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Review

Bacillus lipopeptides as key players in rhizosphere chemical ecology

Guillaume Balleux[1,4,*], Monica Höfte[2,4], Anthony Arguelles-Arias[1], Magali Deleu[3], and Marc Ongena[1,*]

Microbial natural products are widely explored for their therapeutic potential. Understanding the underlying evolutionary and adaptive forces driving their production remains a fundamental question in biology. Amphiphilic cyclic lipopeptides (CLPs), a prominent category of bacterial specialized metabolites, show strong antimicrobial activity, particularly against phytopathogens. It is thus assumed that these compounds are deployed by soil- or rhizosphere-dwelling bacteria as microbial weapons in competitive natural environments. Here, we challenge this reductionist perspective and present evidence that *Bacillus* CLPs are prominent chemical mediators of ecological interactions. They help *Bacillus* to communicate, compete, defend against predators, or cooperate and establish mutualistic relationships with other (micro)organisms. Additional parallel examples are highlighted in other genera, such as *Pseudomonas*. This broader perspective underscores the need for further investigation into the role of CLPs in shaping the adaptive strategies of key rhizobacterial species.

Highlights

Cyclic lipopeptides (CLPs) represent unique bacterial natural products with an amazing chemical diversity driving multiple potent bioactivities.

More than just bacterial lubricants, solubilizers or killing agents, CLPs retain an unsuspected array of natural functions and feature as private tools or shared goods produced on demand.

Resolving the molecular rules driving CLP selectivity for a given function requires combined expertise in molecular biology, structural chemistry, and biophysics to comprehensively understand their intricate interactions with biological membranes.

Discovering new CLPs and new functions should exploit multilevel microbial interactions and involve recent advances in metagenomics, genome mining, and community metabolomics

Deciphering the ultimate and proximate aspects in the lipopeptide science highlights the relevance of natural products for microbial chemical ecology.

CLPs as key components of the bacterial specialized metabolome

Lipopeptides represent a large group of microbial natural products made of a hydrophobic moiety comprising predominantly fatty acyl units of various lengths, isomery, and functionalization, linked to a hydrophilic peptide portion that is most often cyclized (CLPs). CLP production is widespread among soil-dwelling bacteria with an amazing chemical diversity [1–6]. These amphiphilic compounds exhibit potent antimicrobial, antitumor, and anti-inflammatory activities, sustaining promising medical applications [7,8]. This particularly concerns CLPs with strong antibiotic properties produced by genera such as *Streptomyces*, *Brevibacillus*, or *Serratia*, which have therefore been almost exclusively considered as therapeutic leads against human infections.

However, the complex chemical structure of CLPs makes their physicochemical properties more diverse compared with other peptides. They are therefore likely to have unique bioactivities and functions in natural settings. This has been mainly described for beneficial plant-associated *Bacillus* and *Pseudomonas* species evolving in the **rhizosphere** (see Glossary) [9,10]. *Bacillus* CLPs are key molecular determinants in **biocontrol** due to their antimicrobial and plant defense eliciting activities [11,12]. Historically, research on CLPs has thus mainly been guided by practical concerns to exploit producers as biopesticides in sustainable agriculture. However, in *Bacillus* and other genera such as *Pseudomonas*, the potential to produce CLPs is widely conserved across species, and CLPs represent prominent components of the core **specialized metabolome** (Figure 1A) [13–15]. They are efficiently produced by *Bacillus in vitro* and *in planta*, indicating that cells allocate massive resources for forming these compounds even under nutrient-limited conditions (Figure 1A) [16,17]. This leads to the ultimate question: why do soil- or rhizosphere-dwelling bacteria produce CLPs? By taking a more evolutionary- and **chemical ecology**-oriented point of view, in this review we explore how prominent members of the soil and rhizosphere **microbiome**, such as *Bacillus*,

[1]Microbial Processes and Interactions laboratory, TERRA Research Centre, Gembloux Agro-Bio Tech, University of Liège, Gembloux, 5030, Belgium
[2]Laboratory of Phytopathology, Faculty of Bioscience Engineering, Ghent University, Ghent, 9000, Belgium
[3]Laboratory of Molecular Biophysics at Interfaces, TERRA Research Centre, Gembloux Agro-Bio Tech, University of Liège, Gembloux, 5030, Belgium
[4]These authors made equal contributions

*Correspondence:
guillaume.balleux@uliege.be (G. Balleux) and marc.ongena@uliege.be (M. Ongena).

