Frenemies of the soil: Bacillus and Pseudomonas interspecies interactions

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Review

Frenemies of the soil: *Bacillus* and *Pseudomonas* interspecies interactions

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Bacillus **and** ***Pseudomonas*** **ubiquitously occur in natural environments and are two of the most intensively studied bacterial genera in the soil. They are often coisolated from environmental samples, and as a result, several studies have experimentally cocultured bacilli and pseudomonads to obtain emergent properties. Even so, the general interaction between members of these genera is virtually unknown. In the past decade, data on interspecies interactions between natural isolates of** ***Bacillus*** **and** ***Pseudomonas*** **has become more detailed, and now, molecular studies permit mapping of the mechanisms behind their pairwise ecology. This review addresses the current knowledge about microbe–microbe interactions between strains of** ***Bacillus*** **and** ***Pseudomonas*** **and discusses how we can attempt to generalize the interaction on a taxonomic and molecular level.**

Highlights

Outcomes of pairwise interactions between bacilli and pseudomonads do not correlate with species-level taxonomy.

Most interaction pairs of *Bacillus* and *Pseudomonas* engage in net negative interactions that stabilize into coexistence.

Strains from both genera are ubiquitous and often coisolated from soil. Understanding why this is the case may translate to other microbial interactions and deepen our insight into microbial ecology in general.

Bacillus and *Pseudomonas* in microbial ecology

Most of the biomass of the earth is made up of microbial life that drives countless natural and biotechnological processes [1]. The environmental distribution of microbes is determined by microbial ecology, that is, the **ecological interaction** (see Glossary) of microbes with their environment, plants, animals, and each other. For a long time, intermicrobial relations were neglected in favor of understanding how individual microbes affected their hosts and surrounding environment. This has been most common for plant–microbe interactions, in which the bacterial genera *Bacillus* and *Pseudomonas* are heavily represented. While the ability of both genera to suppress plant pathogens was appreciated, little attention was paid to how they affect the rest of the microbiome. Additionally, although both genera are environmentally ubiquitous and often coisolated [2–7], it remains ambiguous whether the two genera generally engage in positive or negative interactions. In the past few decades, this problem has come closer to being solved, and not only do we now have genomic, transcriptional, and metabolic information on the interspecies interactions of many bacilli and pseudomonads, but studies are also starting to map the molecular mechanisms behind intermicrobial interactions.

Therefore, the aim of this review is to provide an overview of the current knowledge of interspecies interactions between *Bacillus* spp. and *Pseudomonas* spp. pairwise and in more complex communities and environments with an emphasis on phylogeny and molecular mechanisms.

Most reported *Pseudomonas–Bacillus* interactions represent competition

The environmental ubiquity of the *Pseudomonas* and *Bacillus* genera provides natural opportunities for individual strains to come into contact and coexist [2–4]. The fact that *Pseudomonas* and *Bacillus* are often coisolated suggests that species of these genera generally settle into a stable coexistence (i.e., they are, in principle, compatible) [5–7]. Indeed, interactions between members of the two genera range from mutualism to competition depending on media, bioactive compound potential, and – to a lesser extent – phylogeny (Figure 1). However, the majority of the currently reported cocultures of bacilli and pseudomonads interacting directly with each other

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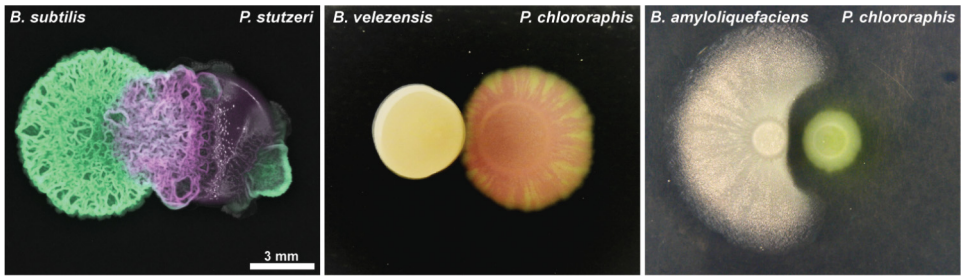

Figure 1. *Bacillus–Pseudomonas* interactions range from mutualism to competition. The pairwise ecology of bacilli and pseudomonads is highly dependent on media and secreted molecules and less dependent on phylogeny. Left: mutualistic coculture with *Bacillus subtilis* 3610 (green) spotted next to *Pseudomonas stutzeri* XL272 (magenta) on tryptic soy agar and incubated at 37°C for 72 h. Scale bar: 3 mm. Center: neutral coculture with *Bacillus velezensis* GA1 spotted next to *Pseudomonas chlororaphis* JV497 on casamino acid agar and incubated at 30°C for 24 h. Adapted from [25]. Right: competitive coculture with *Bacillus amyloliquefaciens* FZB42 spotted next to *P. chlororaphis* PCL1606 on King's B agar and incubated at 30°C for 24 h. Adapted from [22].

