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REVIEW

Bacillus lipopeptides as powerful pest control agents for a more sustainable and healthy agriculture: recent studies and innovations

Rafaela O. Penha[1] · Luciana P. S. Vandenberghe[1] · Craig Faulds[2] · Vanete T. Soccol[1] · Carlos R. Soccol[1]

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Abstract

Main conclusion **Lipopeptides could help to overcome a large concern in agriculture: resistance against chemical pesticides. These molecules have activity against various phytopathogens and a potential to be transformed by genetic engineering.**

Abstract The exponential rise of pest resistances to different chemical pesticides and the global appeal of consumers for a sustainable agriculture and healthy nutrition have led to the search of new solutions for pest control. Furthermore, new laws require a different stance of producers. Based on that, bacteria of the genus *Bacillus* present a great agricultural potential, producing lipopeptides (LPs) that have high activity against insects, mites, nematodes, and/or phytopathogens that are harmful to plant cultures. Biopesticide activity can be found mainly in three families of *Bacillus* lipopeptides: surfactin, iturin, and fengycin. These molecules have an amphiphilic nature, interfering with biological membrane structures. Their antimicrobial properties include activity against bacteria, fungi, oomycetes, and viruses. Recent studies also highlight the ability of these compounds to stimulate defense mechanisms of plants and biofilm formation, which is a key factor for the successful colonization of biocontrol organisms. The use of molecular biology has also recently been researched for continuous advances and discoveries of new LPs, avoiding possible future problems of resistance against these molecules. As a consequence of the properties and possibilities of LPs, numerous studies and developments as well as the attention of large companies in the field is expected in the near future.

Keywords Lipopeptides · *Bacillus* · Biocontrol · Antimicrobial · Antifungal · Antibacterial

Introduction

For many decades, the genus *Bacillus* has been used in different studies in the fields of genetics and biochemistry. During this time, several strains were isolated, with the potential to produce more than two dozens of antibiotics with an impressive array of structures. Therefore, an average of about 4–5% of a *Bacillus subtilis* genome was employed in antibiotic production. Part of these antibiotics are composed of lipopeptides—LPs (Stein 2005). These compounds have been seen as biological control agents as an alternative to chemical pesticides, which generate strong environmental impacts by selecting resistant pests and contaminating the environment (Torres et al. 2017).

Lipopeptides are biosurfactants that are synthesized non-ribosomally by large multi-enzyme complexes, the non-ribosomal peptide synthetases (NRPS) (Chen et al. 2009). These synthesis mechanisms lead to a great diversity among LPs with regard to the type and sequence of amino acid residues, the nature of the peptide cyclization, and the nature, length, and branching of the fatty acid chain (Ben Abdallah et al. 2015). There are three major families of *Bacillus* LPs, namely iturin, surfactin, and fengycin (Dimkić et al. 2017). The amphiphilic nature of these molecules provides a capacity of interfering with biological membrane structures,

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✉ Carlos R. Soccol
soccol@ufpr.br

[1] Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, Centro Politécnico, CP 19011, Curitiba, PR 81531-908, Brazil

[2] Aix-Marseille Université, POLYTECH Marseille, UMR 1163 Biotechnologie Des Champignons Filamenteux, 163 Avenue de Luminy, 13288 Marseille Cedex 09, France

making LPs key factors for the biological control of microorganisms. Lipopeptides are also responsible for systemic resistance induction in plant and biofilm formation (Fig. 1) (Ongena and Jacques 2008).

Iturins are neutral or monoanionic lipopeptides, and have the chiral sequence LDDLLDL with a restricted number of residues (Asx, Glx, Pro, Ser, Thr, Tyr). Also, they share a common sequence (β-hydroxy fatty acid-Asx-Tyr-Asx) and show a variation at the other four positions (Fig. 2a) (Bonmatin et al. 2003). This family is mainly composed of the following compounds: iturin A and C (Besson et al. 1978, 1986), bacillomycin D (Peypoux et al. 1984), bacillomycin F (Mhammedi et al. 1982), bacillomycin L (Peypoux et al. 1984), bacillopeptin (Kajimura et al. 1995), and mycosubtilin (Peypoux et al. 1986). In terms of activity, iturin has a powerful antibiotic

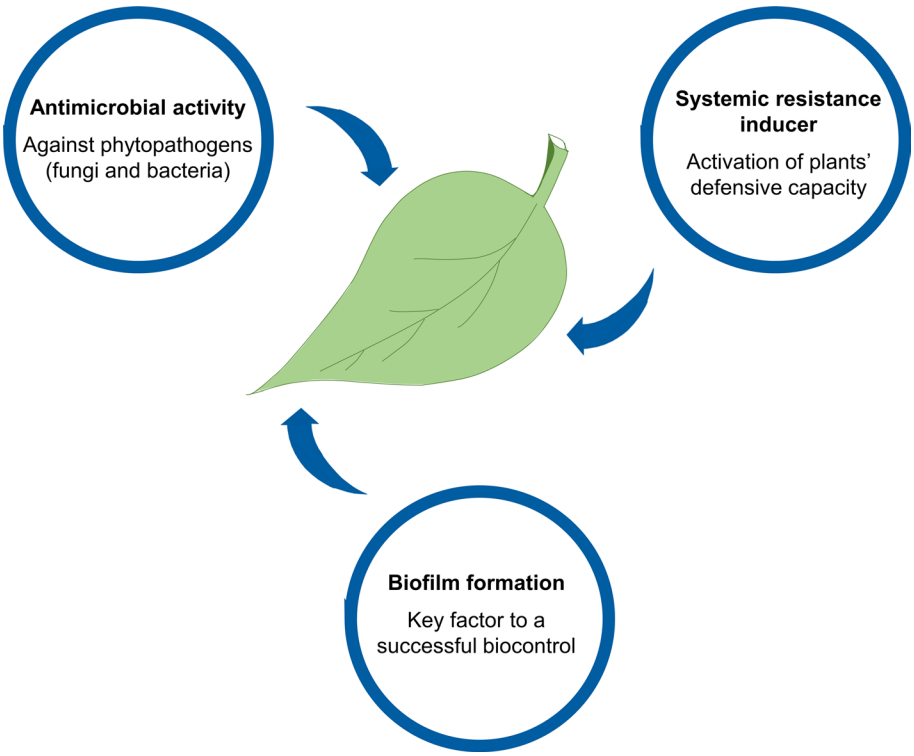

Fig. 1 Effects of LP production by *Bacillus* species. These molecules can act in two ways, namely by directly acting against different phytopathogens and by inducing the systemic resistance of the plant. For the success of the action of these two abilities, biofilm formation is essential, since it overcomes difficulties in the colonization of the microorganisms

