylation domain-containing protein	2.2
C2W64_03090	SagB/ThcOx family dehydrogenase	-5.2

Figure 2. Genes and gene clusters regulated in response to *X. campestris*. Genes with green labels are upregulated during interaction while that with indigo blue labels are downregulated. Yellow, transport-related genes; red, core biosynthetic genes; orange, additional biosynthetic genes; grey, unknown genes.

Response of *B. laterosporus* MG64 to *P. chartarum*

During the interaction with *P. chartarum*, stress relevant genes were not induced, which is different from the response to *X. campestris*. Furthermore, only three sporulation genes were altered, one gene of which upregulated and two genes downregulated (Table 3). The downregulation of the anti-sigma F factor (C2W64_01829) and stage IV sporulation protein A (C2W64_01902) is similar to the response to *X. campestris*. However, the upregulation of the CotS family spore coat protein (C2W64_03567) is in contrast to other sporulation relevant genes and cannot be explained thus far. We also reveal that a total of six inorganic ion transport genes are altered (Table 3). Among them, four genes form the high-affinity phosphate ABC transporter system Pst [53-55]. A study on *Escherichia coli* revealed that this transport system is functional when the extracellular inorganic phosphate is below 20 μM [53]. Therefore, the presence of *P. chartarum* likely altered the concentration of inorganic phosphate, and to compete, *B. laterosporus* MG64 activates the high-affinity phosphate ABC transporter system.

Figure 3. Gene cluster downregulated by *P. chartarum*. Genes with indigo blue labels are downregulated. Orange, additional biosynthetic gene; red, core biosynthetic gene; grey, unknown genes.

A total of 13 genes relevant to the biosynthesis of secondary metabolites are downregulated from 2 to 3.6 folds in response to *P. chartarum* (Table 3). These genes are from the same gene cluster, which is a hybrid of NRPS and polyketide synthetase (PKS). Ten PKS domains and thirteen NRPS domains are encoded by this gene cluster. The presence of four acyltransferase domains suggests the incorporation of four PK moieties while four adenylation domains indicate the incorporation of four amino acid residues (Figure 3). This gene cluster showed a 44% similarity to zwittermicin A, an NRP-PK hybrid produced by *B. cereus* that has potent antifungal activity [56, 57]. However, the arrangement of the genes is different from that of zwittermicin A, indicating that this BGC produces a potential novel compound. The downregulation of this BGC suggests that this potential novel compound is likely not active against *P. chartarum* and to antagonize this fungal pathogen, *B. laterosporus* MG64 concentrates on the production of other active antimicrobials.

Taken together, inorganic ion transport proteins, especially the high-affinity phosphate transporters are altered during the interaction with *P. chartarum*. Moreover, an NRPS-PKS hybrid BGC is downregulated by the fungal pathogen.

Conclusion

In this study, we investigated the transcriptional response of *B. laterosporus* MG64 to two different pathogens, i.e. plant pathogen *X. campestris* and animal pathogen *P. chartarum*. We show that *B. laterosporus* MG64 altered the expression of sporulation genes and secondary metabolite BGCs in order to cope with other bacteria. Sporulation genes are downregulated possibly to support the vegetative growth, during which bacteria produce antimicrobials to compete with other bacteria. Besides, *B. laterosporus* MG64 is likely able to adjust its strategy on the production of secondary metabolites when it encounters different competitors, namely to upregulate the specifically active

antimicrobials and downregulate inactive ones. As a result of this potential strategy, two NRPSs were found upregulated and one LAP downregulated in response to *X. campestris*, and one NRPS- PKS hybrid downregulated by the *P. chartarum*. Our study leads us to a better understanding of the interaction of *B. laterosporus* MG64 and different pathogens, and sheds light on potential novel secondary metabolites. In particular the upregulated ones are interesting because they are likely to have potent activity to the microbe they were exposed to.

