Chemical Crosstalk between the Common Soil Bacteria *Pseudomonas chlororaphis* and *Bacillus subtilis*

Zohar Tik,*[a] Shaked Uzi-Gavrilov,[a] Bat El Hagbi-Lazar,[a] David C. Nwobodo,[a] Orit Sivan,[b] and Michael M. Meijler*[a,c]

[a]Department of Chemistry, Ben-Gurion University of the Negev, Be'er Sheva, Israel,

[b]Department of Earth and Environmental Sciences, Ben-Gurion University of the Negev, Be'er Sheva, Israel,

[c]The National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Be'er Sheva, Israel.

Abstract

Chemical conversations between soil inhabitants are of tremendous importance to the health of many ecosystems, and at the same time detailed molecular knowledge underlying these conversations is surprisingly scarce. One of the major bacterial genera inhabiting the rhizosphere is *Pseudomonas*, of which most species are known to produce phenazines, which carry antibiotic properties. *Pseudomonas chlororaphis*, a common rhizosphere dwelling species with plant growth-promoting traits, produces phenazine-1-carboxamide (PCN). This study examines how the production of PCN by *Pseudomonas* affects another common species in soil that it often encounters, namely *Bacillus subtilis*. When both species were cultured at close distance, distinct and visible changes in colony morphologies were observed without changes in growth rates. Interestingly, a clear transformation occurred in the morphology of *B. subtilis* colonies in the presence of supplemented PCN, indicating the role of phenazines in affecting colony morphology. In addition, untargeted metabolomics analyses showed a decrease in the production of plipastatin and surfactin by *B. subtilis* in the presence of *P. chlororaphis*. Our results indicate that PCN induces changes in morphology and signaling of *B. subtilis* without significantly affecting its growth. We hypothesize that *P. chlororaphis* and *B. subtilis* sense one another and act to conserve energy while avoiding competition.

Introduction

The rhizosphere is the narrow region of soil directly influenced by root secretions and associated soil microorganisms.[1] As such, the rhizosphere contains higher concentrations of compounds that are secreted by microorganisms and roots than bulk soil,[2] creating this area a desirable habitat for many microorganisms. Therefore, many intricate microbial interactions occur in the rhizosphere,[3] creating a

fascinating and complicated network of connections between the microorganisms living in the rhizosphere and nearby plants.[2] Among the rhizosphere microbiome both plant-growth-promoting bacteria (PGPB) and harmful organisms can be found.[4] PGPBs are beneficial to plants for various reasons, such as promoting nutrient availability[5] and antibiotic production that protect the plant from pathogens.[6] Moreover, the diversity of the rhizosphere microbiome allows the plant to adapt to changing environments. Antibiotic production by bacteria is not only taking part in protection against pathogens, but also have a significant effect on the population, and it is a part of its social interaction.[7] Furthermore, it have a decisive role in the bacteria ability to compete and survive in its surrounding.[7]

Bacillus spp., a Gram-positive bacteria, are among the most beneficial PGPB.[8] A well-studied model organism for this group is the non-pathogenic *Bacillus subtilis*. About 4% of its genome is dedicated to producing specialized metabolites – a relatively high percentage;[9] this can be indicative of its versatile mechanisms in response to different signals from its neighbors. In *B. subtilis* three different non-ribosomal peptides (NRPs) systems were found, and one hybrid NRP/polyketide.[10] NRPs distinguish from ribosomal peptides as they contain non-proteinogenic amino acids,[11] and their structures are quite often macrocyclic.[11,12] NRPs have diverse biological activities[13] and are a major chemical family of secondary metabolites.[14] Several specialized metabolites produced by *B. subtilis* are antibiotics belonging to the family of NRPs. Two of the antibiotics that are produced by those systems are plipastatin and surfactin.[15] Both plipastatin and surfactin are groups of compounds that differ from their family members in their fatty acid chain length and degree of saturation.

Another common and well-studied bacterium is the Gram-negative PGPB fluorescent *pseudomonas* spp. *Pseudomonas* is among the most common bacterial genera on earth, and can colonize a wide range of ecological niches, including aquatic areas, plants, animals and soil (i.e. the rhizosphere). *Pseudomonas* are known for their production of phenazines, a family of N-containing heterocyclic compounds, usually pigmented.[16] Phenazines have important and versatile roles in the soil, as they can function as signaling compounds, antibiotics, electron shuttling and anti-virulence agents.[16–18] Although this family is highly studied, in most cases their mechanism of action is not fully known.[17] Not surprisingly, phenazines are known to improve the competence of their producers.[16] *P. chlororaphis* is a common soil-dwelling bacteria considered a prime PGPB.[19–21] *P. chlororaphis* is known to produce mainly two different phenazines, phenazine-1-carboxamide (PCN) and phenazine-1-carboxylic acid (PCA), and PCN is produced in significantly higher quantities. *P.chlororaphis* was found to inhibit the fungal pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici*, due to its secretion of PCN.[25] PCN (purified from a different *Pseudomonas* species) was also found to inhibit *Rhizoctonia solani* and *Xanthomonas oryzae* pv. *Oryzae*.[27]