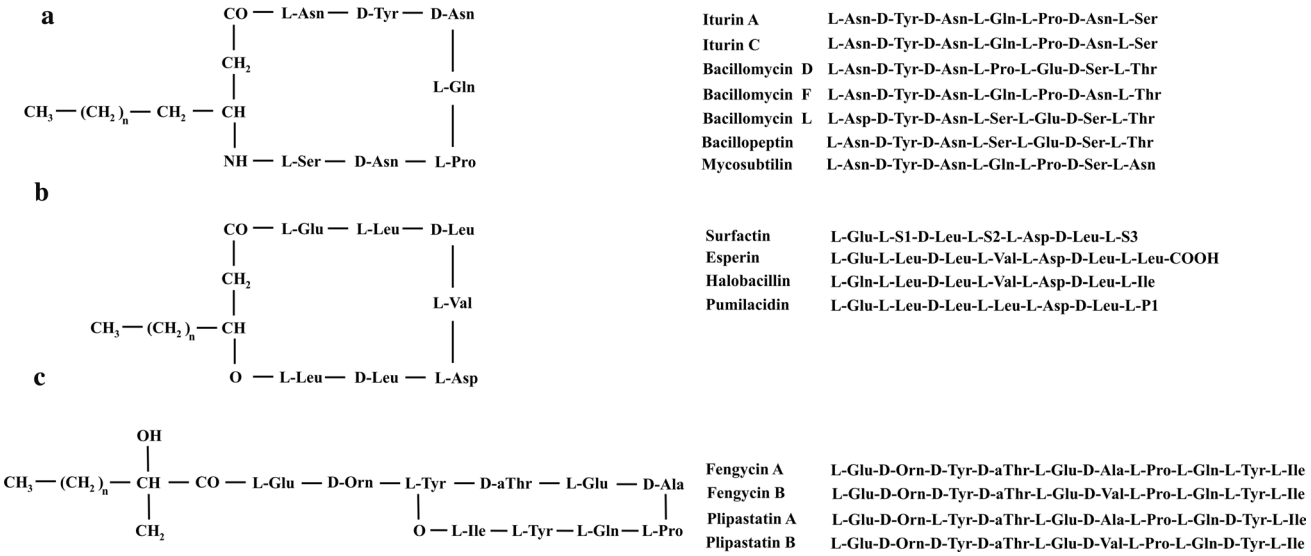

Fig. 2 Structures of **a** iturin variants ($n = 9–12$), **b** surfactin variants ($n = 9–11$; S1 = Val, Leu, or Ile; S2 = Ala, Val, Leu, or Ile; S3 = Val, Leu, or Ile; P1 = Val or Ile), and **c** fengycin variant ($n = 11–14$) (adapted from Ongena and Jacques 2008)

activity against a wide fungal spectrum (Arrebola et al. 2010).

The structure of surfactin was elucidated 49 years ago by Kakinuma et al. (1969); it was found to be constituted by a heptapeptide with a chiral central sequence LLDLLDL, interlinked by a β-hydroxy fatty acid to form a cyclic lactone ring structure (Fig. 2b). The main members of the surfactin group are surfactin (Arima et al. 1968), esperin (Thomas and Ito 1969), halobacillin (Trischman et al. 1994), and pumilacidin (Naruse et al. 1990). Surfactin displays antiviral, antimycoplasma, and antibacterial activities (Ongena and Jacques 2008).

The first fengycin was identified by Vanittanakom et al. (1986) and is composed of lipodecapeptides with an internal lactone ring in the peptidic moiety and with a β-hydroxy fatty acid chain that can be saturated or unsaturated (Fig. 2c) (Ongena and Jacques 2008). This family is mainly composed of fengycin A and B (Vanittanakom et al. 1986) and plipastatin A and B (Umezawa et al. 1986). Fengycin mostly displays antimicrobial activity against a range of yeasts and filamentous fungi (Zihalirwa et al. 2017).

This review highlights the recent studies about the ability of LPs to stimulate defense mechanisms of plants and biofilm formation, which is a key factor for the successful biocontrol against organisms that are harmful for plant cultures and a serious economic problem in agriculture worldwide.

Risk of pesticide resistance around the world and significance of LPs on the pesticide market

The current market for agricultural biologicals is around 2.9 billion dollars. Although it represents a great amount of money, it is still dwarfed by the total agrochemical market, which is around 240 billion dollars. Regarding pesticides, the biological product market is about 2 billion dollars annually, while the chemical product market amounts to 44 billion dollars. Even though still in its starting point, microbiome-based products will have a market size comparable to that of agrochemicals in the next few years. In Europe, it is expected that by 2020, there will be more biopesticides than chemical ones (Singh 2017). The biopesticide markets of Europe and South America are the ones projected to grow most rapidly in the next years, driven by tightening regulatory restrictions, and rapidly emerging disease resistance, respectively (Olson 2015).