Table 3. Secondary metabolites biosynthetic genes in response to *P. chartarum*

Genes	Annotation	fold change
Sporulation relevant gene		
C2W64_03567	CotS family spore coat protein	4.7
C2W64_01829	anti-sigma F factor	-2.6
C2W64_01902	stage IV sporulation protein A	-2.8
Inorganic ion transport protein		
C2W64_03167	phosphate ABC transporter permease PstA	6.5
C2W64_03166	phosphate ABC transporter ATP-binding protein	5.7
C2W64_03168	phosphate ABC transporter permease subunit PstC	4
C2W64_03169	phosphate ABC transporter substrate-binding protein	3.5
C2W64_01056	zinc ABC transporter substrate-binding protein	2.5
C2W64_04357	metal ABC transporter permease	-2.5
Secondary metabolites biosynthetic genes		
C2W64_01692	acyltransferase domain-containing protein	-2
C2W64_01693	3-hydroxybutyryl-CoA dehydrogenase	-2.2
C2W64_01689	HAD-IIIC family phosphatase	-2.4
C2W64_01701	GNAT family N-acetyltransferase	-2.4
C2W64_01690	acyl-CoA dehydrogenase	-2.5
C2W64_01699	LLM class flavin-dependent oxidoreductase	-2.7
C2W64_01695	acyl-CoA dehydrogenase	-2.9
C2W64_01691	acyl carrier protein	-3
C2W64_01694	acyl carrier protein	-3.1
C2W64_01696	amino acid adenylation domain-containing protein	-3.2
C2W64_01698	SDR family NAD(P)-dependent oxidoreductase	-3.4
C2W64_01697	amino acid adenylation domain-containing protein	-3.6
C2W64_01700	hypothetical protein	-3.6

Material and methods

Interaction assay

Two different pathogens, *Xanthomonas campestris* pv. *campestris* NCCB92058 and *Pithomyces chartarum* CBS485.71, were used for the interaction assay. *B. laterosporus* MG64 was grown on

the LB plate for 24 h (28 °C). Colonies were resuspended in phosphate-buffered saline (PBS, pH = 7.4) buffer. The OD₆₀₀ of cells was adjusted to 1.0 to achieve inoculum. For the interaction with *Xanthomonas*, fresh pathogen cells were suspended in PBS buffer and added to the melted TSB agar (around 45 °C) to a final concentration around 1×10^6 CFU/mL. After plates solidified, 10 µL of MG64 inoculum were spotted onto the center. As a control, the same volume of inoculum was inoculated onto blank TSB plates. Plates were sealed with parafilm and incubated at 28 °C for 2 days. For interaction with *Pithomyces*, fungal were grown on TSB plates for 5 days. An agar plug (5 mm diameter) containing fungal hyphae was placed at the center of fresh TSB plates. Plates were sealed with parafilm and incubated at 28 °C to allow the slow-growing fungi to grow for 2 days. After 2 days, *B. laterosporus* MG64 (10 µL inoculum) was inoculated on the plates at a distance of 2 cm to the fungal plug. As a control, blank TSB plates were treated in the same way as the treated strain. Plates were grown at 28 °C for another 2 days. For sampling, cells were scraped off and suspended in DEPC-treated water before snap freezing in liquid nitrogen.

RNA extraction

The RNA was extracted using the Roche RNA isolation kit with some modifications. *B. laterosporus* MG64 cells were suspended in 400 µL DEPC-treated water. Then 500 µL chloroform-isoamyl alcohol (24:1 v/v), 50 µL 10% SDS, and 0.5 g glass beads (0.5 µm) were added to the tube. Cells were lysed with a Mini-BeadBeater (607, BioSpec, USA) by three rounds of 45 s pulse. Samples were centrifuged at 10,000 rpm (4 °C) for 10 min. The upper phase was transfer to a new tube containing 500 µL of chloroform- isoamyl alcohol (24:1). The mixture was centrifuged at 10,000 rpm (4 °C) for 10 min and the upper phase was collected in a new tube. Two volumes of lysis/binding buffer were added and mixed with the sample gently. The mixture was transferred to the column provided by the manufacturer and centrifuged at 8,000 g for 30 s. DNA was digested by adding 100 µl DNase buffer, 10 µl DNase I, and 5 µl Ribolock (Fermentas; RNase inhibitor) and incubated at room temperature for 30 min. The samples were washed with 500 µL wash buffer I, 500 µL wash buffer II, and 200 µL wash buffer II accordingly. The RNA was eluted with RNase free water and the quality was checked with the Bioanalyzer (Agilent Technologies, Wilmington, DE, United States)