Both *B. subtilis* and *P. chlororaphis* are common in the rhizosphere microbiome, and thus, unsurprisingly, are often co-isolated.[15] Different interactions were previously reported between different strains from these species, ranging between beneficial, natural, and harmful.[15] *B. subtilis* can sense the presence of

P. chlororaphis, leading to increase in *B. subtilis* sporulation.[28] Furthermore, *B. subtilis* colonies are protected from infiltration by *P. chlororaphis* due to their extracellular matrix.[28] Substances in the *B. subtilis* extracellular matrix, such as surfactin, participate in the interaction between them.[27,28] Although interactions between these two bacteria significantly affect the rhizosphere, the microbiome of the rhizosphere is of course composed of many more bacteria. It is reasonable to assume that their additional neighbors also affect interactions between *P. chlororaphis* and *B. subtilis*, and vice versa. Moreover, conclusions from culture experiments on the natural environment should not be made easily, as it was established that different growth media and conditions in the lab can change the interactions between those two bacteria.[30] Nevertheless, further studies on the interactions among those two important PGPBs could extend and deepen our understanding of natural soils.

In this study we examined chemical interactions between *B. subtilis* and *P. chlororaphis*, in order to understand how regulation of these interactions affects their coexistence. We focused on measurable changes in colony morphology, growth and metabolite secretion of both bacteria.

Results and discussion

The interaction between *B. subtilis* and *P. chlororaphis* results in colony morphology changes

Wild-type *B. subtilis* strain NCIB 3610 (from here named *B. subtilis*; the microbial strains used in this research are listed in table 1) and wild-type *P. chlororaphis* strain PCL 1391 (from here *P. chlororaphis*) are both prominent members of the rhizosphere; therefore, we set out to examine potential interactions between those two species on solid media (agar) in order to best mimic natural conditions. We incubated the bacteria at varying distances (0.4 cm to 1.8 cm) (Figure S1) on LB agar. LB agar was chosen, and not a more selective medium, in order to allow for robust and equal growth of both species. At the shortest distances, 0.4 cm and 0.6 cm, a small inhibition zone in the *B. subtilis* colony was visible, indicating growth inhibition of *B. subtilis* by *P. chlororaphis*. Although this limited inhibition of *B. subtilis* growth occurred in this experiment, the bacteria do often co-exist in nature.[28] In soil, this inhibition might be prevented due to various reasons such as environmental conditions supporting *B. subtilis* growth, lower concentration of *P. chlororaphis*, and more complicated interactions involving additional bacteria.

As our main goal was to study the chemical interactions that actually support their co-existence, we conducted our experiments at 0.8 cm separation between the bacteria, the shortest distance where there was no visible inhibition. Although no inhibition was observed when *B. subtilis* and *P. chlororaphis* were grown on the same plate at 0.8 cm separation, there was a distinct and visible change in the edges of colonies of both bacteria, (Figure 1A) with an extensive change in *B. subtilis* morphology, represented as a change in the wrinkled characteristic morphology,[31] mainly in the edges of the colonies. This observation is supported by previous studies that established that *P. chlororaphis* has an effect on *B. subtilis*

biofilms by activation of the *P. chlororaphis* type IV secretion system (T6SS).[29] T6SS, via its effectors, causes degradation in the *B. subtilis* peptidoglycan layer,[32] which forms an important component of the *B. subtilis* cell wall. Previous studies have shown that interactions vary with the specific strain involved,[28] as well as the bacterial growth medium.[29] Moreover, there was a visible change in *P. chlororaphis* colony edges. Those morphological changes suggest that the bacteria are sensing one another at this distance. Interestingly, certain interactions between members of those genera were reported by others in diverse conditions.[28,30,33] Most of those interactions were found to be competitive, while others were found to be beneficial.[28] Lyng and Kovács suggest that most of the interactions found are more negative than positive due to research methods bias, and not necessarily a reflection of processes in the rhizosphere.[28] Interestingly, morphological changes were detected all around the colonies, including the farthest zones of the bacteria from one another. It implies that the signals secreted by *P. chlororaphis* are either reaching all of the *B. subtilis* area, either as volatile compound or by diffusion, or the signal is detected by *B. subtilis* near the *P. chlororaphis* colony, and then changes propagate throughout the whole *B. subtilis* colony, as discussed more thoroughly in the next section.