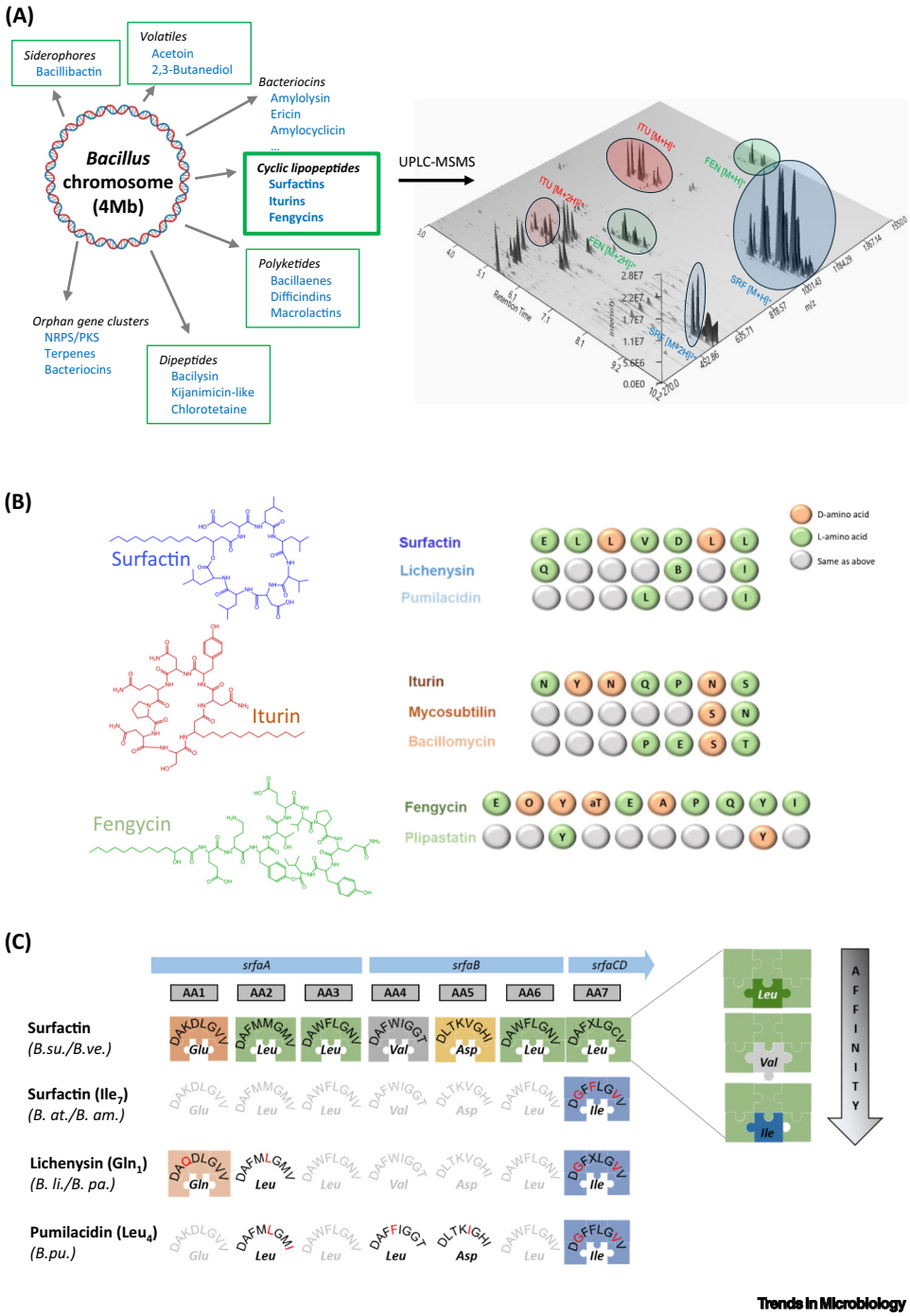


Figure 1. Chemical diversity of cyclic lipopeptides (CLPs) as major components of the *Bacillus* specialized metabolome. (A) Left, diversity of secondary metabolites produced by multiple species of the *Bacillus subtilis* clade including *Bacillus velezensis* [14]. Green boxes: compounds widely conserved across species and referenced as core metabolites. Right, CLPs are efficiently produced by *Bacillus* and correspond to most of the compounds detected upon *in vitro* growth. This is illustrated by the 3D representation of ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS) analysis of crude cell-free extract from *B. velezensis* co-producing

(Figure legend continued at the bottom of the next page.)

Glossary

Biocontrol: the use of living organisms or natural substances to control plant diseases, pests and weeds.

Biofilm: structurally and dynamically complex multicellular systems in which a secreted extracellular matrix holds cells together and provides robustness to the biofilm architecture.

Chemical ecology: a scientific discipline that focuses on the study of the chemical interactions between living organisms in their natural environment. It explores how organisms use and produce chemical compounds with ecological roles and how these chemicals impact the behavior, evolution, and population dynamics of organisms within ecosystems.

Competition sensing: microbes modulate/adapt antibiotic production upon sensing nutrient limitation or cell damage caused by competitors or in response to detection of toxins secreted by the competitors which also warns of imminent danger that does not yet cause harm.

Global regulators (GRs): transcription factors acting on several genes/operons, which primarily regulate developmental traits such as quorum sensing, sporulation, biofilm formation, and abiotic stress sensing.

Induced systemic resistance (ISR): a resistance mechanism in plants that is activated systemically (in all organs) following local interaction with microbes (or their metabolites) and that results in enhanced defense against pathogens and pests.

Lipopeptidome: represents the whole set of CLP-related molecules produced by a single strain in a given condition and includes all variants in all families.

Microbiome: the community of microorganisms living together in a particular habitat. The rhizobiome represents the microbiome associated with the root surface and the rhizosphere. It hosts a complex blend of phylogenetically diverse species that are adapted to, or selected by, the plant.

Nano- or micro-domains: small-scale regions (nm or μm in size) within the membrane that exhibit distinct properties or compositions. These domains contain specific lipids, proteins, and carbohydrates and play crucial roles in organizing cellular membranes and regulating cellular processes.

Quorum sensing (QS): a population-driven signaling system which depends

produce and use CLPs not only to improve their fitness but also to compete and establish mutualistic relationships with other (micro)organisms.

Building on recent insights, and considering their structural diversity, we exemplify the many and unsuspected ecological roles of these chemicals in multitrophic interactions with the host plant and other microbes sharing the natural rhizosphere niche. The focus is mainly on *Bacillus*, but when appropriate we draw parallels with CLPs from other genera to support and expand the role of these molecules in the chemical ecology of other bacterial species.

Multilevel and species-related structural diversity in *Bacillus* CLPs

CLPs are formed via multimodular mega enzymes referred as non-ribosomal peptide synthetases (NRPSs) encoded by cognate biosynthetic gene clusters (BGCs). NRPSs are sophisticated machineries allowing different types of cyclization, and integration of both L and D stereoisomers of a range of polar and non-polar amino acids that can also be non-proteogenic [13]. The genetic sequence and organization of the cognate BGCs determine a specific NRP synthetase that will form a particular type or family of CLP characterized by the fatty acid (FA) structure, the number and type of amino acids, and the mode of cyclization [18]. The three main and best characterized CLP families produced by soil-dwelling *Bacillus* species are iturins, surfactins (both cyclic heptapeptides), and fengycins (decapeptide with an internal cycle of eight amino acids) (Figure 1B) [19]. Two additional families are locillomycins formed by specific strains within the *Bacillus. subtilis* and *Bacillus cereus* clade, and kurstakins isolated from *Bacillus thuringiensis*, a species from the *B. cereus* clade widely used to control insect pests [20,21]. Some other CLPs with distinct peptide compositions – such as bamylocin (heptapeptide) [22] and licheniformin (heptapeptide with internal cycle) [23] – have been identified from species in the *B. subtilis* clade, indicating that new *Bacillus* CLPs still remain to be discovered. However, these latter families do not represent major compounds in the specialized metabolome of the producers, and thus will not be further discussed. A second level of diversity has been described for each of the iturin, fengycin, and surfactin families in which multiple variants differ in amino acids at specific positions (Figure 1B). This diversity primarily results from genetically encoded modifications in binding pockets of the mega enzyme and is mostly species-specific (Figure 1C). At the strain level, and as a third degree of diversity, the lipopeptide chemistry is even more complex, with multiple variants co-produced for each family differing in the length and isomery of the FA chain and/or in the nature of amino acids incorporated at specific positions in the peptide due to the flexibility of some adenylation domains (Figure 1C).

Genome inspection of BGCs via mining tools such as AntiSMASH or PRISM [24] highlights, to some extent, the species specificity of the **lipopeptidome** (Box 1), although the resolution

on local concentrations of specific signaling molecules.
Rhizosphere: the thin layer of soil (few millimeters zone) around the roots that is directly influenced by root-exuded chemicals. The rhizosphere is enriched in microorganisms compared with the bulk soil considering abundance, but has lower species diversity.
Root exudates: the totality of molecules actively or passively released by plant roots into the soil surrounding roots.
Sliding motility: a flagella-independent and cell division-dependent type of bacterial motility.
Social interactions: interactions can either be positive (such as commensalism and mutualism) or negative – such as antagonism and competition, where microbes invest in offensive molecules to gain a competitive advantage.
Specialized metabolome: a group of specialized metabolites, secreted by an organism, which are not required for growth or reproduction but are assumed to retain specialized functions offering specific ecological or physiological advantages to their producers.

the three CLP families (single- and doubly-charged peaks of iturins, fengycins, and surfactins are encircled in red, green, and blue, respectively). The different peaks within each family represent variants with the same peptide moiety but differing in the fatty acid length and/or isomery. (B) Main peptide variants identified within the three CLP families and isomery of their amino acids. D-amino acids are represented as orange balls, and L-amino acids are green. Gray balls imply no variation compared with the main variant of the family (i.e., surfactin, iturin, and fengycin). (C) Illustration of the amino acid sequences of binding pockets in the A-domains of the non-ribosomal peptide synthetases (NRPSs) responsible for surfactin synthesis. Changes in amino acids between species are highlighted in red. Each binding pocket responsible for the selection of the same amino acid is represented in the same color. Binding pockets in gray mean no changes compared with those of *B. velezensis*. In general, this genetically encoded second level of diversity is species related (*B.su., B.ve., B.at., B.am., B.li., B.pa., B.pu* stand for *B. subtilis, B. velezensis, Bacillus atrophaeus, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus paralicheniformis, Bacillus pumilus*, respectively) (see Box 2 in the main text). Besides this interspecies diversity, thanks to the flexibility of some adenylation domains, intraspecies variation can also occur depending on the balance of precursors in the intracellular pool (indirectly due to nutrients) [18]. It happens only at specific positions and always between amino acids of the same 'group' (Val vs Leu or Ile; Asp vs Asn).