reveal either competition or amensalism mediated by bioactive natural products (Table 1; see Box 1 for an overview of how interactions are studied). It is important to recognize that the vast majority of published interactions between bacilli and pseudomonads are from cocultures of isolates originating from plants (either the phyllosphere or rhizosphere) and soil (Table 2). Pairwise interactions with microbes from these environments generally follow the distribution obtained by Kehe *et al*. (2021) [8], although they similarly cocultured soil isolates and not isolates from other niches. Although, the number of *Bacillus–Pseudomonas* interactions is sparse, interactions with pairs from other niches do not follow a similar distribution, possibly due to the diverse levels of nutrient availability in other niches. Still, negative interactions seem to be common when coculturing bacterial species in pairs [8,9], but the debate is currently heavy on whether these findings fail to reflect natural systems which are often much more complex than cultivations in the laboratory. Natural environments harbor multitudes of species, all engaging in a vast network of higher-order interactions [10]. Indeed, what may seem like a negative interaction in a pairwise system, can equilibrate into a competitive balance where numerous species coexist [11,12] or when intraspecific competition is greater than interspecific competition [13], though it remains to be experimentally proven if that is the case for *Bacillus–Pseudomonas* interactions as well. An example of stable competition is observed in the case of the THOR tri-species **synthetic community** (**SynCom**), where a *Bacillus cereus* isolate was found to contain the 'hitchhikers' *Pseudomonas koreensis* and *Flavobacterium johnsonii* [7]. This community synergistically increases biofilm formation when all three species are present, even though *P. koreensis* chemically restricts the growth of *F. johnsonii*, as *B. cereus* limits the concentration of the koreenceine antibiotic from *P. koreensis*. Intriguingly, the interactions in this SynCom are highly temperature-sensitive; a seemingly positive effect at one temperature becomes negative at a different temperature [14].

Studies reporting a net negative interaction between *Bacillus* and *Pseudomonas* therefore might potentially conceal a coexistence when abundances equilibrate in the long term. In addition to this complexity, interaction outcomes seem to lack conservation at the species level, resulting in interplays between pseudomonads and bacilli as diverse as the genera themselves (Table 1). Rather, interactions associated with the metabolomic arsenal of the interacting strains and strains of identical species, but with differing natural product potential, can yield distinct interaction outcomes [15]. This arsenal is mostly composed of bioactive secondary metabolites, but also by other – less obvious – molecules, proteins, and pseudo-organelles. Only some of these molecules

Glossary

2,4-diacetylphloroglucinol (DAPG): a plant-growth-promoting polyketide produced by certain pseudomonads of the *P. fluorescens* group.
Autoinducer II (AI2): a signaling molecule involved in quorum sensing.
Cyclic lipopeptides (CLPs): a group of nonribosomal peptides often exhibiting bioactivity.
Ecological interactions: relationships between pairs of organisms are described by the effect of the relationship on each partner; that is, positive (+), neutral (0), or negative (–). Ecological interactions are divided into mutualism (+/+), competition (–/–), predation/parasitism (+/–), commensalism (+/0), and amensalism (–/0).
Elongation factor G (FusA): a prokaryotic elongation factor involved in protein translation.
***N*-acylhomoserine lactone (AHL):** a signaling molecule involved in quorum sensing.
Plant-growth-promoting rhizobacterium (PGPR): a bacterial strain associated with plant growth promotion.
***Pseudomonas* quionolone signal (PQS):** a *Pseudomonas*-specific quorum sensing system.
Quorum sensing: a bacterial signaling system dependent on local signaling molecule concentrations.
Synthetic community (SynCom): a combined population of microbial species, the composition of which has been designed synthetically.
Type VI secretion system (T6SS): a needle-like pseudo-organelle used by some bacterial strains to inject toxins into competitors.
White-line-inducing principle (WLIP): a CLP encoded by some *Pseudomonas* spp.

Table 1. Outcome of pairwise cocultures with *Bacillus* and *Pseudomonas*[a]