Table 1. Microbial strains used in this study

Bacterial strain	Description	Source
B. subtilis	Wild type NCIB 3610	Branda *et al.*[22]
B. subtilis Δ*pps*	deficient in plipastatin synthesis	Maan *et al.*[23]
B. subtilis Δ*srfAA*	deficient in surfactin synthesis	López *et al.*[24]
P. chlororaphis	Wild type PCL 1391	Chin-A-Woeng *et al.*[25]
P. chlororaphis Δ*phz*	deficient in phenazine synthesis PCL 119	Chin-A-Woeng *et al.*[25]
F. oxysporum	*F. oxysporum* f. sp. *radicis-cucumerinum*	Dror *et al.*[26]

In this study, we examined the potential effects of PCN, the main phenazine produced by *P. chlororaphis*,[34] on *B. subtilis*. To further investigate those interactions, we also cultured the *P. chlororaphis* non-phenazine producing mutant strain *P. chlororaphis* 1119 (from here *P. chlororaphis* Δ*phz*). We cultured *B. subtilis* and *P. chlororaphis* Δ*phz* as mono- and co-cultures (Figure 1B), similarly as the wild-type co-cultures. Phenazines are known as colorful compounds, and PCN has a yellow color, a difference that is evident in the *P. chlororaphis* Δ*phz* cultures (Figure 1A,B). However, when *B. subtilis* grew next to *P. chlororaphis* Δ*phz,* there was still a change in the bacterial morphology, although the change was smaller compared to *B. subtilis-P. chlororaphis* co-cultures (Figure 1A,B). Not surprisingly, this suggests, that while PCN clearly affects *B. subtilis* morphology, it is not the only compound produced by *P. chlororaphis* that affects *B. subtilis*. This correlates with previous studies showing that other compounds are interacting with *Bacillus* and *Pseudomonas* species, such as 2,4-diacetylphloroglucinol,[35] and lipopeptides.[36]

120 **PCN has a significant role in the interaction between *B. subtilis* and *P. chlororaphis***

121 The clear observation of *B. subtilis* different reaction to *P. chlororaphis* and *P. chlororaphis* Δ*phz* indicates
122 the role of PCN in the interaction among these two species. To gain a better understanding of the role
123 that PCN plays in guiding *B. subtilis* and *P. chlororaphis* interactions, we examined whether addition of
124 10 μM PCN to agar would restore the activity of *P. chlororaphis* Δ*phz* to the level of its wild-type counter-
125 part. The PCN concentration was determined to be 10 μM based on PCN concentrations produced by *P.*
126 *chlororaphis* (Figure S2). Co-cultures with the Δ*phz* mutant on media with PCN displayed similar effects
127 on *B. subtilis* morphology as a co-culture with wild-type *P. chlororaphis*, indicating that changes in *B.*
128 *subtilis* morphology are dependent on the presence of PCN. (Figure 1A,C). For further discussion of the
129 spatial effect of PCN on *B. subtilis* morphology, see the supporting information.

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131 **The interaction between *B. subtilis* and *P. chlororaphis* results in *B. subtilis* metabolic changes**

132 In order to further our understanding of how the two soil bacteria affect one another, and in particular
133 how PCN affects *B. subtilis* behavior, we set out to examine differences in production of *B. subtilis* me-
134 tabolites in the presence of *P. chlororaphis* and PCN alone on solid media, and compared with effects
135 caused by the presence of the PCN mutant. To start our analysis, first a principal component analysis by
136 MetaboAnalyst[37] based on MS^1 data showed that each treatment clustered by itself (Figure 2A). This
137 result indicates that clear changes in overall metabolite production between the different experimental
138 treatments could be observed. *B. subtilis* grown together with *P. chlororaphis* Δ*phz* was more similar to
139 *B. subtilis* grown on mono-culture than *B. subtilis* co-culturing with *P. chlororaphis*. Moreover, *B. subtilis*
140 cultured together with *P. chlororaphis* Δ*phz* was more similar to *B. subtilis* rather than when cultured
141 together with PCN, indicating a more prominent effect on *B. subtilis* metabolites when the phenazine was
142 present. This observation supports our hypothesis that phenazines alter *B. subtilis* metabolite production.
143 In addition, it should be considered that the metabolic profile of the *P. chlororaphis* Δ*phz* culture with PCN
144 is similar to *P. chlororaphis* Δ*phz* rather *than P. chlororaphis*. The *P. chlororaphis* Δ*phz* mutant used in
145 this study has a transposon insertion at the PhzB homolog gene,[25] resulting in obstruction of the bacterial
146 phenazine production pathway. That means that *P. chlororaphis* and *P. chlororaphis* Δ*phz* are different in
147 more metabolites than only PCN, which can explain the differences between the mono-cultures treat-
148 ments shown in figure 2A. After establishing differences in the metabolic profiles, we continued investi-
149 gating which *B. subtilis* metabolites are the most affected by PCN.