does not reach the level 'Tell me which species and I will predict its lipopeptidome'. For instance, plant associates such as *Bacillus velezensis* display the potential to co-produce the three families (Box 1). The lipopeptidome of *Bacillus* species may thus somehow be used as an ID card. This is reminiscent of the situation in *Pseudomonas* where there is a strong correlation between CLP type produced and species diversification [9]. Most rhizosphere-associated *Pseudomonas* strains of the *Pseudomonas putida* and *Pseudomonas fluorescens* groups produce CLP variants that belong to a single family. Rhizosphere-colonizing poly-CLP producers are found within specific subgroups of the *P. fluorescens* group. They typically produce a short-chain CLP (nine amino acids) from the mycin family and a long-chain CLP (19 to 25 amino acids) from the peptin family. In addition, they often produce a third linear or cyclic lipopeptide [25,26]. Besides these well-described genera, most *Burkholderia* CLPs identified so far are produced by members of the *Burkholderia cepacia* complex isolated from the rhizosphere or as plant endophytes [5]. Even if such phylogeny-driven CLP potential is difficult to establish for other genera like *Streptomyces*, it is thus tempting to speculate that the CLP potential is related to specific lifestyle and environmental niche. However, this hypothesis must be taken with care since most CLP-producing species from the *B. subtilis* group and of other genera are ubiquitous in nature.

CLP production occurs at specific growth phases and may be costly for the producer, especially upon growth under nutrient-limited conditions [27]. This implies a tight control of the expression of cognate BGCs, which is mainly governed by **global regulators** (**GRs**) in *Bacillus*. Additional components are also involved, allowing specific regulation of the production of each type of CLP (Box 2). Likewise, in rhizosphere *Pseudomonas,* CLP production is under a hierarchical control involving the two-component GacS/GacA system and LuxR-type proteins in addition to other regulators [26].

Complementary functions for ecological fitness

CLP production is thus widespread and finely controlled in *Bacillus* and other species, which supports the importance of these specialized metabolites in bacterial fitness and adaptation to the environment. This section provides an overview of the various roles of CLPs both for intrinsic development of the producer and as chemical mediators of **social interactions** with other organisms in the rhizosphere.

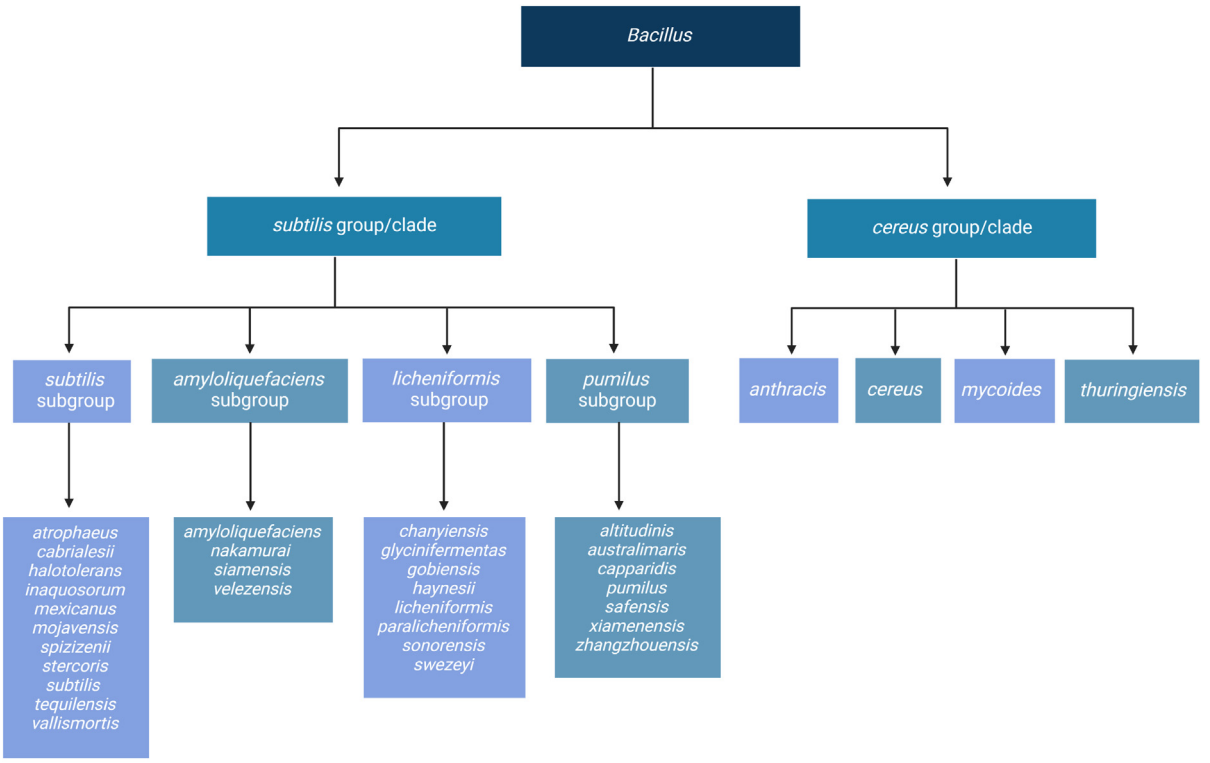
CLPs as facilitators of key developmental traits

Surface motility is a crucial trait for plant-associated bacilli as they must compete for space with a myriad of other microbes in the rhizosphere and on root tissues. By lowering surface tension and

Box 1. *The Bacillus* taxonomy and species-related lipopeptidome

The genus *Bacillus* is currently composed of two phylogenetically unrelated clades or groups, hereafter called the '*subtilis* group' and the '*cereus* group'. Many former *Bacillus* spp. have been moved to other genera. The well-known species *B. thuringiensis* used to control insect pests, the human pathogen *B. anthracis*, and the foodborne pathogen *B. cereus* belong to the *cereus* group, while most plant-associated species that are widely used as biocontrol agents against plant pathogens belong to the *subtilis* group. Four subgroups can be distinguished within the *subtilis* group: *subtilis, amyloliquefaciens, licheniformis*, and *pumilus*, each containing several species (Figure I). The closely related species *Bacillus amyloliquefaciens, B. velezensis*, and *Bacillus siamensis* form the so called 'operational group *B. amyloliquefaciens*'. While *B. amyloliquefaciens* is soil-borne, *B. siamensis* and *B. velezensis* are plant-associated. *B. velezensis* is a taxonomic synonym of *B. amyloliquefaciens* subsp. *plantarum*, *Bacillus methylotrophicus* and *Bacillus oryzicola.* Most *Bacillus* spp. commercialized as biocontrol agents to control plant pathogens are strains of *B. velezensis*, although they are often registered as *B. subtilis* or *B. amyloliquefaciens* [99].