Net Interaction	Direction	*Bacillus* sp.	*Pseudomonas* sp.	Effector	Refs
Negative	Amensalism against *Bacillus*	*B. cereus*	*Pseudomonas.* sp.	Unknown antagonist	[12]
		B. cereus	*P. fluorescens*	Unknown antagonist	[76]
		B. subtilis	*P. chlororaphis*	T6SS effector	[20,21]
		B. thuringiensis	*P. fluorescens*	Unknown antagonist	[93]
		B. velezensis	*P. sessilinigenes*	Lipopeptide	[25]
		B. velezensis	*P. kilonensis*	Lipopeptide	[25]
		B. velezensis	*P. chlororaphis*	Lipopeptide	[25]
		B. subtilis	*P. protegens*	DAPG	[24]
		B. subtilis	*P. putida*	DAPG	[24]
	Amensalism against *Pseudomonas*	*Bacillus.* sp.	*P. fluorescens*	Pr. Metabolism	[66]
		B. stratosphericus	*P. syringae*	Unknown antagonist	[94]
		B. subtilis	*P. syringae*	Lipopeptide	[95]
		B. subtilis	*P. aeruginosa*	Quorum quencher	[68]
	Competition	*B. amyloliquefaciens*	*P. chlororaphis*	Bacillaene and unknown antagonist	[22]
		B. thuringiensis	*P. fluorescens*	Pr. Metabolism	[11]
		B. velezensis	*P. protegens*	Lipopeptide	[25]
		B. velezensis	*P. tolaasii*	Lipopeptide	[25]
		B. cabrialesii	*P. syringae*	Unknown antagonist	[15]
		B. subtilis	*P. syringae*	Unknown antagonist	[15]
		B. velezensis	*P. syringae*	Unknown antagonist	[15]
Neutral	Neutralism	*B. aerius*	*P. monteilii*		[6]
		B. aerius	*P. aeruginosa*		[6]
		B. cereus	*P. fluorescens*		[89]
		B. pumilus	*P. monteilii*		[6]
		B. pumilus	*P. aeruginosa*		[6]
		B. subtilis	*P. putida*		[33]
		B. subtilis	*P. monteilii*		[6]
		B. subtilis	*P. aeruginosa*		[6]
		B. thuringiensis	*P. fluorescens*		[96]
		B. velezensis	*P. protegens*		[25]
		B. velezensis	*P. lactis*		[25]
		B. velezensis	*P. chlororaphis*		[25]
		B. velezensis	*P. mosselii*		[25]
Positive	Mutualism	*B. cereus*	*P. koreensis*	Pr. Metabolism	[7,14,67]
		B. cereus	*P. fluorescens*	Pr. Metabolism	[77–80]
		B. flexus	*P. azotoformans*	Pr. Metabolism	[86]
		B. velezensis	*P. stutzeri*	Pr. Metabolism	[73]

[a]Pr. Metabolism: An interaction characterized by resource competition and not bioactive secondary metabolites.

Box 1. Experimental coculture setups

Research of the interactions between microbes has generally followed quite similar experimental setups (Figure I). For growth kinetics, partners are coinoculated into a medium of interest and cultivated planktonically to determine synergy in growth rate, carrying capacity, or total live colony-forming units (CFUs). In planktonic cocultures, the direction and strength of an interaction is often derived from the carrying capacity of each pair compared to monoculture growth. An increased abundance of both partners is interpreted as mutualism, while a decreased abundance of both implies competition. In cases where a study aims to determine the effect of a natural product, the focal species is inoculated into a medium containing either a concentrated crude extract from the producer or the purified effector molecule. This approach has been useful in determining minimal inhibitory concentrations and in providing evidence for whether an effector molecule is secreted at concentrations that are bioactive against the focal strain. However, planktonic cultures often do not represent natural systems; thus, a favored and appropriate alternative would be to culture strains on solid agar where interacting molecules can diffuse through the agar at concentrations that mimic natural systems more closely. Many bacterial species activate transcription of biofilm-related genes on solid media and differentiate into spatial heterogeneity, increasing the accuracy (and complexity) of the interaction system. Solid medium interaction assays typically involve culturing strains in close proximity as macrocolonies or on top of each other as variations of the Burkholder plate assay. Experiments like these are valuable for insight on how molecules secreted in ecologically relevant concentrations affect a partner, and the experimenter uses inhibition zones or growth-free 'halos' to infer competition. For information on natural product bioactivity, variations of the Kirby–Bauer disc assay are a common choice, where the size of a growth-free 'halo' correlates with bioactivity; however, a researcher must interpret the strength of bioactivity in the context of the tested concentration which often is comparable to natural concentrations. For especially intricate hypotheses, researchers often establish a complex model biofilm system imitating the natural environment where the interacting strains are likely to meet. This can be achieved through substrate mimicking, host colonization, etc.

While antagonism and agonism are often identifiable through coculturing, the effector molecules and molecular mechanisms are not. Therefore, researchers use a multi-omics approach comprising transcriptomics and metabolomics. RNA-sequencing is used to analyze the transcriptomic responses to effector molecules or interaction partners, while chromatography and mass spectrometry are utilized for identification of effector and response molecules. In recent years, developments in mass spectrometry imaging have made it possible to visualize the spatial localization of metabolites during interspecies interactions, allowing for novel hypotheses regarding the distribution of metabolites in addition to the quantity.