150 Several metabolites were found to be produced in altered amounts among the different treatments. We
151 used several metabolomics platforms, MZmine3,[38] GNPS[39] and Sirius,[40] to annotate those affected me-
152 tabolites; the molecular network produced obtained via the GNPS platform is shown in figure 2D. We
153 aimed to explore compounds that were altered as a result of the different treatments. Although metabolite

154 annotation has progressed much in recent years, unequivocal annotation with a high degree of confi-
155 dence is still a major bottleneck in untargeted metabolomics research.[41] Thus, as was expected, we were
156 not yet able to identify all the compounds of interest. For example, we found a compound with an m/z
157 value of 227.1754, which production was significantly inhibited in the presence of *P. chlororaphis*, and
158 also upon the addition of PCN, but its production was not inhibited when *B. subtilis* and *P. chlororaphis*
159 Δ*phz* were co-cultured (Figure S4). The structure with Sirius's highest annotation score for this mass was
160 the cyclo-di-peptide cyclo-Leu-Leu. Although we did not validate the structure of this compound, we will
161 continue to examine this possibility, as cyclo-di peptides are known to participate in quorum sensing
162 processes and have antimicrobial and antifungal properties.[42] Furthermore, cyclo-di-peptides were iso-
163 lated from *Bacillus* spp.[43,44] Thus, it is not unlikely that a compound from this family can serve to mediate
164 interactions between *B. subtilis* and *P. chlororaphis*. Thus, it appears that the secretion of PCN by *P.*
165 *chlororaphis* results in inhibition of this compound with unknown structure. However, some of the metab-
166 olites whose concentrations were changed due to PCN addition could be annotated by GNPS, and,
167 interestingly, several of these are known *Bacillus*-produced antimicrobial substances, such as plipastatin
168 and surfactin (Figure 2B, 2C, 3A, 3B).

169 The presence of *P. chlororaphis* results in strongly decreased plipastatin quantities secreted by *B. sub-*
170 *tilis*, while the presence of *P. chlororaphis* Δ*phz* does not significantly affect the production of plipastatin
171 by *B. subtilis* (Figure 3A). Furthermore, when *B. subtilis* was grown on PCN-containing media, both alone
172 and together with *P. chlororaphis* Δ*phz*, a similar decrease in plipastatin production was observed (Figure
173 3A), implying PCN involvement in a mechanism lowering this antimicrobial substance production in *B.*
174 *subtilis*. The same effect was detected for other plipastatin derivatives (supporting information). Similarly,
175 the production of surfactin, another antimicrobial compound produced by *B. subtilis*, is strongly inhibited
176 by the presence of PCN, although in the co-culture experiments the differences were smaller (Figure
177 3B), indicating the involvement of additional signals of *P. chlororaphis* that may antagonize the effects of
178 PCN on *B. subtilis*. It was previously suggested that surfactin is involved in the interactions between
179 those two species,[29] as we observed as well in this study, and it appears that different mechanisms tightly
180 control the production of surfactin. *B. subtilis* was shown to decrease the production of both plipastatin
181 and surfactin when interacting with the fungus *Setophoma terrestris*.[45] The interaction between *B. subtilis*
182 and *S. terrestris* resulted in growth inhibition of the fungus only when exposed to *B. subtilis* cell-free
183 supernatant after exposure of *B. subtilis* to *S. terrestris*.[46]

184 To verify that the decrease in *B. subtilis* antibiotic production was not caused by inhibition of the bacterial
185 growth, we analyzed growth (by counting CFUs) in the various treatments (Figure 3C, D). Both *B. subtilis*
186 and *P. chlororaphis* (WT and Δ*phz*) did not show a significant change in their CFU count while cultured
187 together, or in treatments where PCN was added. Moreover, we cultured *B. subtilis*, its non-plipastatin-
188 producing mutant, *B. subtilis* Δ*pps*, and its non-surfactin-producing mutant, *B. subtilis* Δ*srfAA*, in the