RNA sequencing and data analyses

RNA sequencing was conducted at GATC Biotech (Konstanz, Germany). The quality of raw RNA-Seq reads was determined with FastQC [22] and the low-quality sequences were trimmed with Trimmomatic [23]. The data set was aligned to the genome with STAR-aligner [58]. Gene counts were summarized with featureCounts [59]. Differential gene expression analyses were conducted with T-REx [24], in which the RPKM values of treatment (interaction) and control (blank) were

compared. $p < 0.05$ and fold-change > 2 were set as thresholds of significance (TopHits). Functional categorization was performed with a web serve genome 2D [60]. The RNA-seq data have been submitted to the NCBI Gene Expression Omnibus (GEO3).

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6

General discussion

According to the World Health Organization (WHO), around 821 million people were undernourished in 2017. This number has been growing in recent years and therefore calls for urgent action to produce more food [1]. Although grass is not a traditional crop, it is the basis for the production of dairy products and meat. Specifically, perennial ryegrass is an important grass for the forage-livestock system [2, 3]. However, plant pathogens and animal pathogens are a great threat to its application. The production of healthy grass relies on the usage of chemical fertilizers and pesticides. However, these chemicals inevitably cause environmental problems. To ensure the sustainable development of agriculture, environmentally friendly alternatives are in demand.

Plant growth-promoting rhizobacteria (PGPR) have been proven to be effective alternatives to chemical fertilizers and pesticides because of their great efficacy on plant growth stimulation and plant disease control. However, studies about the interaction between PGPR and grass, especially perennial ryegrass, are relatively rare. Shoebitz et al. isolated a bacterial strain of *Enterobacter ludwigii* from the rhizosphere of perennial ryegrass, which was able to produce nitrogenase and indole-3-acetic acid (IAA) and inhibit mycelial growth and spore germination of *Fusarium solani* [4]. Castellano-Hinojosa et al. isolated a list of nitrogen-fixing bacteria and tested their ability to produce IAA, ACC deaminase activity as well as biocontrol activity against *Fusarium oxysporum* [5]. Nevertheless, these studies did not investigate the antimicrobials, which play a key role in pathogen control, produced by the isolated PGPR. The work presented in this thesis thus aimed to isolate *Bacillus* species and closely related species from the perennial ryegrass, which have great advantages (i.e. antimicrobials production and endospore formation) to be PGPR, and to investigate their antimicrobials that potentially involved in biocontrol (Figure 1).

The biosynthetic potential of *Brevibacillus laterosporus* is underestimated

Bacillus spp. are well known for their production of antimicrobials, which have been used in different applications. In **chapter 2**, we described the isolation and screening of 7 potential PGPR strains (out of 90 initially isolated), i.e. *Bacillus subtilis* MG27, *Bacillus velezensis* MG33 and MG43, *Bacillus pumilus* MG52 and MG84, *Brevibacillus laterosporus* MG64 and *Bacillus altitudinis* MG75, from the rhizosphere of healthy perennial ryegrass. We also reported abundant biosynthetic gene clusters (BGCs) responsible for the production of potentially novel secondary metabolites from their genomic sequences, especially that of *B. laterosporus* MG64. *Brevibacillus* is a genus of bacteria reclassified from *Bacillus* based on the analysis of 16S rRNA sequences [6]. Species of this genus have been reported to display great potential in biocontrol [7]. For example, *B. laterosporus* ZQ2 strongly inhibits the growth of apple pathogens such as *Rhizoctonia solani*, *Fusarium oxysporum*, *Fusarium solani* and *Physalospora piricola* [8]. *B. brevis* SVC(II)14 is able to enhance the growth of cotton and inhibit fungal pathogens [9]. Biocontrol properties of *Brevibacillus* spp. are associated with the production of antimicrobials, many of which have been identified and characterized [10]. However, only 5 out of 26 BGCs of *B. laterosporus* MG64 were assigned to known products, suggesting that