The high number and good quality of *Bacillus* genomes deposited in recent years allows more accurate assignment of the species in the complex taxonomy of this genus. This allows a more reliable correlation with the potential to produce CLPs based on genome inspection. Within the *B. subtilis* species complex, all species retain the potential to form a CLP from the surfactin family, some can also produce a CLP from the fengycin or iturin family, and a limited number can co-produce the three CLP families (Figure I). Some species form one (*Bacillus pumilus*, *Bacillus licheniformis*) or two (*B. subtilis, B. amyloliquefaciens*) types of CLP (specific variants). The plant-associated *B. velezensis* and *B. siamensis*, however, considered as the archetypes of rhizosphere-dwelling and plant-associated species based on specific genetic traits and population-related phenotypes, are best described as co-producers of the three CLP families and thus have the richest lipopeptidome (Figure I, based on [24]).



Species	Fengycin family		Iturin family			Surfactin family		
	Fengycin	Plipastatin	Iturin	Mycosubtilin	Bacillomycin	Surfactin	Lichenisin	Pumilacidin
subtilis	0	59	0	0	0	100	0	0
inaquasorum	0	80	0	0	100	100	0	0
spizizenii	0	0	0	100	0	100	0	0
atrophaeus	0	100	0	100	0	100	0	0
velezensis	97	0	51	0	49	100	0	0
amyloliquefaciens	11	0	79	0	11	100	0	0
siamensis	100	0	70	0	30	100	0	0
(para)licheniformis	0	0	0	0	0	0	100	0
pumilus	0	0	0	0	0	0	0	100

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Figure I. Phylogeny and cyclic lipopeptide (CLP) production in selected species from the *Bacillus subtilis* clade. The table indicates the percentage of producers for each species according to the genomic content in cognate biosynthetic gene clusters (BGCs). Color intensity increases according to the percentage values. Adapted from [24].

reducing friction between producing cells and the solid support, surfactin (and to a lower extent iturin and fengycin) plays an essential role in **sliding motility** as demonstrated via reverse genetics (loss of function in *srfA* knock-out mutants) and chemical complementation (restoration of phenotype by adding pure surfactin) [27–30]. Surfactin also favors flagellar-dependent swarming

by increasing flagella synthesis [31]. High motility allows *Bacillus* cell populations to reach new sites on root surface and enhances rhizosphere colonization. Such a CLP-assisted motility has been reported for other genera including *Burkholderia* [4] and for root colonization by plant-beneficial *Pseudomonas* spp. as demonstrated for amphisin and viscosin [26,32]. Interestingly, motility may also aid in the evasion of competitors. In response to signals emitted by harmful challengers, *Bacillus* stimulates surfactin-dependent motility as an escape mechanism in microbial interactions [33].

Another essential adaptive trait is the formation of robust **biofilms**. Biofilm formation on roots facilitates efficient colonization, contributes to fitness and persistence in the environment and allows *Bacillus* to reach threshold populations necessary to provide beneficial effects on the host plant [34,35]. Surfactin triggers biofilm formation by causing limited and transient membrane permeabilization leading to potassium leakage. This is sensed and integrated by *Bacillus* cells via a phosphorylation cascade to induce the expression of genes responsible for the synthesis of matrix components [28]. In *B. velezensis*, defect in surfactin production results in reduced pellicle-type biofilm formation *in vitro* and a lower root colonization rate [36]. However, this role of surfactin may differ across species, and surfactin-independent biofilm formation on roots has been reported for some undomesticated *B. subtilis* strains [37]. CLPs from the iturin family also display some synergistic effect with surfactin for robust biofilm formation [38]. The relative importance of different CLPs in biofilm formation is strain-dependent and could be directly linked to the ecological niche of the producer. CLP-dependent biofilm can also act as a shield to protect the embedded cell community from toxins or from infiltration by competitors [39,40]. Similarly, *Pseudomonas* CLPs such as massetolide, sessilin, and xantholysin also regulate biofilm development and can either promote or disperse biofilms depending on the CLP type [26].

The role of surfactin in root colonization and competitor evasion may explain why this CLP is the best conserved in soil bacilli. Interestingly, a recent study proposed an additional role for surfactin as facilitator of genetic transformation [41]. Surfactin affects cell membrane permeability in close relatives, which provokes eDNA release from part of the cell population concomitant with enhanced competence [41]. As transformation mediates horizontal gene transfer, surfactin may thus favor acquisition of genetic material from closely or distantly related organisms, a process crucial in bacterial evolution and ecology.

Box 2. Regulation of CLPs in species of the *B. subtilis* group

Two **quorum sensing** (**QS**) systems, ComQXPA and Rap-Phr, and the global regulator Spo0A play a major role in surfactin regulation as described in *B. subtilis*. The *comX* gene encodes a precursor peptide (PP) which is processed and modified by the membrane protein ComQ leading to the mature ComX secreted outside the cell. Accumulation of ComX in the extracellular space is sensed by the histidine kinase ComP leading to phosphorylation of the response regulator ComA (ComA~P) and transcriptional activation. The *phr* genes encode a pre-pro peptide (ppP) which is secreted via the Sec system upon removal of the signaling sequence. pP is then further cleaved to release the mature Phr signaling peptide which re-enters the cell via Opp. Phr stimulates expression of surfactin because it inhibits Rap phosphatases that inactivate ComA~P by dephosphorylation. Optimal production of Phr requires the sigma factor H, which is negatively regulated by AbrB. Phosphorylated Spo0A inhibits AbrB, thus indirectly stimulating Phr expression [100]. Spx is a redox-responsive transcription factor that binds to RNA polymerase and prevents activation by ComA~P. The two heat-shock proteins ClpX and ClpP facilitate ComA~P binding by proteolysis of Spx [101]. CodY competes with RNA polymerase and inhibits transcription of the surfactin operon [102]. Other pleiotropic transcription factors (TFs) are involved but their impacts are less defined [103].

Regulation of iturin and fengycin BGCs is more species-related. Iturin is not synthesized by *B. subtilis*, and less information about the regulation of its operon is available. Some GRs are common with surfactin regulation but do not necessarily act in the same way. ComA~P is indirectly involved by favoring P transfer via DegQ from DegS to DegU, and DegU~P plays a central role as transcriptional activator by binding the promoter region of iturin and fengycin operons, while it represses the biosynthesis of surfactin in *B. amyloliquefaciens* [104,105]. AbrB is the only GR identified so far as a repressor for both iturin and fengycin synthesis, respectively in *B. amyloliquefaciens* and *B. subtilis* [106,107]. Still, there are specificities in the regulation of these two CLPs. In *B. amyloliquefaciens*, GlnR known to control glutamine synthesis, as well as the membrane protein YczE, are involved in iturin regulation either at the transcriptional or post-transcriptional levels [105]. CodY improves bacillomycin production but the mechanism remains unknown [102]. Two specific mechanisms are involved in fengycin regulation: the LutR and SinR proteins in *B. subtilis*, driving various cellular processes at stationary growth phase and the PhoR–PhoP two-component regulatory system in the *subtilis* group, that positively regulates fengycin production in low-phosphate conditions by regulating the synthesis of branched chain amino acids [108,109]. Regulation mechanisms for the three CLPs are illustrated in Figure I.