presence of different PCN concentrations (Figure 4). A similar trend was observed among the different bacterial strains. At the highest PCN concentrations examined, 32.3-250 μM, all three types of bacteria were inhibited (Figure 4). Although these results are exciting, especially given the general importance of such phenazines, further studies are needed to determine their concentrations, and thus relevance to microbial growth inhibition, in natural environments.[47] PCA, an additional phenazine abundant in *Pseudomonas* species, was more comprehensively studied than PCN. Mavrodi et al.[47] have extracted PCA with the highest concentration of 1.6 μM gr^{-1} of roots, suggesting that higher concentrations used in our study are higher than natural relevant concentrations, and, as such, are not contradicting previous observations. However, further studies is warranted to determine accurate concentrations of PCN and other phenazines in natural environments.[47] At 15.6 μM of PCN, a slight growth inhibition was shown only in *B. subtilis* Δ*srfAA* towards the end of the experiment (Fig. 4C). At lower PCN concentrations, there was no difference between the treatments and the control that did not contain PCN. This indicates that at medium-low concentrations of PCN, which are naturally relevant, plipastatin and surfactin have no major part in *B. subtilis* protection mechanism against PCN.

Plipastatin and surfactin affect *P. chlororaphis* phenotypes

Given that the presence of *P. chlororaphis*, through secretion of PCN, results in a decrease of plipastatin and surfactin production in *B. subtilis* (Figure 3), we propose two hypotheses to explain this phenomenon: (i) competitive interactions: as *B. subtilis* antibiotics such as surfactin and plipastatin inhibit the growth of *P. chlororaphis*, the latter may have evolved to inhibit the production of those compounds. However, *B. subtilis* could potentially have evolved to overcome this inhibition through upregulation of antibiotics production upon sensing PCN, which we did not observe; (ii) this leads us to propose that we may observe here cooperative interactions: the presence of *P. chlororaphis* antibiotics (that do not inhibit *B. subtilis* significantly) in the environment may trigger a sensing mechanism in *B. subtilis*, that leads to downregulation of antibiotic production pathways, thereby effectively conserving energy by avoiding redundant antibiotic synthesis, as it can coexist with *P. chlororaphis*.

To investigate these hypotheses further, we examined differences in metabolites produced by *P. chlororaphis* while growing adjacent to *B. subtilis* or *B. subtilis* Δ*pps*. We observed no significant differences in the production of *P. chlororaphis* metabolites. To further examine the mechanism underlying decreased *B. subtilis* antibiotics production, we isolated a derivative of plipastatin, plipastatin A C_{19} (Figure S5), from *B. subtilis* cultures, along with a mixture of surfactins (Figure S6). We incubated *P. chlororaphis* with increasing concentrations of these isolated metabolites. We did not observe growth inhibition in the presence of these metabolites, in fact we did observe a slight increase in *P. chlororaphis* growth, with surfactin displaying a stronger effect than plipastatin (Figures 5A, B, S7). As a result, we assume that additional signals (and not only PCN) are involved in the interactions between the bacteria.

To examine additional interactions between the bacteria and the effects of plipastatin and surfactin on *P. chlororaphis*, we measured the production of reactive oxygen species (ROS) by the bacteria in the presence of the different *Bacillus* antibiotics. ROS are a group of highly reactive molecules derived from molecular oxygen. Bacteria can produce ROS and inhibit other microorganisms in their surrounding, and *Pseudomonas* spp. are especially adept at their production.[48,49] To quantify the ROS production by *P. chlororaphis*, we used 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). Upon exposure to ROS, DCFDA undergoes oxidation to 2',7'-dichlorofluorescein (DCF), which results in an increase of fluorescence. The addition of both plipastatin and surfactins to the bacteria resulted in an increase in ROS production (Figure 5C, D), in line with previous studies demonstrating that *B. subtilis* employs plipastatin as a defense strategy against the plant pathogens *Magnaporthe grisea*[50] and *Rhizopus stolonifer*.[51] This mechanism involves enhancing ROS production, which contributes to the antifungal activity of *B. subtilis*. However, our results show that these increases do not appear to significantly limit the growth of *B. subtilis* (Figure 3C). We propose, however, that ROS production by *P. chlororaphis* could benefit both species by creating an environment that is less attractive for other species to establish colonies, and *B. subtilis* has effective tools to cope with ROS.[52]

We further examined this hypothesis by evaluating the effects of *B. subtilis* and *P. chlororaphis* mono- and co-cultures on the growth and motility of *Fusarium oxysporum*, a soil-borne fungal plant pathogen. While the co-cultures grown on agar did not significantly affect the growth of *F. oxysporum*, supernatants from the bacterial co-cultures did inhibit the growth (both measured by examining *F. oxysporum* growth inhibition on supernatant-containing agar as well as the fungal radial growth rate), albeit less than each of the monocultures (Figures S8-S11). We tested thus far one of many potential fungal soil co-inhabitants that these bacteria encounter, and these effects may well differ of course when other fungi will be examined, which is part of our ongoing studies.