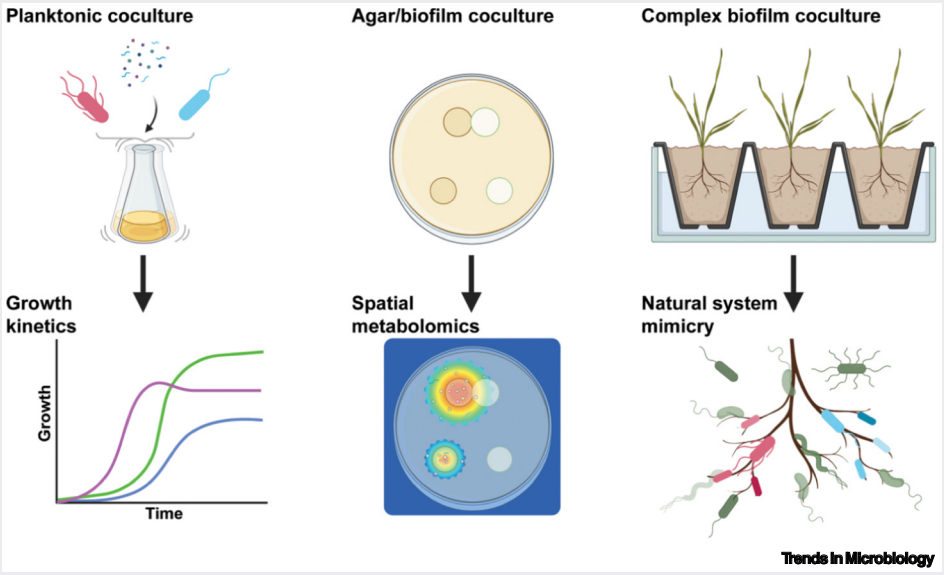

Figure I. Experimental coculture setups. Pairwise interactions are typically assessed via three experimental setups: planktonic cocultures, agar cocultures, and complex biofilm cocultures. Each methodology seeks to understand different aspects of a pairwise interaction from growth kinetics to spatial metabolomics to higher-order interactions in natural systems. Figure created with BioRender.com.

Table 2. Niches of isolates and the outcomes of pairwise interactions[a]

Isolation niche	Positive	Neutral	Negative	Total (*n*)
Plant	13% (4)	20% (6)	67% (20)	30
Soil	10% (1)	40% (4)	50% (5)	10
Animal	0% (0)	70% (7)	30% (3)	10
Marine	0% (0)	0% (0)	100% (4)	4
Laboratory standard	50% (1)	0% (0)	50% (1)	2
Other	50% (4)	0% (0)	50% (4)	8
Combined	15% (10)	26% (17)	58% (38)	65

[a]Sixty-five interactions (cocultures or challenges with cultured media) were summarized based on the isolation environment of the interacting pairs. Interactions with pairs from different origins were excluded except in cases with standard laboratory strains. In these cases, the niche is denoted as that of the isolate.

are conserved among members of a species and even fewer within members of a genus. Instead, many are likely inherited through horizontal gene transfer, possibly originating from a distantly related organism [16]. Few studies report interactions mediated by primary metabolism, though all positive interactions reported in Table 1 are products of cross-feeding or other ways to share resources. This finding may be biased by the underwhelming number of competition assays examining mechanisms such as resource competition.

With the current body of literature on *Bacillus–Pseudomonas* interactions, it is impossible to accurately predict the outcome of a pairwise contact from the phylogeny of the interacting partners. In addition, recent evidence shows that phylogeny might not be the best predictor for pairwise interactions [17], and therefore, the undergoing intensification in research on bioactive compound potential could provide a stronger understanding on *Bacillus–Pseudomonas* ecology.

Physical interactions

The literature investigating interactions between *Bacillus* and *Pseudomonas* has generally focused on how secreted chemicals from one strain affect the interacting partner (Box 1). As such, the number of examples describing physical cell-to-cell interactions are scarce.

Several species of *Pseudomonas* are well known for carrying a **type VI secretion system** (**T6SS**) [18,19], which works as a toxin-injecting syringe, activated upon cell-to-cell contact with competitive species. When cocultured next to *Bacillus subtilis*, *Pseudomonas chlororaphis* uses a T6SS to compete for space, triggering *B. subtilis* cells to undergo sporulation as a defense mechanism [20]. This is most evident in situations where *Bacillus* is unable to produce biofilm matrix components, which it employs as a defensive barrier. The effector toxin was recently defined as Tse1, a peptidoglycan hydrolase, whose gene is located in a T6SS gene cluster that is shared across a number of Gram-negative bacteria [21]. Interestingly, T6SS might be a last line of defense, provided that chemical inhibitors prove ineffective against a competitor allowing for cell-to-cell contact. Close-by macrocolonies of *Bacillus amyloliquefaciens* and *P. chlororaphis* result in a distinct zone of inhibition with no physical contact between colonies. In this case, *P. chlororaphis* downregulates T6SS gene expression in favor of the type II secretion system and production of several secondary metabolites [22].

Chemical interactions

Both *Pseudomonas* spp. and *Bacillus* spp. are well known for their massive arsenal of secondary metabolites (Box 2). Many secondary metabolites display antimicrobial activity, though some are used to gain colonization advantages in competitive niches without killing other microbes.

Box 2. Secondary metabolites

Secondary metabolites (also known as natural products) are non-essential compounds produced by an organism to confer a selective advantage. Two molecular families dominate the secondary metabolite group: nonribosomal peptides and polyketides. The functions of these metabolites range from toxic antagonism [97] to signaling molecules [98,99], cellular differentiators [24], or entries into novel nutritional niches [100].