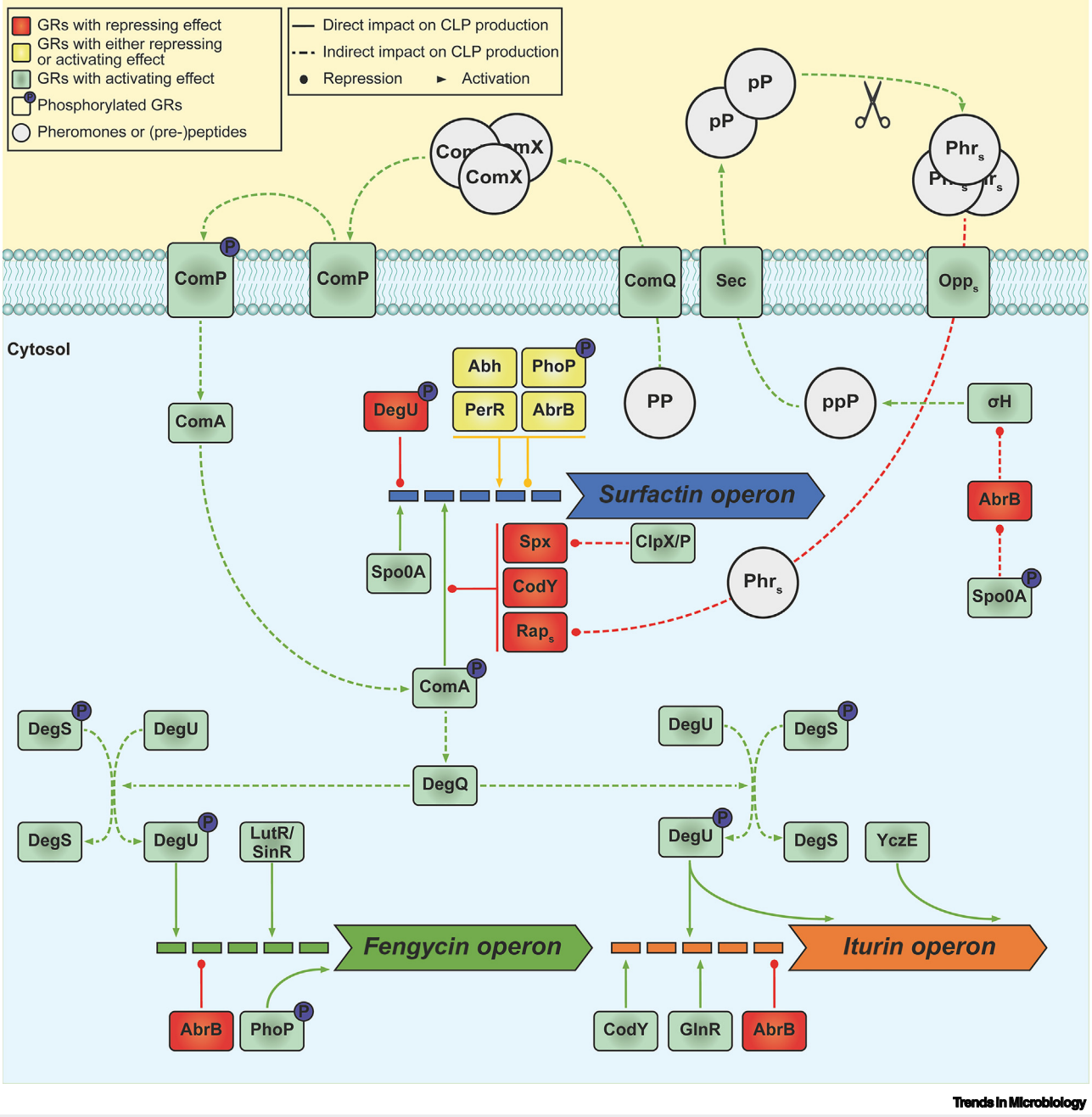

Figure I. *Bacillus* intracellular regulation of lipopeptides. Schematic representation of the influence of global regulators (GRs) on the expression of *Bacillus* main cyclic lipoproteins (CLPs).

CLPs in interactions with competitors and predators

As for *Pseudomonas*, *Burkholderia*, and cyanobacteria, *Bacillus* CLPs have been best described for their antifungal activities. Mainly iturin and fengycin have been reported for their toxicity against

a wide range of soil-borne phytopathogenic fungi and oomycetes. This features these two CLPs as main weapons of the specialized metabolome used by bacilli to inhibit fungal competitors in the rhizosphere [14,19]. From a mechanistic point of view, most antimicrobial activities of CLPs rely on their strong amphiphilic properties allowing prompt insertion into the lipid bilayer of cellular membranes [42,43]. This thermodynamically spontaneous process driven by hydrophobic interactions leads to membrane disruption and cytosolic leakage from target cells [5,6]. Via this mechanism, the development of resistance to lipopeptides is likely to be slow and limited [44]. Beyond this generic mode of action based on pore formation, CLPs display selective bioactivities according to the target microorganisms. Fengycin and iturin are not necessarily toxic for the same fungi, and surfactin is not or poorly antifungal at biologically relevant concentrations (low micromolar range). This reflects the high specificity of CLPs for membranes with precise lipid composition as evidenced via biophysical approaches involving biomimetic membranes [45]. Experimental biophysics also revealed the impact of CLPs on membrane domains and the importance of sterols for CLP–membrane interaction [46,47] but also shed light on the process underpinning ion channel formation by fengycin that requires at least fengycin dimers and the presence of negatively charged lipids [48]. Comparison of the inhibitory potential of CLP homologues in a single family revealed that antifungal activity of CLPs is also impacted by tiny changes in their chemistry (Box 3). In addition, different CLPs co-produced by some species may act in synergy to enhance antimicrobial functions. For instance, the combination of mycosubtilin (iturin variant) with fengycin and/or surfactin improves the control of *Fusarium oxysporum*. Likewise, inhibition of *Botrytis cinerea* by *B. subtilis* isolates is driven by both surfactin and plipastatin (fengycin variant) [15]. Surfactin and fengycin also showed a synergistic antifungal activity on a tebuconazole reduced-sensitivity strain of *Venturia inaequalis* [49].

That said, CLP interference with fungal development may rely on mechanisms other than membrane disruption. Surfactin does not display marked permeabilization activity on fungal membranes [50] but can alter *Aspergillus niger* hyphae morphology and/or impact key processes such as secretory vesicle trafficking, hampering proper cell wall synthesis at the tip of the hyphae [51]. Abnormal fungal cell wall assembly and other effects such as chromatin damage, inhibition of conidial sporulation or suppression of the synthesis of ergosterol and fatty acids, have been described for CLPs produced by other species [5,6,52–54].