A great example of losses due to disease resistance is Brazil, one of the largest producers of soybean in the world, contributing around 30% of the total production worldwide. The country faces significant problems with the Asian soybean rust (*Phakopsora phachyrhizi*). The chemical control of this fungus began in Brazil in 2002/03, and the total costs to control the disease are estimated to be around 2 billion dollars per year (Kawashima et al. 2016). Fungicide resistance has been observed in the past few years, and the main reason is the frequent exposure of the pathogen to chemical fungicides (Godoy et al. 2016). The problem of resistance to chemical pesticides is also faced by the rest of the world, with a wide range of diseases which developed pesticide resistance over the decades, such as citrus storage rot, cereal

Table 1 Recent deals on the biopesticide market

Company	Type of deal	Target	Value	Years	References
Bayer CropScience	Acquisition	AgraQuest	$425 M	2012	Kling (2012)
	Partnership	Flagship ventures	Not disclosed	2015	Birkett (2015)
Monsanto	Development partnership	Novozymes	$300 M	2013	Novozymes (2016)
	Start-up founding	Preceres LLC	Not disclosed	2014	Kalaitzandonakes et al. (2018)
BASF	Acquisition	Becker underwood	$1.02 B	2012	Burger et al. (2012)
Syngenta	Acquisition	DevGen	$526 M	2012	Sa'Pinto et al. (2012)
	Acquisition	Pasteuria	$113 M	2012	Thompson (2012)
DuPont	Acquisition	Taxon Biosciences	Not disclosed	2015	Hopkins (2015)
	Development partnership	Hexima	Not disclosed	2014	Hexima (2016)
Platform Specialty Products	Acquisition	Arysta LifeScience	$3.5 B	2014	Chakravorty et al. (2014)
Dow AgroSciences	Development partnership	Radiant genomics	Not disclosed	2015	Dow AgroSciences (2016)
Sumitomo Chemical (Valent BioSciences)	Development partnership	Evolva	Not disclosed	2015	Evolva (2016)

Adapted from Olson (2015)

leaf spot, apple scab, powdery mildews, potato blight, grape downy mildew, cereal powdery mildew, and others (Lucas et al. 2015).

As a consequence of the adversities faced by chemical pesticides, large companies are working on multiple acquisitions, licensing agreements, and partnerships on the agro-biological market (Table 1). The combination of both government agencies and private sectors, including a large number of start-up companies, are also evidence that the use of microbiome products for agriculture will exponentially increase in the near future (Singh 2017).

Recent studies about *Bacillus* LPs and their use in agriculture

Production and recovery

In terms of recent scientific studies, *B. subtilis* is still one of the most used species for LP production among bacteria of the genus *Bacillus* (Gong et al. 2014; Cawoy et al. 2015; Farace et al. 2015). Another species commonly studied is *Bacillus amyloliquefaciens* (Chen et al. 2016b; Soares et al. 2016). Different microorganisms and media that are used for LP production at laboratory scale are shown in Table 2.

Agro-based low-cost products or wastes are being sought as alternative media to minimize the production costs. For example, cassava wastewater was tested in a pilot scale

Table 2 LP production from different *Bacillus* species at laboratory scale

Microorganisms	Medium	Parameters	Inhibition (μg/mL[A], (%)[B], mm[C])	References
Bacillus sp.	Brain heart infusion broth	125 rpm; 42 °C; 48 h	80 mm	Perez et al. (2017)
B. amyloliquefaciens	Nutrient broth	150 rpm; 28 °C; 72 h	MIC 12 μg/mL; 62.7%	Soares et al. (2016)
	Landy medium	200 rpm; 30 °C; 64 h	87.5%	Chen et al. (2016b)
	YPG medium	28 °C; 48 h	MIC 50 μg/mL	Gong et al. (2015)
	40 g/L glucose; 4 g/L NH_4NO_3; 7.098 g/L Na_2HPO_4; 6.805 g/L KH_2PO_4; 0.332 g/L $MgSO_4.H_2O$; 0.0017 g/L $MnSO_4.H_2O$; 0.002 g/L $FeSO_4.7H_2O$; 0.001 g/L $CaCl_2 4H_2O$	1L bioreactor 250 rpm; pH 6.8; 30 °C; 0.8 vvm	17.63 mm	Pretorius et al. (2015)
B. subtilis	60 g/L glucose; 1 g/L yeast extract; 20 g/L $NaNO_3$; 0.333 g/L KH_2PO_4; 1 g/L $Na_2HPO_4.12H_2O$; 0.15 $MgSO_4.7H_2O$; 0.0075 g/L $CaCl_2$; 0.006 g/L $MnSO_4.H_2O$, 0006 g/L $FeSO_4.7H_2O$	200 rpm; 37 °C; 48 h	–	Yang et al. (2015)
	LB medium	130 rpm; 37 °C	35%	Farace et al. (2015)
	Landy medium	180 rpm; 33 °C; 50 h	96.63%	Gong et al. (2014)
	Nutrient broth	150 rpm; 30 °C	45.8%	Guo et al. (2013)
B. megaterium	25 g/L glucose; 6 g /L NH_4NO_3; 0.028 g/L KH_2PO_4; 1.6 g/L K_2HPO_4; 0.3 g/L $MgSO_4$; 0.2 g/L $CaCl_2 \bullet 2H_2O$;	180 rpm; 32 °C	–	Rangarajan et al. (2012)
Bacillus mojavensis	Landy medium	160 rpm; 30 °C; pH 7.0; 72 h	22 mm	Ayed et al. (2014)
Bacillus methylotrophicus	LB mendium and Landy medium	150 rpm; 30 °C; pH 7.0; 72 h	–	Jemil et al. (2017)

[A]*MIC* minimum inhibitory concentration

[B]Percentage inhibited

[C]Diameter of pathogen inhibition

process and proved to be a good substrate for the production of LPs (Barros et al, 2008). Palm oil mill effluent (POME) (Abas et al. 2013), soy flour, molasses (Yánez-Mendizábal et al. 2012a), soybean meal, wheat flour (Song et al. 2013), and desizing wastewater (Li et al. 2011b) have also been used for the production of LPs by *Bacillus* species.