Taken together, our observations support the hypothesis that co-evolution of *B. subtilis* and *P. chlororaphis* may have led to mutualistic rather than competitive interactions, through down-regulation of antibiotic agents such as plipastatin and surfactins, as depicted in Figure 6.

Conclusions

B. subtilis and *P. chlororaphis* evolved together and interact with one another. Our study reveals that while the interaction seems to alter production of *B. subtilis* metabolites, it is not the case for *P. chlororaphis*. However, morphological changes were observed in both species. This interaction resulted in lower antibiotic production by *B. subtilis*. Our results support the hypothesis that lowering antibiotic production is part of a cooperation mechanism, not a competititive one. The production of plipastatin and surfactins is costly for *B. subtilis*, and as it appears to be able to cope with physiological concentrations of PCN (up

to 15 μM), it may be advantageous for *B. subtilis* to lower its antibiotic production and allow for the effects of *P. chlororaphis* virulence factors to provide a 'protective zone'. Nevertheless, environmental interactions are obviously much more complex than interactions under controlled laboratory conditions, examining only a few species. Further research is needed to examine those interactions under conditions resembling those of the natural environment, such as addition of specific soil minerals and additional fungal species.

Associated content

Experimental procedures and supporting figures 1–11, including additional experiment photos, metabolites distributions and mass spectrometry chromatograms.
The MS data are accessible at the UCSD Center for Computational Mass Spectrometry's server (access with password) which will become public upon publication:
ftp://MSV000096742@massive.ucsd.edu

Author contributions

All authors have given approval to the final version of the manuscript.

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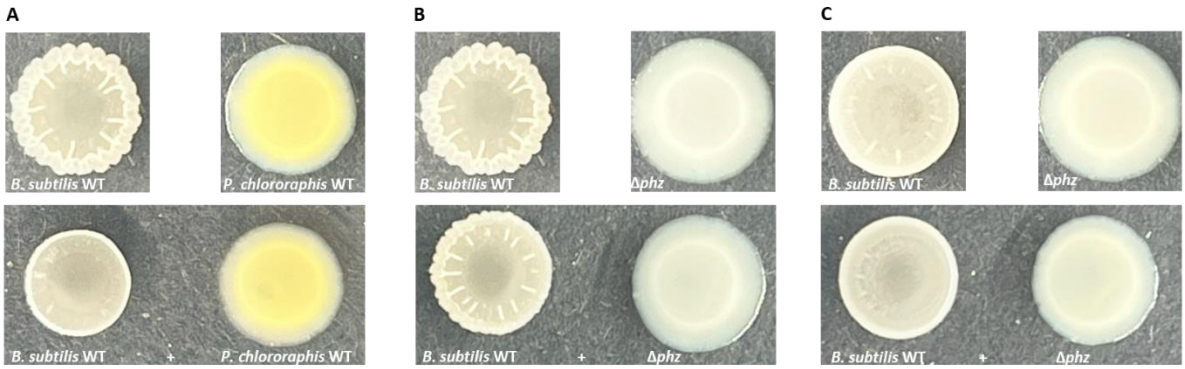

Figure 1. Cultures of *B. subtilis*, *P. chlororaphis* wild type (WT) and a *P. chlororaphis* non-phenazine producing mutant (Δ*phz)*. Top – mono-cultures. Bottom – co-cultures. (A,B) Agar plates (C) Agar + 10 µM PCN plates.