the biocontrol potential of the *B. laterosporus* species is likely underestimated. The transcriptomic analysis in **chapter 5** showed that a linear azole-containing peptide (LAP) and a nonribosomal peptide (NRP) harbored by *B. laterosporus* MG64 are potentially functional during the interaction with other microbes. However, these compounds were not identified to date possibly because the yield of these compounds is too low, making it difficult to extract and identify. Future research will be directed to express these compounds in other hosts such as *Escherichia coli* and *B. subtilis*. Recent research using a miniBacillus PG10 as a host successfully expressed several lanthipeptides [11]. PG10 is derived from *B. subtilis* by deleting 36% of its genome [12]. It has a clean background of metabolites, thus making it easier to identify the compounds. Therefore, PG10 is a promising host for heterologous expression of BGCs identified from *B. laterosporus* MG64.

***B. laterosporus* MG64 is a good producer of cationic lipopeptides**

Cationic peptides are found in many organisms and display an enormous variety of structures. Consensus features of these peptides are the overall net positive charge and their amphiphilic nature. Both features make this class of peptides easier to interact with the bacterial membranes, which are anionic and constituted with hydrophilic head groups and hydrophobic cores [13, 14]. Therefore, they are considered good antibiotic candidates to cure diseases caused by bacterial pathogens, especially Gram-negative ones, which have caused significant public health problems in the whole world. We reported a class of cationic circular lipopeptides (relacidines) in **chapter 3** and two classes of cationic linear lipopeptides (bogorols and succilins) in **chapter 4**. These cationic peptides are produced by the same strain, i.e. *B. laterosporus* MG64.

Relacidines are constituted with a fatty acid tail and 13 amino acid residues, 3 of which are positively charged (Lys/Orn). They are structurally similar to brevicidine and laterocidine, two lipopeptides produced by *B. laterosporus* DSM25 and *B. laterosporus* LMG15441, respectively [15]. Brevicidine and laterocidine show great potential as antibiotics, because 1) they display potent bioactivity against Gram-negative human pathogens; 2) they have a low risk of antibiotic resistance; 3) they do not cause lysis of erythrocytes; and 4) they have no toxicity against the human cell line HeLa [15]. Relacidines have similar structures, inhibitory spectra and mode of action to brevicidine and laterocidine. Therefore, they are also considered antibiotic candidates. Moreover, relacidines are active against numerous Gram-negative plant pathogens including *Xanthomonas* and *Pseudomonas*. This suggests their potential application in agriculture, which was not shown for brevicidine and laterocidine.

The bogorol family of peptides (bogorols, brevibacillins, BT peptide and BL-A60) form a class of cationic linear lipopeptides that are active against a wide variety of human pathogens and food pathogens, especially the Gram-positive ones [16-21]. In **chapter 4**, we reported four novel variants of bogorols (bogorol I-L) and four succilins (succilin I-L). Bogorol I-L has at least one different amino acid residue compared to the known ones. Moreover, we showed their bioactivity against plant pathogens, including both Gram-positive and Gram-negative ones. This implies their potential in biocontrol, which is not shown for this class of peptides before. Succilins are a class of modified

bogorols, in which a succinyl group is attached to the third amino acid residue (Lys/Orn) of bogorols. This is the first report of succinylated lipopeptides and this new group of compounds was found to display only modest activity against species of *Xanthomonas*, *Bacillus* and *Staphylococcus*.