Some protozoa share many similarities in their cellular structures with fungi. Accordingly, the fungitoxic keanumycin CLP produced by *Pseudomonas nunensis* also confers protection against predation by social amoeba like *Dictyostelium discoideum* [55]. In addition, keanumycin reduces

Box 3. Structure-dependent activity among CLP variants

Different bioactivities are observed for the different families of CLPs, but for some of the key natural functions there is a clear structure-dependent activity according to the type of variant which is co-produced in a particular family. As a first example, biofilm formation in *Bacillus atrophaeus* and *B. subtilis* was more robust after addition of the cognate surfactin homologue than upon supplementation with a structurally close derivative. The CLP thus seems to act as a species-specific signal molecule, possibly to avoid hijacking by close relatives [110]. Antimicrobial activity based on membrane disruption can also be strongly impacted by minor changes in the structure of a given CLP. This has been recently illustrated for traits like fatty acid chain length and charge by the good correlation between results from antibiotic assays and from liposome permeabilization measured via Time-Correlated Single-photon Counting based on calcein release assays [111,112]. The ISR potential of a given CLP also tightly depends on key structural traits. Among surfactin congeners, only long-chain fatty acid homologues are active at triggering immune-related events in Solanaceae plants, and a single amino acid substitution at the last position in the peptide (Val instead of Leu) is sufficient to significantly decrease the eliciting activity. Moreover, linearized surfactin is fully compromised in its plant immune activation potential [113,114]. Binding experiments via isothermal titration calorimetry and leakage assays performed on plant membrane-mimicking liposomes revealed that long-chain fatty acid homologues have a higher affinity for these vesicles than the short fatty acid forms and display a higher destabilizing effect on the lipid bilayer. This correlated quite well with the contrasting elicitor activities of longer and shorter surfactin homologues [16].

proliferation of the nematode *Oscheius myriophilus*, further supporting an important ecological role of CLPs in defense against predators [56]. Such anti-predator activity has not yet been reported for *Bacillus* CLPs.

CLPs from *Streptomyces* sp., *Pseudomonas* sp., *Brevibacillus* sp., and *Serratia* sp. have been described for their direct antibiotic activity against pathogens, but toxicity towards rhizosphere competitors remains to be further investigated [2]. The antibiotic activity of *Bacillus CLPs* against soil bacteria is poorly documented. Surfactin displays antibacterial activity against human pathogens but at concentrations much higher than those possibly occurring in natural settings. Antibacterial properties of iturin or fengycin have been occasionally reported [57] but globally, the real involvement of *Bacillus* CLPs as killing agents in interbacterial antagonism in the rhizosphere can be questioned [58]. However, surfactin inhibits the growth of bacterial competitors via mechanisms other than direct toxicity – such as interference with the formation of aerial mycelium in *Streptomyces* [59], inhibition of multicellular development of *Pseudomonas syringae* and *Ralstonia solanacearum* [60,61], or inactivation of the heat shock protein Hsp90 with ATPase activity as key cellular function in cyanobacteria [62]. Surfactin also antagonizes closely related species, in synergy with cannibalism toxins [63]. As an additional illustration of indirect interference mediated by CLPs, orfamide A secreted by *Pseudomonas protegens*, triggers a Ca^{2+} signal causing rapid deflagellation of the soil-dwelling microalga *Chlamydomonas reinhardtii*. This CLP can thus be viewed as a toxin causing immobilization and preventing the algae from escaping bacterial attack [64].

Interestingly, new roles for *Bacillus* CLPs in counteracting the toxicity of compounds emitted by competitors have been reported. Surfactin acts as a chemical shield that inactivates toxic CLPs secreted by *Pseudomonas* via coaggregation into insoluble complexes [33]. Also, surfactin disrupts extracellular vesicles isolated from its own producer *B. subtilis* and, interestingly, from other species. One may thus assume that targeted lysis of antibiotic-laden vesicles by surfactin could serve as a defensive mechanism against competing organisms [65,66].

CLPs in commensal/mutualistic interactions

Bacilli also interact positively with other bacteria for beneficial outcomes. In the context of commensal interactions, surfactin secreted by *B. subtilis* is perceived by *Paenibacillus dendritiformis* to increase motility and space invasion. Surfactin may thus be used as signal sent out by *Bacillus* to recruit other bacteria to its ecological niche and establish favorable mixed colonies [67]. In interaction between *B. subtilis* and *Pseudomonas chlororaphis*, surfactin promotes colony spread of *Pseudomonas* and may act as facilitator of interspecies interaction, with potentially positive outcome leading to coexistence and/or cooperation *in planta* [68]. Bacilli also associate in a mutualistic interaction with arbuscular mycorrhizal fungi with shared benefits [69]. Little is known about the molecular basis driving cross-talk between the two microbes but here again, the surfactin lipopeptide plays a role as signal to boost fungal vitality [70].

Mutualism is also the basis of the interactions between plant-associated *Bacillus* species and their host. In return for nutrients and physical support provided by the plant, the bacterium helps its host to grow and protects it against pathogens. A role in plant growth promotion has been recently reported for fengycin on melon, cucumber, and soybean [71]. The CLP mediates not only a short-term effect on radicle growth by causing the disaggregation of seed oil bodies and mobilization of their fatty acid content, but also a long-term growth promotion of adult plants associated with the accumulation of specific lipid molecules and antioxidants [72]. In addition, CLPs secreted by plant-beneficial bacilli have emerged as an important category of elicitors of plant immune activation, which ultimately leads to **induced systemic resistance** (**ISR**) against

various pathogens [71,73]. Surfactin is the best described CLP for that function and induces ISR in several dicot plant species [71]. Binding experiments via isothermal titration calorimetry and leakage assays based on the release of fluorescent probe revealed that surfactin perception by plant cells relies on direct interaction with sphingolipid-enriched domains in the plasma membrane but not through a process leading to permeabilization and pore formation [16,71]. ISR stimulation has also been occasionally reported for fengycin and iturin in different pathosystems [71] as well as for *Pseudomonas* CLPs such as orfamide, massetolide, WLIP, entolysin, and tolaasin [74,75]. Iturin and fengycin were also reported to act in synergy for ISR elicitation [76]. Various CLP families with different sizes, cyclization and amphiphilic character can thus act as triggers of plant immunity. Immune activation efficiency may also differ widely among the multiple fatty acid homologues or peptide variants co-produced within a given CLP family (Box 3). In addition, the elicitor activity of a particular CLP also depends on the host plant species, again illustrating the importance but also the complexity of CLP-membrane interactions at the molecular level (Box 4). Indeed, surfactin is the best trigger of plant immunity in various dicots, but is not active on monocots while iturin was reported as elicitor for ISR elicitation in wheat and in rice [76,77].

CLPs as common goods exploited by other microbes

Due to cyclization and alternation of L- and D-amino acids in the peptide, CLPs have long been viewed as chemicals unlikely to be degraded by other microbes. However, some competitors may secrete the enzymatic arsenal necessary for breaking down these compounds. *Streptomyces* sp. can degrade iturin, fengycin, and surfactin as well as a range of *Pseudomonas* CLPs. Degradation occurs to different extents and according to specific mechanisms depending on the CLP [78,79]. Enzymatic linearization of surfactin is deployed by *Streptomyces* as a detoxification mechanism to counteract the inhibitory effect of the CLP on aerial mycelium formation [78]. Moreover, complete breakdown of the three *Bacillus* CLP families occurs in interaction with other *Streptomyces* sp. expressing the proper enzymatic equipment. The free amino acids released are used to sustain growth

Box 4. Unveiling the physicochemical rules driving CLP–membrane interactions

Further insights into the molecular basis of CLP interactions with membranes at the supramolecular, molecular, and atomic levels are necessary if we want to better comprehend and even predict the functional selectivity of CLPs. Small structural variations in one type of CLP can drastically impact its functions. Such apparently minuscule changes may modify the 3D conformation of the CLP molecule and/or significantly influence its polarity and amphipathic character with strong impact on membrane interaction [13,42]. In addition, CLP antimicrobial bioactivities and ISR potential clearly differ according to the target species with specific lipid compositions and organizations in their cell membrane. CLPs are known to interact with sterols and sphingolipids, and some CLP–membrane interactions depend on the intrinsic membrane lipid organization in terms of **nano- or micro-domain** size and distribution [115,116]. Further investigation requires combining biological assays with other approaches such as solid-state nuclear magnetic resonance (NMR) spectroscopy to study the spatial conformation of a CLP and its orientation in simple membrane mimics such as mono-lipid micelles or bicelles [117]. Experimental biophysical approaches using biomimetic membranes allow quantification of CLP binding affinity to specific lipid membranes and/or to determine their effects on fluidity, integrity, and permeabilization, taking into account the importance of membrane domains and asymmetry [45,118]. However, for optimal use of biophysics, a better knowledge on the specific lipid content of native cell membranes is mandatory to generate the most relevant biomimicking artificial membrane systems [119]. Tremendous progress is being made on those aspects thanks to advanced lipidomics [120] and *in silico* modeling [121]. *In silico* biophysics using docking methods and molecular dynamics can be exploited to determine the affinity of CLPs for individual lipids at the resolution of single molecules, to dissect the behavior of a given CLP in terms of aggregation and insertion into defined bilayers [122], or to predict the impact of CLP on membrane deformation, curvature or pore formation.