In pseudomonads, production of secondary metabolites is controlled by the global Gac/Rsm regulon which is responsible for regulating the expression of approximately 10% of *Pseudomonas* genes [101]. Though the signaling pathway is activated by the sensor kinase GacS, it is currently unknown which environmental stimulus causes the initial activation. In bacilli, secondary metabolite regulation is more decentralized, with specific regulons for different classes of compounds [102].

Notable examples are the cyclic lipopeptides (CLPs): surfactin, iturins, and fengycin/plipastatin from *Bacillus*, and viscosin, tolaasin, and syringopeptin from *Pseudomonas* which are known for their bioactivity against fungi and/or other bacteria. In addition, the *Pseudomonas* polyketide 2,4-diacetylphlorogucinol (DAPG), produced by some members of the *P. fluorescens* group, is of great interest due to its apparent plant-growth-promoting and antimicrobial activities. Secondary metabolism of *Bacillus* is reviewed more extensively in [102,103], and of *Pseudomonas* in [104].

In the case of interactions between *Bacillus* and *Pseudomonas*, a general view suggests that *Pseudomonas* spp. are most often offensive (i.e., they produce bactericidal or bacteriostatic molecules) [20,22–24], while *Bacillus* spp. more often defend themselves (i.e., they neutralize offensive molecules or prevent being overtaken by a competitor) [25–27] (Figure 2).

Several studies have demonstrated the antimicrobial effect of secreted molecules through variations of the disk diffusion assay (Box 1). This approach allowed researchers to identify saponins and alkaloids (specifically phenetyl acetamide) produced by *B. cereus, Bacillus thuringiensis,* and *B. subtilis* that inhibit *Pseudomonas aeruginosa* [28,29], while **2,4-diacetylphloroglucinol** (**DAPG**), pyocyanin, and several bacteriocins from *Pseudomonas protegens*, *Pseudomonas putida*, and *P. aeruginosa* have been able to inhibit *B. subtilis*, *Bacillus licheniformis*, *B. cereus*, and *B. thuringiensis* [24,30–33]. While these studies are valuable in identifying effector molecules and their range of effect, their contribution to ecological or mechanistic insight is sparse as they did not report the concentrations of the molecules in the environments where they were produced.

Pseudomonas cyclic lipopeptides are deadly (and) specific

The **cyclic lipopeptides** (**CLPs**) comprise one of the most structurally diverse groups of secondary metabolites produced by both bacilli and pseudomonads [34]. *Bacillus* spp. are

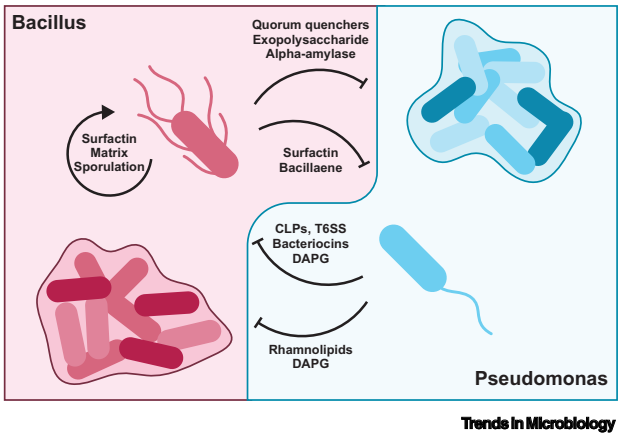

Figure 2. Pseudomonads are offensive, bacilli are defensive. *Pseudomonas* and *Bacillus* use a range of bioactive compounds to interact with each other, mainly in net negative relations. Especially *Pseudomonas* cyclic lipopeptides (CLPs) and 2,4-diacetylphloroglucinol (DAPG) directly antagonize multiple *Bacillus* spp. In cell-to-cell contact, *Pseudomonas* utilizes type VI secretion systems (T6SS) to puncture *Bacillus* and inject toxins intracellularly. *Bacillus* spp. rarely directly antagonize *Pseudomonas*. Instead, they either sequester offensive molecules, produce protective matrix, or undergo sporulation to preserve part of the population. However, bacilli do contain multiple molecules to prevent or disrupt biofilm formation of pseudomonads.

primarily known for surfactin, iturins, and fengycins, while *Pseudomonas* spp. carry the potential for producing up to 14 distinct groups of CLPs [35].

The bioactivity of *Pseudomonas* CLPs is specific to their molecular structure, and mainly CLPs from the viscosin, tolaasin, and syringopeptin groups have been demonstrated to inhibit growth of bacilli. Syringopeptin 22A, syringopeptin 25A, corpeptin, tolaasin, and **WLIP** (**'white-line-inducing principle'**) all inhibit *Bacillus megaterium* [36–38], while massetolide A, syringomycin E, orfamide A, arthrofacin, and entolysin B do not [39,40]. Even within CLP groups, bioactivity varies. Though massetolide A is unable to antagonize *B. megaterium*, massetolide E, from *Pseudomonas lurida*, strongly inhibits *B. thuringiensis* [39]. Within the viscosin group, a hierarchy of effect has been established on the antagonism of *B. subtilis* and *B. cereus* with pseudodesmin being the most active, closely followed by viscosinamide and finally viscosin and WLIP showing almost no inhibition [41].