Figure 1. A summary of the works presented in this thesis. We first isolated PGPR strains from perennial ryegrass and further mined for secondary metabolites BGCs (chapter 2). Antimicrobials were extracted and characterized (including their biosynthesis and mode of action) from the isolated PGPR *Brevibacillus laterosporus* MG64 (chapter 3 and chapter 4). A comparative transcriptomic analysis was used to study the interaction of *B. laterosporus* MG64 and pathogens (chapter 5).

Mode of action of cationic lipopeptides produced by *B. laterosporus* MG64

Many cationic peptides were reported to form pores in cell membranes. However, there is growing evidence showing that some cationic peptides are targeting intracellular macromolecules. In **chapter 3**, we investigated the mode of action of relacidines, which is not yet clear for the structurally similar peptides brevicidine and laterocidine. We showed that relacidines do not form pores in the bacterial cell membrane. This is similar to brevicidine and laterocidine and different to other cationic peptides such as polymyxins [15]. Relacidines were also found not to affect the biosynthesis of peptidoglycan and RNA. Instead, they affect the oxidative phosphorylation process of cells. Oxidative phosphorylation is an important cellular process to produce ATP, which provides energy

to the cells. Therefore, it has become a promising target space for drug development. Several compounds were reported to inhibit the oxidative phosphorylation of cells. For instance, bedaquiline and delamanid, two recently approved drugs for curing tuberculosis, were shown to target the F_1F_0 ATP synthase and respiratory cytochromes, respectively [22-24]. In addition, lansoprazole, a prodrug displaying intracellular activity against *Mycobacterium tuberculosis*, targets the cytochrome bc_1 , the complex III of the electron transport chain [25]. However, reports of lipopeptides targeting oxidative phosphorylation are rare. Relacidines are, to the best of our knowledge, the first ones. Different from relacidines, the bogorol family of peptides form pores in the cellular membranes of both Gram-positive and Gram-negative bacteria (**chapter 4**). This result is in line with the study on brevivacillins [26].

Biosynthesis of the bogorol family of peptides employ novel modifications

Many peptides from the bogorol family have been identified in recent years. However, their biosynthesis has never been investigated. In **chapter 4**, we systemically studied the biosynthetic pathway of bogorols and succilins. Both classes of compounds are synthesized by the same BGC and undergo two modifications: lipoinitiation and reduction. Moreover, succilins also undergo a succinylation using bogorols as substrates.

Lipoinitiation is typically found in the biosynthetic pathway of lipopeptides. The usual pattern of lipoinitiation involves two steps: activation of the substrate by ligating it to a coenzyme A (CoA) by catalysis of fatty acid CoA ligases (FACL) and incorporation of the activated substrate into the NRPS assembly line by catalysis of a starter condensation (C) domain [27]. However, the lipoinitiation of bogorols and succilins is different from this usual pattern. It employs an adenylation (A) domain to activate the fatty acid. Unlike the FACL encoding genes that are located distantly from the BGC and are usually difficult to find, the module responsible for the lipoinitiation of bogorols and succilins is encoded by the BGC. Therefore, it is possible to engineer the lipid tail of any NRPs by incorporating this module into their BGCs. Moreover, by changing the substrate specificity of the A domain of this module, the activity-related lipid tail can be very diverse. However, there is a long way to go before realizing this great blueprint.

The formation of valinol of bogorols and succilins is catalyzed by two different reductases: a terminal reductase domain (TD) and a keto-reductase (Bogl). The TD is responsible for the release of a peptide from the peptidyl peptide carrier protein. This release mechanism is different from that mediated by a thioesterase and usually yields an aldehyde-form peptide, which will be further reduced to an alcohol form [28]. The second reduction is catalyzed by the keto-reductase Bogl in the cases of bogorols and succilins. It has been reported that such alcohol-form peptides are resistant to proteases [21]. In this study, we also showed that the alcohol-form peptide is more active than the aldehyde form. These are likely the biological functions of the two consecutive reductions in NRPs.