Co-produced CLPs also work in synergy to boost some key functions but the molecular basis of such synergistic effect is poorly known. Different CLPs may tightly interact with each other resulting in a higher membrane activity (perturbation or disruption) or each CLP retains a distinct mechanism of action which is reinforced/triggered by the effect of the other CLP. For example, one CLP creates pores and facilitates transport of the other CLP across the cell membrane acting on intracellular targets. Experimental and *in silico* biophysics can also help to determine the molecular basis of the synergistic effects observed between co-produced CLPs by analyzing the formation of specific CLP assemblies (in terms of molar proportions).

in a foraging strategy [79]. Such biotransformation of CLPs leads to loss of functions that may impact negatively competitiveness of the producing strain. As an example, degradation of iturin by *Streptomyces* generates products that are no longer fungitoxic [79]. *Mycetocola* spp. detoxify tolaasin produced by the mushroom pathogen *Pseudomonas tolaasii* via linearization and destroys the swarming factor pseudodesmin, thereby restricting *Pseudomonas* motility [80].

However, degradation of CLPs may generate new products potentially displaying unsuspected bioactivities. Degradation of the *Pseudomonas* lipopeptide syringafactin by *Paenibacillus* leads to products toxic to their common amoeba predators [81]. Surfactin is also actively degraded by *Paenibacillus*. The resulting lipopeptidic tail product better acts as deterrent while integral surfactin facilitates motility of *P. dendritiformis* colonies towards *B. subtilis* [67]. Some *Pseudomonas* CLPs such as orfamide and lokisin are biotransformed by other species and serve as scaffolds to generate unique metabolites in interaction with more complex microbial communities [82,83]. CLP remodeling or destruction may be detrimental for the fitness of the producers since both developmental traits and competitiveness rely on native conformation.

Modulation of lipopeptidome expression upon interactions

New insights into the ecological functions of microbial natural products may also come from understanding how the producer responds to exogenous signals from its biotic environment. In that context, *Bacillus* CLP production can be modulated upon perception of cues from the host or other microbes sharing the niche. Growth in **root exudates** impacts the production of surfactin both quantitatively [11,84] and qualitatively with changes in the relative proportions of variants [16]. Upon contact-dependent interaction, perception of plant cell wall polysaccharides such as homogalacturonan, xylan, and arabinogalactan stimulate production of surfactin in *B. velezensis* and *B. subtilis* [16,36,85]. This correlates with the stimulation of differentiation programs leading to biofilm formation, motility, and sporulation, indicating that these *Bacillus* species may coordinate phenotypical traits and CLP production to maximize root colonization [36]. In a different context, but also reflecting modulation of CLPs upon plant perception, the production of mycin and peptin-type CLPs by *P. syringae* leaf pathogens is triggered by plant signals such as the phenolic glucoside arbutin and sugars that are abundant in leaf tissues such as D-fructose [86].

However, microbial interactions are considered the main factors affecting the production of specialized metabolites [87]. Accordingly, enhanced biosynthesis of the antifungal iturin and fengycin CLPs has been reported in response to a range of phytopathogenic fungal and oomycete species. *Bacillus* may sense the presence of fungi in a species-specific way and reacts by overproducing the most active antifungal CLP [88,89]. A recent experimental evolution study also revealed that interaction with *A. niger* leads to the emergence of *Bacillus* cell lineages displaying enhanced surfactin production [51]. This illustrates another aspect of CLP boost as adaptative response facilitating spreading behavior of *Bacillus* upon microbial interaction. Interestingly, upon co-cultivation with nonpathogenic fungi such as *Trichoderma* sp. or arbuscular mycorrhizae, CLP production is dampened, most likely to favor mutualistic relationships [70,90]. Surfactin and plipastatin productions are also strongly reduced in *B. subtilis* upon interaction with the soil fungal pathogen *Setophoma terrestris*. This occurs in rapidly adapting cells as part of a global genetically stable phenotypic variation [91]. Explaining the specificity of the response is difficult since our knowledge about the chemical dialogue between *Bacillus* and fungi is limited. No diffusible signal actively secreted by fungi and sensed by *Bacillus* has been conclusively identified so far even if exosmotic glycerol from *Fusarium* triggers fengycin production. Enniatin peptides, lateropyrone, and fusaric acid from the same fungus may be other good candidates for triggering some **competition sensing**-associated response at subinhibitory concentrations [92].

Few papers report changes in lipopeptidome expression upon interaction with other bacteria. *Bacillus* can boost biofilm formation and motility upon sensing siderophores or antibiotics from competitors but this has not been associated with enhanced CLP production [93,94]. In response to *R. solanacearum* and *Pseudomonas fuscovaginae*, *Bacillus* increases expression of surfactin and iturin BGCs but this was not correlated with higher amounts of these CLPs [95]. *B. velezensis* mobilizes a substantial part of its bioactive secondary metabolome by sensing the siderophore pyochelin produced by its *Pseudomonas* competitor [17]. This includes stimulation of surfactin, which contributes to motility and biofilm formation, possibly favoring rhizosphere fitness. It is worth noticing that, in this study, CLPs with antifungal properties such as iturin or fengycin are not stimulated upon interaction with *Pseudomonas*. *B. velezensis* thus induces a subset of its chemical weapon arsenal according to the nature of the microbial challenger, probably by relying on differential regulation of the three CLP families. However, it is not known how signal perception at the cell surface is integrated intracellularly via the GR regulatory network to lead to enhanced or repressed BGC expression.

Nevertheless, it is thus clear that some *Bacillus* species have developed detection systems to eavesdrop on their biotic environment and may respond appropriately by modulating lipopeptidome expression. Some specificity of the CLP response has also been reported in other bacteria. Production of antimicrobial mycin- and peptin CLPs in *P. nunensis* is not triggered by plant signals but by fungal extracts and fungal associated molecules such as trehalose and glycerol [96,97]. It illustrates the concept of CLP production on demand where these metabolites can be boosted or diminished when needed for the benefit of the bacterium and according to the interacting organism. By identifying the nature of the (micro)organism and the chemical signaling that modulate CLP production in *Bacillus*, we may anticipate why these compounds are involved in the interaction and deduce new functions.