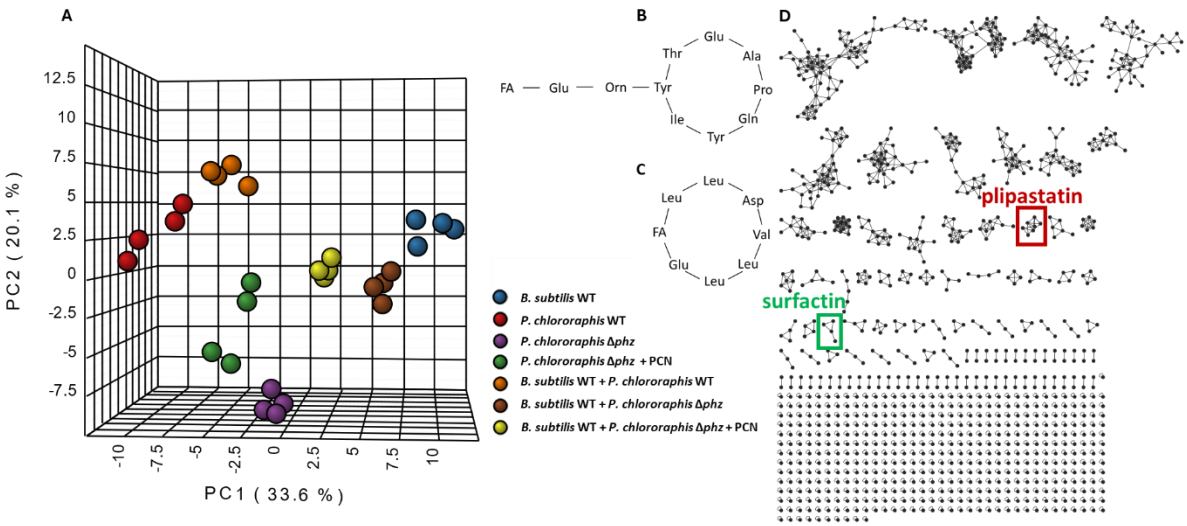

Figure 2. Effects on metabolite secretion in the bacterial co-cultures. (A) Principal component analysis of MS^1 data from single and co-culture extracts, created with use of MetaboAnalyst. The data is based on four replicates of cultures. Structure of (B) plipastatin and (C) surfactin. (D) Based on MS^2 data obtained from extracts of the various co-cultures and data treatment using MZmine, a network could be generated using the feature-based molecular network workflow (Nothias et al. 2020) on the open source GNPS platform (Wang et al. 2016). Each node represents a metabolite in the sample, and any edge connecting two nodes suggests that they have a similar structure. Plipastatin and surfactin clusters are indicated in red and green, respectively. FA = fatty acid. For the specific fatty acids identified in this study, see Figures S5, S6.

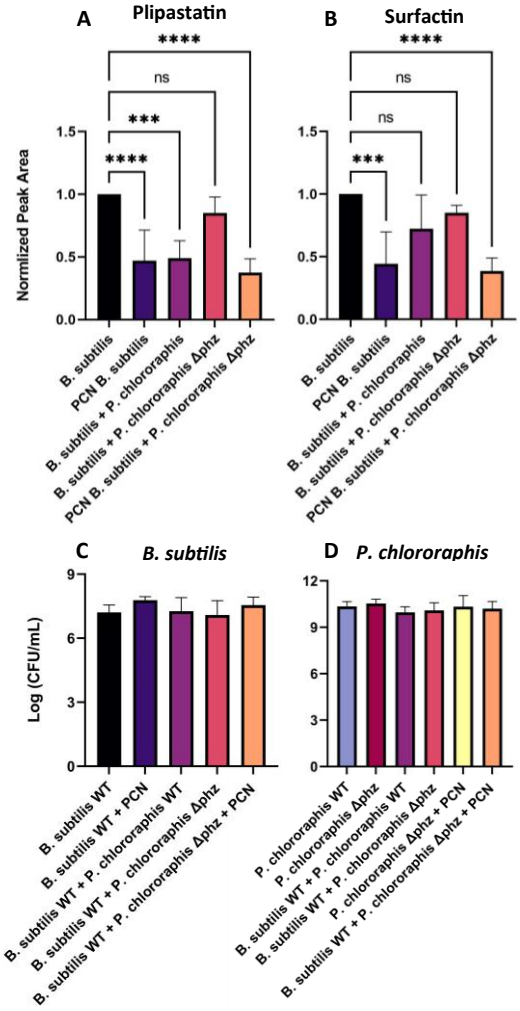

Figure 3. Production of (A) plipastatin, and (B) surfactin by *B. subtilis* in various treatments. The data represent the average of normalized peak area of three biological replicates by *B. subtilis* secretion in mono-culture, consist of four technical replicates, based on relevant compound production in mono-culture of *B. subtilis*. Colony forming unit of (C) *B. subtilis*, and (D) *P. chlororaphis* WT and *P. chlororaphis* Δ*phz*. Statistical analysis was performed using one-way ANOVA, with $p<0.0001$, $p<0.001$, are marked by ****, ***, respectively.