Succinylation is a modification frequently found in many proteins of both prokaryotes and eukaryotes [29-31]. It usually occurs at the lysine residue and likely depends on the succinyl-CoA, which is involved in the tricarboxylic acid cycle [30]. Succinylation is also found in bacterial secondary metabolites. Subtilin-like ribosomally synthesized and posttranslationally modified peptides (RiPPs) contain a succinylated tryptophan at the N-terminus [32, 33]. Succinylation of subtilin-like peptides is influenced by the concentration of glucose in the growth medium as well as by the transition state regulator AbrB [33]. Our work demonstrates for the first time the succinylation of a lipopeptide. However, the responsible enzymes have not been identified yet. An in-depth study using comparative genomics and transcriptomics will be needed for their discovery.

Interaction between *B. laterosporus* MG64 and pathogens

In **chapter 5**, we investigated the response of *B. laterosporus* MG64 to two different pathogens: the Gram-negative plant pathogen *X. campestris* pv. *campestris* and the fungal mammalian pathogen *Pithomyce chartarum*. We reveal that the expression of sporulation-relevant genes and secondary metabolite biosynthetic genes are altered by both pathogens. This is likely a strategy employed by *B. laterosporus* MG64 to combat competitors. A similar response was observed in the interaction between *Serratia plymuthica* AS13 and the plant pathogen *Rhizoctonia solani* [34]. In-depth studies are needed to reveal the function of specific genes in the microbe-microbe interaction.

We also showed that relacidines are induced by the plant pathogen *Xanthomonas campestris* pv. *campestris* and *Pseudomonas syringe* pv. *tomato* at the transcriptional level (**chapter 3**). This implies that relacidines play an important role during the interaction of plant pathogens and are potentially able to control plant diseases. Also, their producer, *B. laterosporus* MG64, has the potential to be developed into a biocontrol agent. However, the production of relacidines is likely affected by temperature (compounds were not detected at 37 °C). This may limit their application to an appropriate region and season. Also, our study is limited to microbe-microbe interaction under laboratory conditions. Therefore, the exact effect of relacidines or their producers on controlling plant diseases *in vivo* still requires further investigation.

In summary, we isolated seven PGPR strains (out of 90 initially isolated) from the rhizosphere of perennial ryegrass and further investigated the antimicrobials produced by one of the most promising strains, *B. laterosporus* MG64. A total of three different classes of compounds were extracted and characterized. The biosynthesis or/and mode of actions of these compounds were also thoroughly investigated. Furthermore, the transcriptional response of *B. laterosporus* MG64 to pathogens was studied by transcriptomics, showing that the sporulation-relevant genes and secondary metabolites biosynthetic genes were altered. All in all, this thesis work provides a comprehensive understanding of the novel potent biocontrol strain *B. laterosporus* MG64 as well as its secondary metabolites.

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Summary

Samenvatting

Acknowledgements

Summary

Plant growth-promoting rhizobacteria (PGPR) form a good alternative to chemical fertilizers and pesticides. Moreover, PGPR can produce antimicrobials that have the potential to be antibiotics, which is in great demand because of the rapidly emerging antibiotic resistance. **The aims of this PhD thesis** were 1) to isolate and screen effective PGPR strains for use in perennial ryegrass, a crucial pasture plant distributed worldwide; 2) to identify novel antimicrobials produced by these PGPR strains; 3) to investigate the biosynthetic machinery and mode of action of the novel antimicrobials.