One of the biggest challenges in LP production is the intense foam production during fermentation due to its surfactant nature. This characteristic affects LP recovery and purification and impedes the continuous production of these compounds (Coutte et al. 2013). The use of rotating disk bioreactors can promote a non-foaming fermentation and can be a good alternative to pilot and industrial processes. The use of this type of bioreactors showed a higher yield of LPs when compared to others bubbleless reactors (Chtioui et al. 2012). Other alternatives are the use of a bubbleless membrane bioreactor (Coutte et al. 2010), a biofilm reactor (Zune et al. 2014), and solid-state fermentation reactors (Ano et al. 2009). High foam production was also experienced by Barros et al. (2008), who produced LPs at pilot scale using a 40 L batch pilot bioreactor adapted for simultaneous foam collection.

Extraction of the LPs from the supernatant can be performed by two classic methods. The first method consists of adding ethyl acetate to the cell-free supernatant in a 1:1.1 ratio, with the addition of NaCl (30 g/L). The suspension must be homogenized, followed by the collection of the ethyl acetate fraction, which will be dried in a rotary evaporator (Dimkić et al. 2017). Another approach for the extraction of LPs is acid precipitation, which consists of adjusting the cell-free supernatant to pH 2 using HCl, resulting in a precipitate. Resuspension in methanol will provide an LP extract (Asari et al. 2017; Dimkić et al. 2017). Dimkić et al. (2017) demonstrated that acid precipitation, followed by a methanol extraction, led to a reduction of biosurfactant activities to only 23%, which is in accordance with earlier studies. The authors recommend ethyl acetate, in which the hydrophobic residues of LP compounds are probably better dissolved than in methanol.

After extraction, a finer purification can be executed for further LP characterization. Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) are alternatives of LP purification. Liquid chromatography (LC) and high-pressure liquid chromatography (HPLC) are also extensively employed, especially using a reverse phase. In this case, C18 columns are highly used. For complete identification, mass spectrometry is recommended (Table 3). Figure 3 presents a summary of the main ways to produce, recover, and identify LPs.

LPs and their producers' application in agriculture

Lipopeptides can be used as antimicrobials against a wide range of organisms including bacteria, fungi, oomycetes, and viruses (Raaijmakers et al. 2010). When it comes to important agricultural crops, such as soybean, wheat, maize, and potatoes, there are several studies about the use of LP producers in the biocontrol of pathogens as well as about the direct application of LPs. As mentioned above, *P. pachyrhizi* is one of the greatest challenges in soybean production. The use of *B. subtilis* QST-713 Serenade from Bayer (producer of lipopeptides), coupled with *Bacillus pumilus*, showed a positive effect on soybean exposed to the Asian rust. In particular, *B. subtilis* was able to reduce disease severity by 98.6% in tests with detached leaves and by 23% under field conditions (Dorighello et al. 2015). In wheat, *Bacillus* LP efficacy was observed against *Zymoseptoria tritici* (Mejri et al. 2017), *Gaeumannomyces graminis* var. *tritici* (Zhang et al. 2017; Yang et al. 2018), and *Fusarium graminearum* (Gong et al. 2015). The latter was also inhibited by *Bacillus* LPs in maize (Chan et al. 2009). In potatoes, *Bacillus* biosurfactants were effective against *Fusarium solani* (Mnif

Table 3 Methods of purification and identification of LPs

LPs	Method of identification and quantification	References
Kurskatin, iturin, surfactin and fengycin	HPTLC; MALDI-TOF MS	Dimkić et al. (2017)
Iturin, surfactin and fengycin	HPTLC (silica gel 60 plates—Merck)	Geissler et al. (2017)
Iturin and surfactin	HPLC (Semi-preparative reverse phase C18 HPLC column); MALDI-TOF MS; LC-ESI MS	Pathak and Keharia (2014)
Surfactin and fengycin	HPLC (C18 column)	Coutte et al. (2010)
Surfactin and iturin	TLC (Precoated plates – Merck); LC–ESI–MS/MS (C18 column)	Caldeira et al. (2011)
Iturin and fengycin	Vacuum flash chromatography; semi-preparative HPLC; NMR spectroscopy; HR ESI MS	Ma et al. (2012)
Iturin, surfactin and fengycin	LC–MS (Zorbax 300A Extend C-18 Column)	Ben et al. (2014)
Iturin, surfactin and fengycin	LC–MS (C8 Column)	Malfanova et al. (2012)
Surfactin	HPLC (Luna C18 reversed phase column, equipped with a Luna C18 pre-column)	Pretorius et al. (2015)
Iturin, surfactin and fengycin	MALDI-TOF	Slimene et al. (2012)

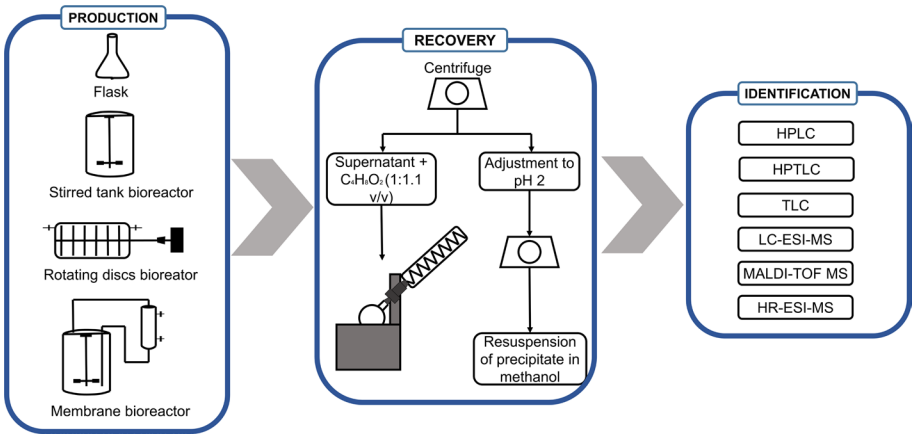

Fig. 3 Different alternatives for the production, recovery, and identification of LPs. The production of these molecules can be performed at smaller scales, such as flasks, as well in different bioreactors (STR, rotating discs, and membrane bioreactor). The recovery is made by two classic methods: (1) acid precipitation, followed by resuspension in precipitate in methanol; (2) addition of ethyl acetate and drying in a rotary evaporator after extraction of the ethyl acetate fraction. Identification can be performed by different analytical methods of chromatography and mass spectrometry

et al. 2015). Different antimicrobial actions of LPs towards different organisms are presented in Table 4.