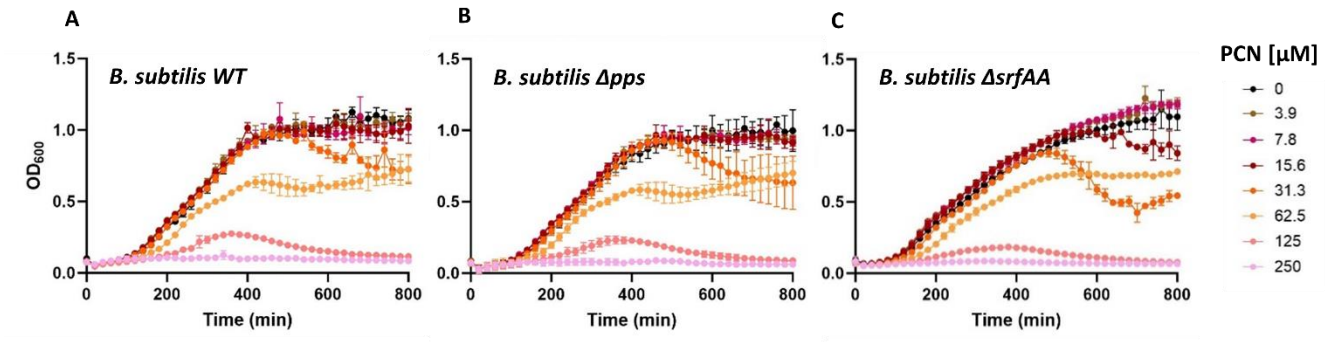

Figure 4. PCN affects *B. subtilis* growth: (A) WT (B) Δ*pps* (C) Δ*srfAA*. Error bars represent the standard deviation of triplicates and are not shown when smaller than the symbol.

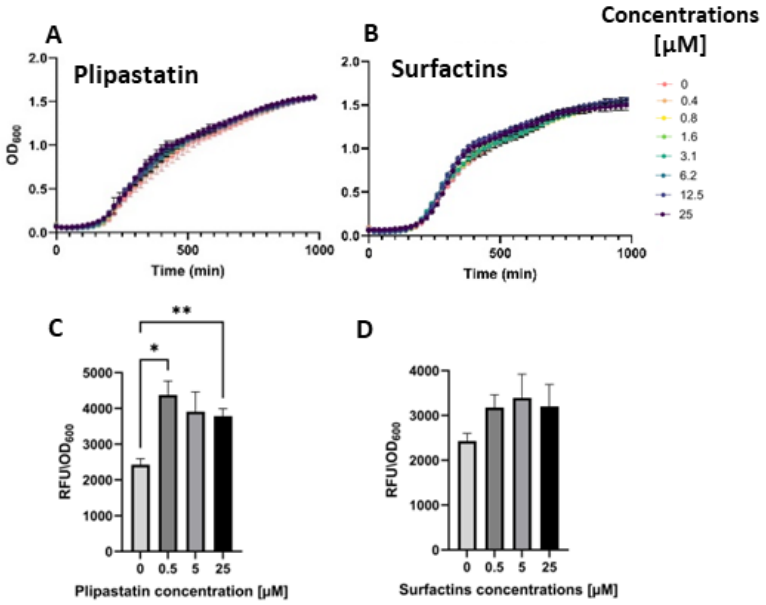

Figure 4. Effects of plipastatin and surfactin on *P. chlororaphis* growth (A, B) and ROS production (C, D). As test compounds we examined plipastatin A C_{19} (A, C) and a mixture of surfactins (B, D). Error bars represent the standard deviation of triplicates. Error bars smaller than the symbol are not shown. Statistical analysis was performed using Brown-Forsythe and Welch ANOVA, with P<0.01 and p<0.05 marked by **, *, respectively.

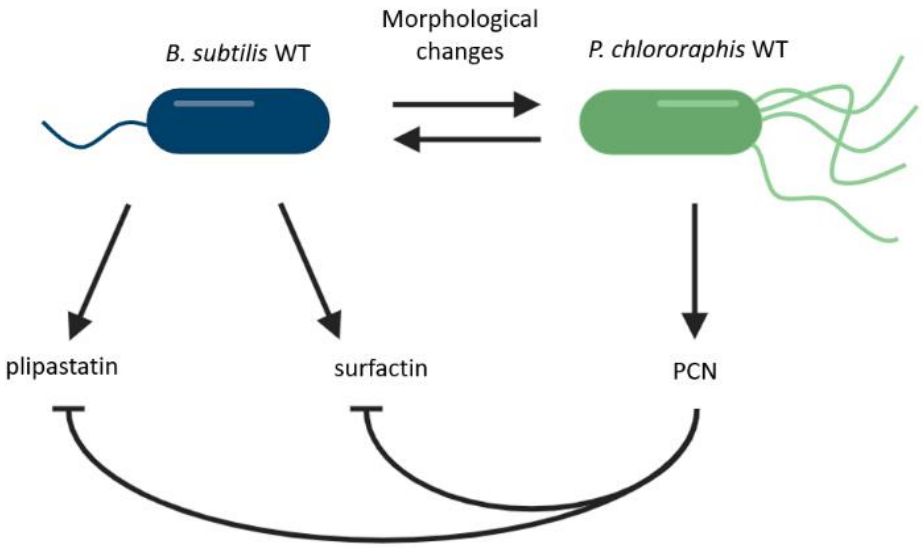

Figure 5. Proposed chemical interactions between *B. subtilis* (NCIB 3610) and *P. chlororaphis* (PCL 1391) and their effects.