A total of 90 *Bacillus* strains were isolated from the rhizosphere of perennial ryegrass and seven of them displayed outstanding biocontrol activity, namely stimulating the growth of plants and antagonizing bacterial and fungal pathogens that are potential threats to the plants and ruminants. Further mining into their genome sequences revealed a vast number of biosynthetic gene clusters (BGCs) of secondary metabolites, some of which remain unknown. One of the seven PGPR strains, MG64, belongs to a relatively less well-known species, *Brevibacillus laterosporus*. Notably, around 80% of the BGCs harbored by MG64 have not been assigned to any known products. Further characterization of the secondary metabolites produced by MG64 revealed three different classes of antimicrobials: relacidines, bogorols, and succilins. Relacidines are a class of cationic circular lipopeptides that constituted with a fatty acid side chain (4-methylhexanoic acid) and 13 amino acid residues. A lactone ring is formed by the last five amino acid residues and three positively charged ornithine residues are located in the linear fragment. Relacidines selectively combat Gram-negative bacteria by affecting the oxidative phosphorylation process. Bogorols belong to a class of cationic linear lipopeptides that comprise a fatty acid side chain and 13 amino acid residues, three of which are positively charged-lysine residues. Bogorols are active against both Gram-positive bacteria and Gram-negative bacteria, which is a result of forming pores in the cell membranes. Succilins are modified from bogorols by succinylation at Orn₃. Succinylation affects the polarity and charges of the peptides. As a result, succilins only display modest bioactivity against bacterial pathogens.

Understanding the biosynthetic machinery of lipopeptides is the basis for further engineering. Bogorols and succilins have interesting characteristics: 1) they possess a fatty acid at the N-terminus but a starter condensation domain is missing; 2) they contain valinol, an alcohol form of valine, at the C-terminus; 3) the occurrence of succinylation at the third amino acid residue (Orn or Lys). These characteristics suggest the involvement of novel modifications. Further investigation revealed that the lipidation of bogorols and succilins is mediated by an adenylation domain, which is different from the usual pattern that employs external fatty acid coenzyme ligase (FACL). The valinol at the C-terminus is formed by a two-step reduction catalyzed by a terminal reductase (TD) and an aldo/keto reductase (Bogl). The occurrence of succinylation requires a high concentration of bogorols. However, its responsible enzyme remains unknown and future research will be directed to discover it.

A comparative transcriptomics study was conducted to investigate the response of *B. laterosporus* MG64 to different pathogens (plant pathogen *Xanthomonas campestris* and animal pathogen *Pithomyces chartarum*). We showed that the expression of sporulation genes and secondary metabolites BGCs are regulated during the interaction with pathogens. Two interesting BGCs, including one nonribosomal peptide (NRP) and one linear azol(in)e-containing peptide (LAP), potentially function during the interaction with pathogens. Further study will be directed to characterize these two compounds.

This thesis provides a comprehensive understanding of the PGPR strain *B. laterosporus* MG64, including some of its antimicrobials, its interaction with different pathogens, and its potential in biocontrol. Moreover, our study of the biosynthesis of bogorols and succilins extends our knowledge on the biosynthetic machinery of lipopeptides, which serves as a basis for the engineering of lipopeptides.

Samenvatting

Plantengroei bevorderende rhizobacteriën (PGPR) vormen een goed alternatief voor kunstmest en bestrijdingsmiddelen. Bovendien kunnen PGPR antimicrobiële stoffen produceren die mogelijk als antibiotica kunnen dienen, waar veel vraag naar is vanwege de snel opkomende antibioticaresistentie. De doelstellingen van dit proefschrift waren 1) het isoleren en screenen van effectieve PGPR stammen voor gebruik op Engels raaigras, een cruciale weideplant die wereldwijd verspreid is; 2) het identificeren van nieuwe antimicrobiële stoffen die door deze PGPR stammen worden geproduceerd; 3) het onderzoeken van de biosynthese en het werkingsmechanisme van de nieuwe antimicrobiële stoffen.