Induced systemic resistance in plants

Bacillus species promote an enhanced defensive capacity to the plant against a wide spectrum of fungi, bacteria, and viruses; this phenomenon is known as induced systemic resistance (ISR). The defense mechanism may be activated through a similar way as the response against pathogenic microorganisms with incompatible interactions (García-Gutiérrez et al. 2013). Induced systemic resistance is often represented by jasmonic acid/ethylene (JA/ET)-dependent signaling pathways. According to Rahman et al. (2015), although ISR is typically independent of salicylic acid (SA), some rhizobacteria may trigger the SA-dependent signaling pathway. Cyclic LPs are key contributors to ISR-eliciting activity (Rahman et al. 2015). The mechanisms triggered by ISR are related to biochemical changes, including reinforcements of plant cell walls, production of antimicrobial phytoalexins, and synthesis of pathogenesis-related (PR) proteins, such as chitinases, β-1,3-glucanases, or peroxidases (García-Gutiérrez et al. 2013). An ISR scheme can be seen in Fig. 4.

The LPs from *B. amyloliquefaciens* mediated plant defense gene expression against *R. solani* in lettuce. In this case, with the presence of the bacteria in the plant, there was a higher expression of the gene PDF 1.2, which encodes for defensin (host defense peptide). The same response was not observed using surfactin-deficient mutants, showing the important role of surfactin in the ISR (Chowdhury et al. 2015). For grapevine, gene expression analysis suggests that mycosubtilin (iturin family) activated the SA and JA signaling pathways, whereas surfactin mainly induced an SA-regulated pathway. Both LPs were responsible for a local long-lasting enhanced tolerance to the pathogen *B. cinerea* in grapevine leaves (Farace et al. 2015). Surfactin and iturin also played a significant role in the plant defense response of strawberry against *C. gloeosporioides*. The LPs played a major role in the expression of chitinase and β-1,3-glucanase in strawberry leaves (Yamamoto et al. 2015). Other recent studies reported *Bacillus* LPs as inducers of defense responses of several plants including rice (Chandler et al. 2015), *Arabidopsis* (Kawagoe et al. 2015), tomato (Abdallah et al. 2017), and maize (Gond et al. 2015).

Biofilm formation induced by LPs

Colonization of biocontrol microorganisms and their maintenance in the plant area are important factors and major challenges. Changes in environmental conditions, such as temperature and relative humidity, are decisive for the colonization of these organisms. *Bacillus* species evolved a mechanism to overcome these challenges by developing a multicellular behavior known as biofilm formation (Zeriouh et al. 2014). The biofilm structure is initially formed by a process called swarming, which is a rapid and massive migration of cooperating groups of bacteria. Basically, a group of cells forms 'buds' at the edge of the original colony, which are then abruptly released forming initial monolayer dendrites. Surfactin is considerably involved in the swarming process, since in a biofilm structure, it was mainly located in the mother colony and along the edges of the dendrites (Debois et al. 2008). In addition, bacillomycin D, a member of the iturin family, also played a role in the expression of the genes involved in biofilm formation of *B. amyloliquefaciens* (Xu et al. 2013). The biofilm formation

Table 4 Antimicrobial action of LPs against different pathogens

Lipopeptide family	Target organism	Test's condition	References
Fengycin, iturin and surfactin	*Monilinia fructicola*	The treatment was tested on peaches and nectarines in laboratory	Yánez-Mendizábal et al. (2012b)
Fengycin, iturin and surfactin	*Sclerotinia sclerotiorum*	Inhibition tested in petri dishes	Alvarez et al. (2012)
Fengycin, iturin and surfactin	*Phoma medicaginis*	Inhibition tested in petri dish and assay in microculture	Ben Slimene et al. (2012)
Fengycin	*Botrytis cinerea, Sphaerotheca fuliginea*	Inhibition tested in petri dishes	Zhang et al. (2013)
Fengycin, iturin and surfactin	*Fusarium oxysporum* f. sp. melonis	The treatment was applied on muskmelon's nursery and pot soil	Zhao et al. (2013)
Iturin and surfactin	*Xanthomonas arboricola, Pectobacterium carotovorum*, *Colletotrichum acutatum, Colletotrichum gloeosporioides, Monilinia fructigena, Alternaria alternata, F. solani, F. oxysporum, Botryosphaeria obtusa, Penicillium expansum, Mucor sp., Aspergillus flavus*	Inhibition tested in petri dishes and on apple fruits (for *P. expansum* and *F. oxysporum*) under laboratory conditions	Dimkić et al. (2013)
Fengycin, iturin and surfactin	*Pectobacterium carotovorum, Xanthomonas campestris, Podosphaera fusca*	Inhibition tested on detached melon leaves	Zeriouh et al. (2014)
Fengycin	*Rhizoctonia solani*	Inhibition tested in petri dishes	Guo et al. (2013)
Fengycin, iturin and surfactin	*Sclerotium rolfsii, S. sclerotiorum, R. solani, F. solani, Penicillium* spp.	The treatment was tested on white and black common beans under laboratory conditions	Torres et al. (2017)
Non-specified	*P. pachyrhizi*	The treatment was tested on soybeans under greenhouse conditions	Dorighello et al. (2015)
Iturin, fengycin and surfactin	*Z. tritici*	The treatment was tested on wheat under greenhouse conditions	Mejri et al. (2017)
Iturin, fengycin and surfactin	*G. graminis var. tritici*	Inhibition tested in petri dishes and on wheat plants under greenhouse conditions	Yang et al. (2018)
Iturin, fengycin and surfactin	*G. graminis* var. *tritici*	The treatment was tested on wheat under greenhouse conditions	Zhang et al. (2017)
Iturin, fengycin and surfactin	*F. graminearum*	Inhibition tested in petri dishes	Gong et al. (2015)
Fengycin	*F. graminearum*	Field test with maize and culture-chamber with wheat	Chan et al. (2009)
Non-specified	*F. solani*	Inhibition tested in petri dishes and on potato tubers	Mnif et al. (2015)