The lipopeptides of *Bacillus* – fengycin, iturin, and surfactin – are all well known for their bioactivities. Fengycin and iturin are generally regarded as antifungal [42], and accordingly demonstrate very weak activity against *Pseudomonas.* Concentrations must reach more than 200 μg/ml, at which point fengycin kills *P. aeruginosa* cells by altering the cell topography, causing leakage of the intracellular content [43]. More examples exist of surfactin bioactivity against pseudomonads, and against *Pseudomonas fluorescens,* it is bactericidal down to 25 μg/ml, inhibiting tomato root colonization [42]. Though best known for enhancing motility during swarming [44] and sliding [43,45], surfactin produced by *Bacillus velezensis* also functions as a chemical trap to sequester the *Pseudomonas sessilinigenes* cyclic lipopeptide sessilin [25]. This reduces the toxicity of sessilin to a level where *B. velezensis* is not eradicated in a coculture. Interestingly, this chemical interplay is specific to sessilin and does not carry over to other *Pseudomonas* CLPs.

Bacillus defends itself by producing biofilm matrix and committing to sporulation

Both *B. subtilis* and *B. amyloliquefaciens* have been shown to defend themselves against competitors using biofilm matrix components and, as a last resort, by inducing sporulation to preserve part of the population [20,22]. In several reported cocultures, bacilli have entered sporulation with no evidence that *Pseudomonas* is able to destroy *Bacillus* endospores. To date, only a single study has provided evidence of *Bacillus* directly inhibiting the growth of pseudomonads with bacillaene produced by *B. amyloliquefaciens* that interacts with *P. chlororaphis* **elongation factor G** (**FusA**) inhibiting protein synthesis without killing *Pseudomonas* [22]. More examples exist that report indirect inhibition of *Pseudomonas* by priming plant systemic resistance, for example with *B. cereus* instigating *Arabidopsis thaliana* systemic resistance against *P. syringae* pv. *tomato* both dependently and independently of salicylic acid, jasmonic acid, and ethylene signaling [46–48].

Bacillus biofilm formation can be inhibited by *Pseudomonas* species of the *fluorescens* and *protegens* subgroups through the production of the antimicrobial secondary metabolite DAPG [24]. Although DAPG-producing pseudomonads are often applied in biocontrol, the compound antagonizes more than just plant pathogens. DAPG at high concentrations suppresses the growth of *B. subtilis*, while subinhibitory concentrations reduce biofilm formation and delay sporulation [24]. The mechanism is unknown but speculated to be due to an interaction with Spo0A, the global regulator of sporulation in *Bacillus*. Interestingly, a combination of *B. subtilis* with DAPG-producing pseudomonads still led to synergistic biocontrol [49]. Additionally, *Bacillus* surface-associated biofilm can be prevented and dispersed by the rhamnolipid biosurfactants of *Pseudomonas* [50–52]. The current consensus suggests an altered surface hydrophobicity without experimental proof so far [53]. However, as most studies assess biofilm functionality *in vitro*, the ecological relevance of biofilm requires further testing.

Bacillus carries a vast arsenal of quorum quenchers

Bacillus spp. have demonstrated the ability to prevent competitors from establishing themselves, particularly by disrupting or inhibiting their biofilm formation. Isolates of *Bacillus pumilus, Bacillus paralicheniformis, B. subtilis,* and *B. thuringiensis* have all demonstrated some sort of *Pseudomonas* **quorum sensing** inhibition [16,26,54–57]. At this point, *Bacillus* isolates have been experimentally shown to quench quorum sensing systems that depend on ***N*-acylhomoserine lactones** (**AHLs**) and ***Pseudomonas* quinolone signal** (**PQS**), but not **autoinducer II (AI2)**-dependent cell–cell communication. Interestingly, supplementation of AI2 seems to inhibit initiation of the sporulation process in *B. velezensis* [58]. Particularly, the lactonase AiiA has been shown to degrade both short-, medium-, and long-chain AHLs, thus disrupting quorum sensing and downstream effects in *P. aeruginosa* and plant pathogenic pseudomonads [54,56,59]. AiiA was originally discovered in endophytic *B. thuringiensis* isolates [56]; however, additional research revealed conservation of lactonase activity in the entire *B. cereus* group. Noor and colleagues reported that 22 out of 64 *B. subtilis* soil isolates carry homologs of AiiA, conserved with 84.4% amino acid identity [57]. A second lactonase, YtnP, was identified in a soil isolate of *B. paralicheniformis* and shown to degrade AHLs from multiple Gram-negative strains, including *P. aeruginosa, Pseudomonas extremeorientalis,* and *Pseudomonas lini* [55]. Similarly, *B. pumilus* disrupts quorum sensing by producing an acylase that enzymatically deacetylates AHLs [26], while a marine *Bacillus* sp. isolate produces a non-enzymatic antagonist of AHL without enzymatically changing the *Pseudomonas* autoinducers [60]. None of the identified quorum quenchers exhibit direct growth inhibition against any tested *Pseudomonas* spp. strains.