Concluding remarks

The diversity of CLP natural functions extends far beyond their role as chemical weapons for microbial warfare and highlight these natural products as prominent mediators of ecological interactions (Figure 2, Key figure). Reminiscent of their importance, these costly metabolites can be produced on demand in response to exogenous cues. We anticipate that more CLP-triggering signals or organisms will be discovered in the near future along with more insights into the molecular basis of their perception (see Outstanding questions). This provides a foundation that should encourage us to further investigate the biology of CLPs to reinforce their relevance in the adaptation to the specific lifestyle of the producer. It applies to *Bacillus* species secreting different CLPs with complementary functions but also to other soil-dwelling bacterial genera known to produce this type of compound (see Outstanding questions).

CLPs are microbial natural products displaying a remarkable chemical diversity and this chemistry drastically impacts key functionalities relying on interaction with membranes. Many studies have reported structure/activity relationships but most are essentially descriptive with few or no insights into the mechanism of membrane activity. Combinatorial studies testing a large catalog of structural derivatives for a given function are needed, and to that end, the natural flexibility of NRPSs, synthetic biology, and chemical synthesis can be exploited [53,98]. That said, resolving the intricate physicochemical rules driving CLP–membrane interactions is extremely challenging since they tightly depend on lipid composition and nano- and micro-domain organization of the membranes. This should be tackled via an interdisciplinary approach integrating expertise in CLP structural characterization, membrane chemistry, and lipidomics supported by multiscale biophysical approaches (Figure 2). This is necessary for predicting functional selectivity of known or new CLPs or to unravel the mechanism(s) driving synergistic activities between known families (see Outstanding questions).

Outstanding questions

A wide range of bioactivities have been described but what are the (physico-)chemical rules determining the specificity of CLP-biological membrane interactions and, therefore, the CLP selectivity for natural functions?

CLPs act as triggers of plant immunity in a structure-dependent and plant species-dependent processes. What are the molecular mechanisms underpinning recognition of CLPs as immunogenic elicitors by plant cells?

The antimicrobial activity of CLPs has been mainly reported against phytopathogens, but what about other epiphytic non-pathogenic microbes sharing the niche?

CLPs' roles in interactions with other (micro)organisms have been mostly studied so far in pairwise co-culture settings but their effect at the community level is not known. What is the importance of CLPs for *Bacillus* competitiveness within the rhizosphere microbiome, and do CLPs impact the composition and dynamics of this rhizobiome?

CLP production can be modulated upon interkingdom and interspecies interactions but our knowledge remains quite limited about the nature of the external signals identified so far. How diverse is the panoply of exogenous triggers (molecules, organisms) impacting lipopeptidome expression?

Most studies have investigated the cellular regulation of CLP synthesis in *B. subtilis* but rules established for this model species may not strictly apply in other species. How does the GR network operate to control and fine-tune CLP expression in less-studied bacilli such as *B. velezensis* co-producing multiple families? Is it possible that changes could result from adaptation to the rhizosphere niche, driven by minute mutations that impact GR functioning and could 'rewire' the regulation network?

To some extent, it seems that the type and diversity of CLPs formed by a given species can be correlated with the

lifestyle and biocontrol potential of that species, but can the lipopeptidome be used as a strong and relevant taxonomic indicator for *Bacillus*?

Key figure

Natural functions of cyclic lipopeptides (CLPs) in the rhizosphere context, highlighting their role as mediators of multitrophic interactions with the host and other soil (micro)organisms

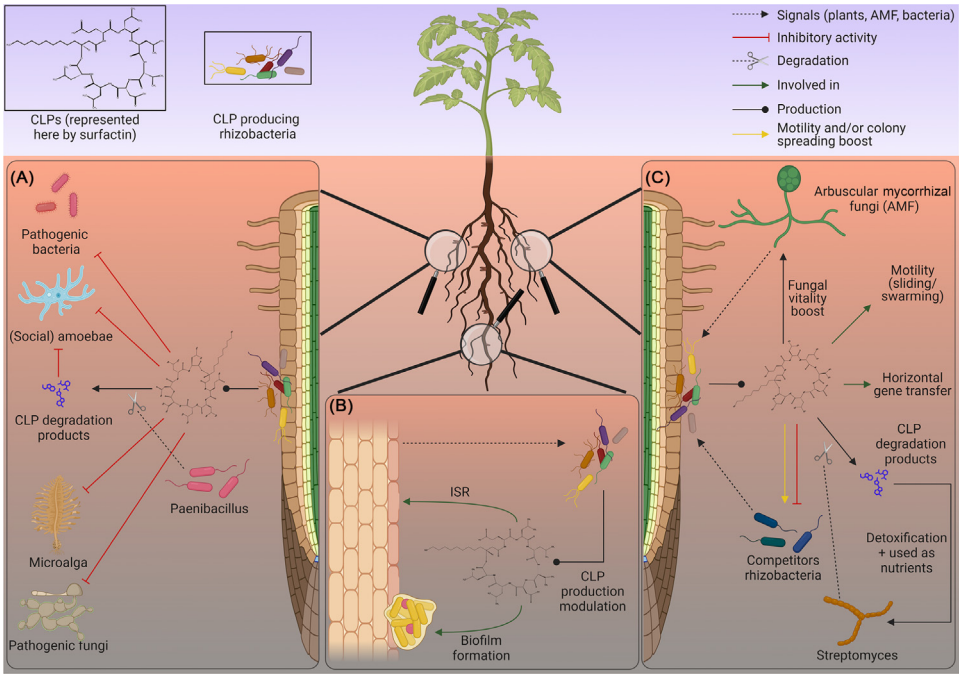

Figure 2. The figure is divided into three sections, each depicting relationships and interactions facilitated by CLPs. (A) Antagonistic effects of CLPs. Pathogenic bacteria, (social) amoebae, microalgae, and pathogenic fungi can be suppressed by CLPs, protecting plant roots from diseases through direct antagonism. In the special case of amoebae, biotransformed CLPs can also be toxic. (B) CLPs in rhizobacteria–plant interactions. The perception of plant cell wall polymers and exudates triggers the production of CLPs, which act as elicitors of host immunity and induce systemic resistance against pathogen infection. CLPs also contribute to biofilm formation on root surfaces, enhancing microbial colonization and plant protection. (C) CLPs involved in social interactions. CLPs that may be stimulated in response to exogenous cues mediate commensal or mutualistic interactions with soil microorganisms such as arbuscular mycorrhizal fungi. Besides their roles in horizontal gene transfer and motility, CLPs can be biotransformed and further used by some *Streptomyces* species to sustain growth. Some signals modulating CLP synthesis have been identified but others still remain to be discovered. Figure created with BioRender.com. Abbreviation: ISR, induced systemic resistance.

CLPs may also be conceptualized as shared goods not solely restricted to host plant immunity reinforcement, but also for the sustainability of key bacterial processes such as moving or foraging. However, further studies are necessary to better appreciate to what extent CLPs can be structurally biotransformed and functionally repurposed upon interspecies interactions in complex communities (see Outstanding questions). In that context, our current knowledge on CLP functions and fate in ecological interactions almost exclusively relies on the study of pairwise interactions. Translating the outcomes of these simple interactions to more complex community settings is challenging but necessary. It should also provide valuable insights into the persistence and fitness of strong producers.

Additional knowledge of the natural functions of key metabolites such as CLPs is needed to better understand the chemical ecology of keystone rhizosphere bacteria such as *Bacillus*. The ultimate objective for scientists is to harness these insights for practical purposes, such as developing environmentally friendly plant-disease management strategies or discovering new (phyto)pharmaceutical hits.

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Declaration of interests

The authors declare no competing interests.

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