of *B. subtilis* is less robust in strains with null mutation in the gene *srfAA*, which is responsible for encoding part of the NRPS, consequently forming the surfactin molecule. Biofilm formation in the root helped to increase the local concentration of LP in root-surrounding areas with further stimulation of biofilm formation and antimicrobial action (Chen et al. 2013). The importance of biofilm formation to the success of the biocontrol activity and the role of the surfactin in this process have also been confirmed by other authors such as Aleti et al. (2016), Bais et al. (2004), and Luo et al. (2015).

NRPS engineering

Non-ribosomal peptide synthetases are composed of multi-modules that are responsible to recognize, activate, modify, and link the amino acid intermediates to the product peptide. These multi-enzymes are capable of synthesizing a variety of peptides by adding unusual amino acids, including D-amino acids, β-amino acids, and hydroxy- or *N*-methylated amino acids. The multi-modular property is another feature that leads these enzymes to produce a great variety of products. Each module is composed of specific domains that catalyze different enzymatic activities (Roongsawang et al. 2010). These modules are responsible for incorporating specific amino acids in the molecules and consist of three major

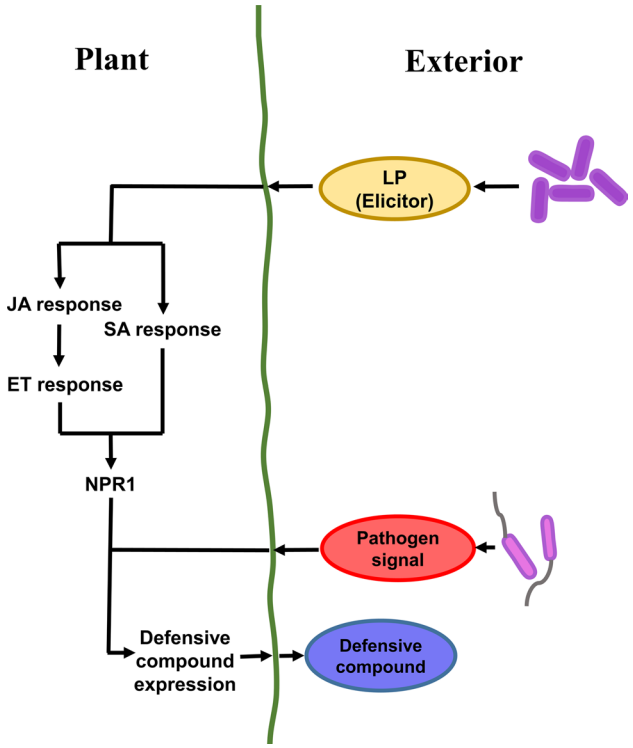

Fig. 4 Systemic resistance induced in plants by LPs. These molecules work as an elicitor, triggering the plant's systemic resistance, by activating a JA/ET response or an SA-dependent pathway. As a result, the plant produces a defensive compound

catalytic domains: condensation (C), adenylation (A), and thiolation (T) domains. Finally, the last module frequently also contains the thioesterase (Te) domain (Gao et al. 2018).

Based on the NRPS's features presented above, studies are being developed on engineering these enzymes to create new LPs. One of the first studies in the field was done by Symmank et al. (2002), who created a lipohexapeptide by genetically engineering the surfactin biosynthesis using a combination of in vitro and in vivo recombination. In their work, a complete amino acid incorporating module was eliminated, creating a modified peptide synthetase. The remaining modules, which are adjacent to the deletion, were recombined at different highly conserved sequence motifs that are characteristic of amino acid-incorporating modules of peptide synthetases. Gao et al. (2018) stated that the entire deletion of an NRPS module, which was a producer of plipastatin, caused the inactivation of the enzyme. However, the authors observed that individual domain deletion (A and T domains) leads to the creation of three novel plipastatin derivatives. Jiang et al. (2016) studied the subunits of the A domain, SrfA-A (responsible for the Glu^1-Leu^2-Leu^3 portion of surfactin) and SrfA-B (responsible for the Val^4-Asp^5-Leu^6 portion of the LP). The authors knocked-out the modules SrfA-A-Leu^3, SrfA-B-Asp, and SrfA-B-Leu from surfactin NRPS in *B. subtilis*, and three novel surfactin products were produced individually, lacking amino acid Leu-3, Asp-5, or Leu-6. Both [ΔLeu^3] surfactin and [ΔLeu^6] surfactin presented reduced toxicity, and [ΔAsp^5] surfactin showed greater inhibition when compared to native surfactin against *B. pumilus* and *Micrococcus luteus*. Also, [ΔLeu^6] surfactin showed a significant antifungal activity against *Fusarium moniliforme*. Liu et al. (2016) were able to create novel LPs by shifting the selectivity of the donor COM domain (communication-mediating domain, essential for coordinating intermolecular communication within NRPSs complexes). Using this technique, and reprogramming the plipastatin biosynthetic machinery, five new LPs were identified. All of the molecules showed antimicrobial activity against five fungal species (*Rhizopus stolonifer, F. oxysporum, Aspergillus ochraceus, Penicillium notatum*, and *A. flavus*). Gao et al. (2016) claim to be the first authors to report truncated cyclic LP production and module skipping by simply moving the TE domain forward in an NRPS system. The authors stated that the plipastatin TE domain could be used to rationally manipulate the ring size of macrocyclic products and could be a potential domain in the engineering of peptide synthetases for generating many new analogues of active peptides.