Recently, *B. subtilis* isolates were shown to encode Stigmatellin Y: an orthosteric antagonist for quinolones in PQS quorum sensing [16,61]. It is speculated that StigY is not conserved in *Bacillus* spp. but rather was horizontally transferred from *Stigmatella aurantiaca*. Other than quorum quenching, some bacilli use specialized exopolysaccharides to reduce the surface hydrophobicity of Gram-negatives and thus suppress adherence and biofilm formation [62].

Certain bacilli are also capable of degrading already established *Pseudomonas* biofilm, as experimentally demonstrated by exopolysaccharide degradation by α-amylase and through an unknown mechanism facilitated by an antimicrobial protein [52,63,64]. However, it is likely that the concentration required for biofilm degradation far surpasses that produced in natural systems. Importantly, these studies recognize the potential of quorum quenching as a treatment option via these *Bacillus* effector molecules, but no experimental test has been reported to prove that quenching occurs in natural settings or even within *in vitro* cocultures.

Transcriptional responses are unique to a coculture partner

An unresolved question in microbial ecology is whether an interaction is caused by a specific response or is simply the passive consequence of microbes being in the vicinity of molecules secreted by other microbes. Additionally, if a strain responds to the presence of an unrelated neighbor, is its metabolism regulated based on the specific interaction partner or is it simply a general response to any microbe or stress condition [65]?

For *Pseudomonas*, evidence of specificity is derived from transcriptomic and gene essentiality studies, where proximal macrocolonies with different neighboring organisms led to distinct transcriptional and gene essentiality profiles [66]. Although differential expression and conditional essentiality are shared between all organisms, most genes are highly dependent on the specific organism. Interestingly, patterns diverge when coculturing one *Pseudomonas* strain next to different bacilli, suggesting that the metabolome of the interacting *Bacillus* strongly influences the response of *Pseudomonas* [20,22], though this, too, may be species- or even strain-specific

[67]. Although fewer examples exist of *Bacillus* transcriptomic responses to *Pseudomonas*, there is evidence that *B. subtilis* alters its metabolome in response to secreted molecules from *P. aeruginosa*, increasing its antibiofilm activity [68]. A recent study using the THOR SynCom also demonstrated that, in the three-species community, *B. cereus* was the member that modulated its expression of biosynthetic gene clusters primarily when cocultured with *P. koreensis*. The metabolome and transcriptome of *P. koreensis* were not altered dramatically in coculture with *B. cereus*, and both *B. cereus* and *P. koreensis* were unresponsive towards *F. johnsonii* [67]. Additionally, *P. chlororaphis* has been shown to produce antagonistic molecules that select for specific genetic variants of *B. amyloliquefaciens* [22].

How these interaction patterns fit together is difficult to conclude based on the current literature. Nevertheless, a candidate regulatory system that is likely involved in interspecies interactions of *Pseudomonas* is the main controller of secondary metabolism, the Gac/Rsm two-component system (or its orthologs – see [69]). In addition to secondary metabolism, Gac/Rsm controls several primary metabolic lifestyles such as motility, quorum sensing, and biofilm formation. Generally, GacS activation results in an increased production of offensive and defensive molecules, secreted or retained intracellularly [70–72].

On secondary metabolism-inducing King's B agar, *P. chlororaphis* GacS was reported to be essential for antagonism against *B. amyloliquefaciens* in a transposon library screen, but transcriptomic analysis of the same interaction revealed no differential expression [22]. When competing *P. fluorescens* Pf0-1 with *Bacillus* sp. V102 on a carbon-limited water-agar medium, *gacS* and *gacA* were also not found to be differentially expressed, though several other two-component systems were transcribed at increased levels in coculture compared to monoculture [66]. In a similar setup, competition of *P. protegens* against *B. subtilis* and several other soil microbes demonstrated that *P. protegens* GacS was not essential in interactions with *B. subtilis* but was essential in coculture with another *Pseudomonas* strain and even disadvantageous in interactions with fungal plant pathogens [23]. Future comprehensive molecular ecology studies will hopefully reveal the emerging and common patterns in *Bacillus–Pseudomonas* interactions.

Mutually beneficial coexistence

Only few *Bacillus–Pseudomonas* coculture studies have been reported with the aim of identifying and understanding positive interactions. This could explain the abundance of net negative interactions and the lack of direct positive interactions. In recent years, however, the field has started to report studies providing evidence for commensalism and even mutualism.