In totaal werden 90 *Bacillus* stammen geïsoleerd uit de rhizosfeer van Engels raaigras. Hiervan toonden zeven uitstekende biocontroleactiviteit, namelijk het stimuleren van de groei van planten en het tegengaan van pathogene bacteriën en schimmels die potentiële bedreigingen vormen voor de planten en herkauwers. Verder onderzoek naar de bijbehorende genoomsequenties onthulde een groot aantal biosynthetische gen clusters (BGC's) van secundaire metabolieten, waarvan sommige onbekend blijven. Een van de zeven PGPR stammen, MG64, behoort tot een relatief minder bekende soort, *Brevibacillus laterosporus*. Opmerkenswaardig, ongeveer 80% van de BGC's in het genoom van MG64 zijn niet toegewezen aan bekende producten. Verdere karakterisering van de door MG64 geproduceerde secundaire metabolieten bracht drie verschillende klassen van antimicrobiële stoffen aan het licht: relacidines, bogorols en succilins. Relacidines zijn een klasse van kationische cirkelvormige lipopeptiden die bestaan uit een vetzuurzijketen (4-methylhexaanzuur) en 13 aminozuren. Een lactonring wordt gevormd door de laatste vijf aminozuren en drie positief geladen ornithine residuen bevinden zich in het lineaire fragment. Relacidines bestrijden selectief Gram-negatieve bacteriën door beïnvloeding van het oxidatieve fosforylatieproces. Bogorols behoren tot een klasse van kationische lineaire lipopeptiden die een vetzuurzijketen en 13 aminozuren bevatten, waarvan er drie positief geladen lysine residuen zijn. Bogorols zijn actief tegen zowel Gram-positieve bacteriën als Gram-negatieve bacteriën, als gevolg van het vormen van poriën in de celmembraan. Modificatie van bogorols via succinylering van Orn3 resulteert in de formatie van succilins. Succinylering beïnvloedt de polariteit en ladingen van de peptiden. Hierdoor vertonen succilins slechts matige biologische activiteit tegen bacteriële pathogenen.

Het begrijpen van de biosynthese van lipopeptiden vormt de basis voor verdere engineering. Bogorols en succilins hebben interessante eigenschappen: 1) ze hebben een vetzuur aan de N-terminus, maar er ontbreekt een startcondensatiedomein; 2) ze bevatten valinol, een alcoholvorm van valine, aan de C-terminus; 3) het optreden van succinylering van het derde aminozuur (Orn of Lys). Deze kenmerken suggereren de betrokkenheid van nieuwe modificaties. Nader onderzoek wees uit dat de lipidatie van bogorols en succilins wordt gemedieerd door een adenyleringsdomein, dat verschilt van het gebruikelijke patroon dat gebruikmaakt van extern vetzuur co-enzym ligase (FACL). Valinol aan de C-terminus wordt gevormd door reductie in twee stappen die wordt gekatalyseerd door een terminaal reductase (TD) en een aldo / keto-reductase (Bogl). Het optreden

van succinylering vereist een hoge concentratie van bogorols. Het verantwoordelijke enzym blijft echter onbekend en toekomstig onderzoek zal zich hierop richten.

Een transcriptoom studie werd uitgevoerd om de respons van *B. laterosporus* MG64 op verschillende pathogenen (plantpathogeen *Xanthomonas campestris* en dierpathogeen *Pithomyces chartarum*) te onderzoeken. We toonden aan dat de expressie van sporulatie genen en BGC's van secundaire metabolieten gereguleerd wordt tijdens interactie met pathogenen. Twee interessante BGC's, waaronder één niet-ribosomaal peptide (NRP) en één lineair azol(in)e-bevattend peptide (LAP), functioneren mogelijk tijdens de interactie met pathogenen. Verder onderzoek zal gericht worden op karakterisatie van deze twee peptiden.

Dit proefschrift biedt een uitgebreid begrip van de PGPR stam *B. laterosporus* MG64, inclusief enkele van de antimicrobiële stoffen, de interactie met verschillende pathogenen, en de mogelijkheid voor biocontrole. Bovendien breidt het bestuderen van de biosynthese van bogorols en succilins onze kennis uit over de biosynthese van lipopeptiden, die als basis kan dienen voor de engineering van lipopeptiden.

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