Studies on engineering of new LP molecules are an important effort to bypass current and future problems of resistance against these molecules. Lipopeptides resistance against a *B. subtilis* surfactin by *Streptomyces* sp. has already been detected. Based on previous study, this species was able to secret an enzyme that could hydrolyse and confer resistance to aerial growth inhibition (Hoefler et al. 2012). The dissemination of the use of these biocontrol agents, along with its produced molecules, could be a triggering agent to new cases of resistance. Therefore, continuous advances and discoveries of new molecules are indispensable for the endurance of this biological technology.

Recent technologies

In terms of recent technologies, major companies such as Bayer and Dupont showed interest in using cyclic LPs as biocontrol agents [e.g. WO2016044529-A1 (Curtis and Thompson 2016), WO2012162412-A2 (Guilhabert-Goya and Margolis 2012), WO2015184170-A1 (Kijlstra et al. 2015), WO2013126387-A2 (Weber et al. 2013)]. In the past 10 years, some technologies related to genetic modified organisms (GMOs), processes, purification and characterization, and formulation have been developed and protected (Table 5).

Regarding new GMOs, the patent WO2017125583-A1 (Lereclus et al. 2017) claims a genetically engineered *Bacillus thuringiensis* capable of overexpressing the *krsE* gene,

Table 5 Different patents on the use of LPs as biocontrol agents

Microrganism	Technology	Targeted plant pathogen	Patent
B.amyloliquefaciens subsp. *plantarum*	An agent for the treatment of microbial diseases in crops with a LP concentration of at least 1 g/L, and a spore content of at least 1×10^{10} spores/g	*R. solani*	WO2012130221-A2 (Borriss 2012)
B. subtilis	Agriculture-grade lipopeptide and potassium fertilizer	–	CN103613434-A (Chen and Qu 2013)
Bacillus spp.	Composition comprising recombinant *Bacillus* cells and lipopeptide	Insects, mites, nematodes and/or phytopathogen	WO2016044529-A1 (Curtis and Thompson 2016)
Bacillus spp.	Synergistic fungicidal combination of a polyene fungicide and at least one lipopeptide	*Penicillium* sp. or *Geotrichum* sp.	WO2012162412-A2 (Guilhabert-Goya and Margolis 2012)
B. amyloliquefaciens	Lipopeptide and nano-titania compound preparation	*Ralstonia solanacearum*	CN105494431-A (He 2015)
B. subtilis	Composition comprising a cell-free fermentation product of one or more *B. subtilis* strains	Bacteria and fungi	WO2013126387-A2 (Weber et al. 2013)
B. subtilis or B. amyloliquefaciens	A fungicidal composition comprising a lipopeptide-producing strain of B. subtilis or *B. amyloliquefaciens* and one of several compounds in a synergistically effective amount	Bacteria and fungi	WO2015184170-A1 (Kijlstra et al. 2015)
B. subtilis	Novel lipopeptides, and engineered polypeptides useful in synthesizing lipopeptides	Insects, bacteria and fungi	WO2008131014-A1 (Keenan et al. 2008)
B. thuringiensis	Genetically modified bacterial strain producing kurstakin	*G. geotrichum, B. cinerea*	WO2017125583-A1 (Lereclus et al. 2017b)
Bacillus spp.	Mutant capable of raising cyclic lipopeptides antibiotic output using RNA polymerase mutation	Wheat powdery mildew, soybean anthracnose, rice sheath blight, *Alternaria* leaf spot, gray mold, peppers *Alternaria* blight and fruit rot	CN102492639-A (Li et al. 2011a)
B. subtilis	Preparation method and usage for novel lipopeptide type biosurfactant surfactin	–	CN101041846-A (Gong et al. 2007)
B. subtilis	A *B. subtilis* strain obtained via mutation breeding and a production method lipopeptide biosurfactant	–	CN103865855-A (Huang 2014)
B. subtilis	A cell culture medium for growing *Bacillus* cells which comprises cellulosic material	–	WO2010039539-A2 (Jarrell et al. 2010)
B. subtilis	A method of semi-solid state fermentation for producing surfactin from a mutant strain of *B. subtilis*	–	WO2016179735-A1 (Lu 2015)
B. licheniformis	A method of producing a lipopeptide against plant pathogenic fungi biocontrol microorganism	*B. cinerea, Botryosphaeria berengriana, F. oxysporum, R. solani, Bipolaris maydis, F. graminearum, F. solani, Verticillium dahlia*	CN104232499-A (Wang et al. 2013)
B. subtilis	Antibacterial lipopeptide of endophytic *B. subtilis* and separation and purification method	–	CN101724014-A (Lin et al. 2009)
B. subtilis	Method for separating and purifying antifungal LP	*R. solani*	CN101851654-A (Liu et al. 2009)

Table 5 (continued)

Microorganism	Technology	Targeted plant pathogen	Patent
Bacillus marinus	The invention provides a new LP compound Maribasin A produced by ocean *Bacillus*	*Alternaria solani, F. oxysporum, Verticillium alboatrum, F. graminearum, Penicillium* sp., *B. cinerea R. solani, Colletotrichum* sp.	CN101838314-A (Tao et al. 2009)

responsible for the production of the LP kurskatin. The molecule showed antimicrobial activity against *Galactomyces geotrichum* and *B. cinerea.* Another molecular technology was developed in the patent CN102492639-A (Li et al. 2011a), which claims a GMO, *Bacillus* spp., with a high yield of antimicrobial LPs (iturin and surfactin) and an antifungal activity increased by 20–60%. Genetic engineering is also applied in the patent WO2008131014-A1 (Keenan et al. 2008); this work alleges an engineered LP synthetase polypeptide useful in synthesizing novel LPs, which are effective against insect or microbial pathogens.