An important recent study supports the coexistence of bacilli and pseudomonads: Sun and colleagues [73] inoculated cucumber roots with the **plant-growth-promoting rhizobacterium** (**PGPR**) *B. velezensis* SQR9 and found that, in particular, *Pseudomonas* spp. were enriched in the rhizosphere compared to non-inoculated controls. With a combination of transcriptomics, metabolomics, and molecular biology, a mutualistic relationship was ultimately identified between *B. velezensis* and a *Pseudomonas stutzeri* soil isolate potentially facilitated by cross-feeding of branched amino acids. Similarly, combinations of bacilli and pseudomonads increase the growth of the plant *Cannabis sativa* by up to 70%, with *Bacillus* spp. being the main modulator of the soil microbiome while *Pseudomonas* spp. are more associated with plant growth promotion [74]. Apparently, *Bacillus* arrives first and subsequently recruits *Pseudomonas* via metabolic byproducts. However, only few studies reporting synergy have also evaluated the compatibility of the consortium members, instead concentrating primarily on the application efficiency and thus lack specific insights on the distribution and the interaction of the mixed species. As such, it is impossible to deduce the net outcome of the pairwise interaction and whether synergy

stems from positive or negative interactions. Some of these studies show positive interactions and subsequent synergy [75], while others report competition and synergy [76]. For instance, in a series of coculture studies with *B. cereus* and *P. fluorescens,* initial planktonic cocultures revealed antagonism by *P. fluorescens* against *B. cereus* [76]. In biofilm reactors, inhibition was absent, and instead, biofilm matrix formation increased synergistically with more protein present in the coculture biofilm [77,78]. Interestingly, despite the synergistic increase in biofilm formation, coculture biofilm could be removed more efficiently with a quaternary ammonium disinfectant [79,80]. The complexity of such interactions means that one should be careful oversimplifying and attempting to generalize pairwise interactions.

Applicability of *Bacillus–Pseudomonas* cocultures

With their status as plant-growth-promoting rhizobacteria, *Bacillus* and *Pseudomonas* have been studied extensively for their potential as alternatives to chemical fertilizers and pesticides. Species from either genus have been successfully applied as growth promoters and biopesticides, but several studies have also shown how consortia of bacilli and pseudomonads act in synergy to produce emergent properties not possible by any of the partners in isolation. Such synergy has led to the control of *Cephalysporium* in maize [81], of *Fusarium* in beans [82], and blight in cucumber, radish, and cotton [49,83]. As with interactions between *Bacillus* and *Pseudomonas*, secondary metabolites are the main mediators of biocontrol, and the cocultivation of species has resulted in additive effects of metabolites produced in monocultures, but also in novel compounds produced only in coculture. In the case of plant growth promotion, synergy may again stem from additive effects of molecules produced by each microbe [84], or the enhanced ability of cocultures to establish themselves on plants compared to pure cultures [85], allowing higher production of effector molecules [5,49,81–87].

Pairs of bacilli and pseudomonads clearly can create biotechnological value, and it will be interesting to see if future formulations will be able to outcompete chemical alternatives.

Concluding remarks

Research into pairwise interactions between *Bacillus* and *Pseudomonas* still leaves much unanswered (see Outstanding questions). With our current understanding, a reductionistic approach using selected single isolates of each genus is insufficient, as we cannot draw clear conclusions and identify overarching patterns. One thing that most research agrees on is that interspecies interactions are more complex than net negative or positive associations.

Thus, the simple hypothesis that successfully applied biotechnological consortia must in general engage in mutualism is flawed. If the scope is limited to look only for isolates that grow synergistically, we risk overlooking emergent properties evident in competitive interactions that settle into a stable equilibrium. It is well known that resource competition is a strong selective pressure that determines species coexistence [88]. Indeed, several examples exist of *Bacillus* and *Pseudomonas* being capable of specifically controlling the differentiation and metabolism of each other independently of secreted molecule toxicity [24,68,73]. However, pairwise interactions do not necessarily translate to higher-order interactions in multispecies consortia. Rather, several examples exist of bacilli and pseudomonads that outcompete each other in dual species coculture but stabilize into coexistence in a more diverse community [67,89]. Importantly, pairwise interactions between *Pseudomonas* and *Bacillus* have greatly contributed to our understanding of the effects of secondary metabolites on model soil bacteria and the relevance of pairwise interactions in biotechnology. However, it is exceedingly rare to find studies reporting concentrations (or even the presence) of effector molecules in natural environments. This is largely due to the complexity of *in situ* quantification. Though examples exist, they currently rely on relatively simple substrates (e.g., the

Outstanding questions

Why are bacilli and pseudomonads so often coisolated?

How do pairwise interactions between *Bacillus* and *Pseudomonas* affect natural microbiomes?

How can knowledge of *Bacillus–Pseudomonas* dynamics be applied in the development of biotechnologically relevant culture formulations?

What are the concentrations of effector molecules in natural systems?

phyllosphere) [90,91], and it has not yet been possible to quantify secondary metabolites directly from soil. An undergoing development in mimicking natural systems and detecting molecules from complex systems [92] means that future studies could potentially report biological functions in the context of naturally occurring concentrations, an important advance in understanding chemical microbial ecology.

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Declaration of interests

No interests are declared.

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