New processes are also being developed, such as in the patent CN101041846-A (Gong et al. 2007), which claims a new pathway for the production of surfactin, using a medium containing soluble starch, sodium nitrate, potassium dihydrogen phosphate, magnesium sulfate, potassium chloride, ferrous sulfate heptahydrate, manganese sulfate, copper sulfate pentahydrate, and yeast extract. For LP purification, the invention employs an acid precipitation at 4 °C by adding hydrochloric acid until pH 2, followed by freeze drying to obtain a purified surfactin preliminary product. The invention CN103865855-A (Huang et al. 2014) also consists of a technology using a different medium composition from standard. For that, the inventors used a mutant *B. subtilis*, able to use glycerol as the main carbon source, for producing LPs. The use of some residues or alternative substrates as carbon source can also be seen in the patent WO2010039539-A2 (Jarrell et al. 2010), which states a medium for growing *Bacillus* cells constituted by cellulosic material as its main carbon source. The cellulosic material is comprised of soybean hulls, which contain cellobiose, xylose, xylan, or a combination thereof. The employment of soybean as substrate in semi-solid state fermentation was developed in the invention WO2016179735-A1 (Lu 2015); the inventors claim a high surfactin yield. The patent CN104232499-A (Wang et al. 2013) claims a production of LPs using *Bacillus licheniformis* in a 10 L bioreactor at 27–40 °C, a pressure of 0.03–0.08 MPa, and an aeration rate of 3–9 L/min.

Different inventions were developed for LP formulation and composition. Seeking to produce an environmentally compatible, storable, and long-acting agent against phytopathogenic microorganisms, the inventors of the patent WO2012130221-A2 (Borriss 2012) developed a product containing *B. amyloliquefaciens* spores. The formula contains at least 1×10^{10} spores/mL and an LP concentration of at least 1 g/L. In the patent WO2016044529-A1 (Curtis and Thompson 2016), the Bayer Company claims a product with a large action range, with the following composition: (1) recombinant exosporium-producing *Bacillus* cells that express a fusion protein comprising at least one plant growth-stimulating protein or peptide as well as a targeting sequence, exosporium protein, or exosporium protein

fragment; (2) at least one biological control agent selected from the group consisting of the following LP-producing strains: *B. subtilis*, *B. amyloliquefaciens*, *Bacillus firmus*, and *B. pumilus* in a synergistically effective amount. Bayer also protected other technologies such as the patents WO2012162412-A2 (Guilhabert-Goya and Margolis 2012) (comprising a synergistic fungicidal combination of a polyene fungicide and at least one *Bacillus* LP) and WO2015184170-A1 (Kijlstra et al. 2015) (comprising a strain of *B. subtilis* or *B. amyloliquefaciens* and one of several compounds in a synergistically effective amount, which includes LPs from the surfactin, iturin, and fengycin families). Dupont is another major company that is interested in *Bacillus* LPs for agricultural application, covered by the patent WO2013126387-A2 (Weber et al. 2013). The invention claims an anti-contaminant composition comprising a cell-free fermentation product of one or more *B. subtilis* strains, containing LPs selected from surfactin, bacillomycin, and fengycin groups and combinations thereof.

Concerning the separation and purification of LP molecules, the extraction method of the patent CN105861602-A (Chen 2016) is based on the classic acid precipitation mentioned earlier in this work. It starts by the preparation of a fermentation broth of *B. subtilis* at 28 °C in a shaker for 72 h. The broth must be then centrifuged, with the pH adjusted to 2.5–3.0, and set at 4 °C overnight. After centrifugation, the precipitate is extracted by methanol (organic solvent), and finally, the solution is centrifuged again for crude LP methanol extraction and concentrated in a rotary evaporator to 1/8 of its original volume. The last step consists of a size-exclusion chromatography using a Sephadex G-100 column for separation to obtain the single antifungal substance. The inventors of the patent CN101851654-A (Liu et al. 2009) also extracted the LPs using methanol. However, RP-HPLC (Column YMC ODS-A 250 mm X 10 mm) was used to prepare and obtain a pure antifungal LP. Another approach can be seen in the patent CN101724014-A (Lin et al. 2009), which uses a 45–55% ammonium sulphate saturation to precipitate these compounds and later purifies the LPs from the iturin, fengycin, and surfactin families through Sephadex G-25 molecular sieve chromatography, cellulose DEAE-52 anion exchange chromatography, and FPLC 300SB-C18 column chromatography successively. The authors allege a distribution range of the molecular weight of the extracellular antibacterial LP between 1000 and 2200 Da.

Conclusions

Different species of *Bacillus* produce LPs with a large spectrum of antimicrobial activity. One of the most used species is *B. subtilis*, a widely adopted bacterial model organism for laboratory studies and one of the most understood prokaryotes in molecular and cellular biology. Therefore, studies relying on LP production by these organisms are provided with a huge literature to base and start a research project. Surfactin, iturin and, fengycin, families of LPs that are produced by *Bacillus*, are strong antimicrobial agents capable of acting synergistically against pathogens in a direct and indirect way by affecting and killing them or by activating an induced systemic resistance and biofilm formation, respectively. These properties of *Bacillus* LPs make them an excellent option for biocontrol systems, which are increasingly being demanded by both tightening regulatory restrictions and rapidly emerging disease resistance. *Bacillus* LPs present an important group of biomolecules that will inexorably contribute to the development of a cleaner and more sustainable agriculture in the next decades.

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